

Regulation of Immunity to Visceral *Leishmania* in a Non-Human Primate Model of Infection

Tese do 3º Ciclo de Estudos Conducente ao Grau de
Doutoramento em Ciências Farmacêuticas –
Especialização em Bioquímica

Trabalho realizado sob a orientação de:

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É AUTORIZADA A REPRODUÇÃO INTEGRAL DESTA TESE APENAS PARA
EFEITOS DE INVESTIGAÇÃO, MEDIANTE DECLARAÇÃO ESCRITA DO
INTERESSADO, QUE A TAL SE COMPROMETE.

This work was performed at the *Parasite Disease* Group of the Institute for Molecular and Cell Biology (IBMC, Porto, Portugal) in collaboration with the *Programmed Cell Death and Signaling* Group (CNRS Unity FR3636) at the Neuroscience Research Federation of the Faculty of Fundamental and Biomedical Sciences of the Paris Descartes University (Paris, France). The author has received financial support through an individual Doctoral fellowship credited by the Foundation for Science and Technology (FCT, Portugal) with the reference SFRH / BD / 64064 / 2009. The experimental research performed in this work has received financial support from FCT, the National Research Agency (ANR, France) and the European Community's Seventh Framework Programme under grant agreement No.602773 (Project KINDRED).



Fundação para a Ciência e a Tecnologia

MINISTÉRIO DA CIÊNCIA, INOVAÇÃO E DO ENSINO SUPERIOR

Author's Declaration

Under the terms of the Decree-law number 216/92 of October 13th, is hereby declared that the candidate afforded a major contribution to the conceptual design and technical execution of the work, interpretation of the results and manuscript preparation of the published articles included in this dissertation.

The following original articles/communications were prepared in the scope of this dissertation:

Articles in International Peer-Reviewed Journals

In the scope of this dissertation:

Vasco Rodrigues, Mireille Laforge, Laure Campillo-Gimenez, Calaiselvy Soundaramourty, Ana Oliveira, Ricardo Jorge Dinis-Oliveira, Ali Ouaiissi, Anabela Cordeiro da Silva, Ricardo Silvestre and Jérôme Estaquier (2014). Abortive T follicular helper development is associated with a defective humoral response in *Leishmania infantum*-infected macaques. PLOS Pathogens **10**(4):e1004096.

Vasco Rodrigues, Mireille Laforge, Laure Campillo-Gimenez, Calaiselvy Soundaramourty, Ali Ouaiissi, Anabela Cordeiro da Silva, Ricardo Silvestre and Jérôme Estaquier (2014). Longitudinal analysis defines the kinetics of CD8 T cell differentiation and chronic exhaustion in rhesus macaques infected with *Leishmania infantum*. (To be submitted).

Participation in publications in related fields:

Vasco Rodrigues, Anabela Cordeiro da Silva, Mireille Laforge, Ali Ouaiissi, Ricardo Silvestre and Jérôme Estaquier (2012). Modulation of mammalian apoptotic pathways by intracellular protozoan parasites. Cellular Microbiology **14**(3):325-33.

Vasco Rodrigues, Anabela Cordeiro da Silva, Mireille Laforge, Ali Ouaiissi, Khadija Akarid, Ricardo Silvestre and Jérôme Estaquier (2014). Impairment of T cell function in parasitic infections. PLOS Neglected Tropical Diseases **8**(2):e2567.

Mireille Laforge, Sophie Limou, Francis Harper, Nicoletta Casartelli, Vasco Rodrigues, Ricardo Silvestre, Houda Haloui, Jean-Francois Zagury, Anna Senik and Jérôme Estaquier (2013). DRAM triggers lysosomal membrane permeabilization and cell death in CD4(+) T cells infected with HIV. PLOS Pathogens **9**(5):e1003328.

Vasco Rodrigues, Tarik Mouttaki, Khadija Akarid, Myriam Riyad and Jérôme Estaquier (2014). Immune transcriptional profiling of skin lesions from Moroccan patients with cutaneous leishmaniasis (In preparation).

Diana Moreira, Vasco Rodrigues, Maria Abengozar, Luis Rivas, Eduardo Rial, Mireille Laforge, Xiaoling Li, Marc Foretz, Benoit Viollet, Jérôme Estaquier, Anabela Cordeiro da Silva and Ricardo Silvestre (2014). *Leishmania infantum* modulates the host macrophage metabolism by hijacking the SIRT1-AMPK axis (Submitted for publication).

Mireille Laforge, Ricardo Silvestre, Vasco Rodrigues, Julie Garibal, Laure Campillo-Gimenez, Shahul Mouhamad, Valérie Monceaux, Marie-Christine Cumont, Bruno Hurtrel, Guido Silvestri, Anna Senik and Jérôme Estaquier (2014). Q-VD-OPH induces effector lytic CD4 T cells and protects SIV-infected rhesus macaques (Submitted for publication).

Mireille Laforge, Vasco Rodrigues, Ricardo Silvestre, Clément Gautier, Robert Weil, Olga Corti and Jérôme Estaquier (2014). NF- κ B pathway and mitochondrial dynamics: minor role of Parkin and NEMO (Submitted for Publication).

Communications

Oral Communications

Vasco Rodrigues*, Mireille Laforge, Anabela Cordeiro da Silva, Ali Ouaiissi, Ricardo Silvestre and Jérôme Estaquier. Abortive T follicular helper development is associated with a defective humoral response in *Leishmania infantum*-infected macaques. Congrès Annuel de la Société Française d'Immunologie, Paris, France, 24-27 November 2013.

Vasco Rodrigues*, Mireille Laforge, Anabela Cordeiro da Silva, Ali Ouaiissi, Ricardo Silvestre and Jérôme Estaquier. Abortive T follicular helper differentiation and loss of activated memory B cells underlie the poor parasite-specific IgG production in *Leishmania infantum*-infected rhesus macaques. Fifth World Congress on Leishmaniasis, Porto de Galinhas, Pernambuco, Brazil, 13-17 May 2013.

Jérôme Estaquier*, Vasco Rodrigues, Mireille Laforge, Anabela Cordeiro da Silva, Ali Ouaiissi and Ricardo Silvestre. Abortive T follicular helper development is associated with a defective humoral response in *Leishmania infantum*-infected macaques. 88th Annual Meeting of the American Society of Parasitologists, Quebec City, Quebec, Canada, 26-29 June 2013.

*Presenting author

Poster Communications

Vasco Rodrigues*, Mireille Laforge, Anabela Cordeiro da Silva, Ricardo Silvestre and Jérôme Estaquier. Acute and chronic immune determinants in *Leishmania infantum*-infected rhesus macaques. A novel model for visceral leishmaniasis. Third I3S Scientific Retreat, Póvoa de Varzim, Portugal, 10-11 May 2012

Vasco Rodrigues*, Mireille Laforge, Anabela Cordeiro da Silva, Ricardo Silvestre and Jérôme Estaquier. Acute and chronic immune response in *Leishmania infantum*-infected rhesus macaques. A novel model for visceral leishmaniasis. Third European Congress of Immunology, Glasgow, Scotland, 5-8 September 2012

*Presenting author

Acknowledgments

Accomplishing a doctoral thesis is a long and hard endeavor. Over the past four years, many were those who contributed in various ways to the realization of my goals. I would like to express my gratitude to every single person that I have crossed along this path, acknowledging and apologizing beforehand those that I may eventually forget to mention.

I would have never been able to finish this work without the guidance and advice from my supervisors. To Professor Anabela my words are of sincere gratitude. Thank you for the advices, the careful revisions and the encouraging words even during the time I was away from the lab. Six years have passed since I was first introduced to a research lab. Six years in which I always felt your care for my personal and scientific wellbeing. To Jérôme, I would like to summarize my gratitude by stating that when I first arrived in Paris I was nothing but a (very) young researcher who had a passion for Immunology learned from textbooks. I see myself now as (not so) young scientist capable of performing tasks that seemed so daunting and complex only a few years ago. Much of this progress was due to your advice, patience and persistence in showing me the right way. I truly admire your far-reaching understanding of science, which I intend to follow throughout my career. To Ricardo, the space here is perhaps scarce to express my gratefulness properly. You have been the supervisor one can only wish for, and I hope I have been up to the expectations of being your first Ph. D. student. Apart from your scientific input (which was extraordinary and key to the success of the work), I would like to refer the friendship built over these 4 years and that I believe will endure for a long time.

I would like to leave a kind word to Dr. Ali Ouaiissi, who, while not directly supervising my work, has always been available to review the papers insightfully, and for his encouragements every time things did not go as expected.

The project's demands and some vicissitudes made me spend substantial time in three different laboratories during my Ph. D. In each of these stays, I came across with great people who in a professional or personal way have contributed to the accomplishment of this thesis.

The *Parasite Disease* group at the IBMC has always been my home lab; the place where I first started to work at the bench and where it is always nice to comeback. While impossible to individually express my gratitude to each one who has passed by the lab in these years I'd like to leave a word to Diana (Dianucha!), Nuno (a great guy! so pure-hearted that he is still convinced that he has any skills to play football), Mariana (Rota!), Lúcia, Inês Mesquita, Joana Cunha, Sofia, Inês Loureiro, Joana Faria, Daniela, Patrícia, Ana Luísa, Begoña, Pedro (Ceci!!), Susana, Joana Tavares, Luís, Alex, Cátia, Marta, Helena and Catarina for

the friendship and nice moments spent together. To Dona Rosa for her everlasting good-mood and kindness.

To everyone at the INSERM Unity 955, where I spent the first year of my Parisian experience. Thank you for the support and kindness. I'd like to leave a special word to those who then became my closest Parisian friends. Ayrin, we started our thesis at the same time and became something like blood brothers. It has been a pleasure to meet the wonderful person you are, and I enjoyed every bit of time we spent together. To Laure (ma Blonde!!) and the ingenious way we found to communicate with each other when there was no language that we both could speak. To Matthieu, Ali (vérrre are da girls!!), Daniel (thank you for the Dark Side, the margaritas, the guitar solos and for curing cancer), Maymouna, Houda, Shahul, Mehwish and Anne-Marie for all the soirées and enjoyable moments together.

To my dear colleagues at Jérôme's lab in Saints-Pères I have a few special words. To my dear Mireille, it has been a pleasure to work alongside you, to learn with you; our insightful discussions during journal clubs and thank you for introducing me to Lebanese food. To Bernard, I can only say that I feel privileged for having met someone as kind as you, always available to solve any problem that comes across. To my sweet Selvy, words are hard to find that can describe my gratitude to you. Your care for me made you my Parisian mother and I cannot move on without referring the marvelous Indian dishes you brought me! To Cécile, Olivia, Chloé and Marine, thank you for making the lab a better place to be. To everyone at the FRE3235 Unity for always being kind and supportive. To Vasco and Aline for doing such a great job in organizing nice *cinelabô soirées*, and to Brigitte Delhomme for her precious help with histological colorations and the use of the cryostat.

I would like to extend my gratitude to all the co-authors of the work presented in this thesis. Their contribution was key to the scientific quality of this work.

To everyone I met at the *Maison du Portugal*, in particular Óscar and Nicole, thank you for the dinners, parties, and barbecues together. The *Cité Internationale* is a wonderful place to live, and I will remember those years as among the best in my life.

Though limited, I always managed to save some time to my closest friends. This thesis is for them as well. To Joca, Cristo, Migas, Claudino, Raposinho, Óscar, Rui Pedro, Sombra, Hélder, Ivo, Carla, Natália, Marta for always being comprehensive even when I didn't show up for a beer or coffee for several months.

Finally, to my mother, father and brother and the rest of my family, for believing me and providing me support, without which none of this would be possible.

Summary

Unicellular eukaryotes of the genus *Leishmania* are collectively responsible for a heterogeneous group of diseases known as leishmaniasis. The visceral form of leishmaniasis, caused by *L. donovani* or *L. infantum*, is a devastating condition, claiming about 50.000 lives annually, with particular incidence in some of the poorest regions of the world. While some immune correlates of protection against visceral leishmaniasis (VL) have been defined, no efficient vaccine yet exists. Such lack of success results, in part, from the difficulty in translating findings obtained with experimental models to clinical application in human patients. For instance, the widely used mouse model, while instrumental in dissecting several immune mechanisms operating during VL, fails to replicate the fatal human condition. In this context, non-human primates constitute unique models of human disease owing to their close phylogenetic relationship and similar physiology. Unfortunately, non-human primates have been underexplored in VL research, contrasting with their wide use as models of other infectious diseases.

In this work, we conducted experimental infections with *L. infantum* in rhesus macaques with the aim of developing a non-human primate model for immunological studies in VL. Our approach involved inducing VL in macaques by intravenous injection of *L. infantum* promastigotes. Groups of infected animals were euthanized at distinct time-points after infection and infected organs recovered for analysis.

Early after infection (first month), parasites were progressively cleared from circulation and contained in visceral compartments such as the spleen and the liver and essentially absent from additional sites. In contrast, at chronic infection (eight months), the splenic and hepatic parasite populations expanded. We further observed extensive parasite dissemination and growth in the blood, lymph nodes and bone marrow.

Despite the absence of an overt clinical disease, macaques developed a set of clinical signs that are reminiscent of human VL, including hypergammaglobulinemia, anemia, elevated serum alanine transaminase and augmented spleen cell numbers. The increased splenic cellularity resulted mainly from an expansion of the CD4 and CD8 T cell pools and was accompanied by differentiation of effector and effector memory cells from naïve precursors, for both T cell lineages. Splenic CD4 T cells adopted a predominant Th1 molecular signature during the first month of infection. However, at the chronic phase, expression of genes encoding for Th1 cytokines decreased, while expression of *IL10* augmented, concomitant with parasite growth and dissemination. Splenic CD8 T cells were relatively modest at expressing Th1 cytokines during the acute phase of infection, but instead expressed high levels of granzyme B. Paralleling CD8 T cell expression of cytotoxic molecule was the

induction of the transcription factors T-bet and Eomesodermin. Remarkably, at the chronic phase, CD8 T cell effector function decreased prominently, with decreased mRNA levels for Th1 cytokines and abrogated expression of granzyme B. Such functional attrition is suggestive of immune exhaustion, prompting us to analyze the expression of inhibitory receptors in splenic CD8 T cells. Indeed, expression of the inhibitory receptor LAG-3 in splenic CD8 T cells, increased towards the end of the acute phase of infection and persisted elevated at the chronic phase, unveiling a possible link with CD8 T cell exhaustion.

One of the most intriguing observations in our studies was the fact that, despite the persistent hypergammaglobulinemia, the serum levels of *Leishmania*-specific IgG increased modestly during the acute phase and decreased at chronic infection. Aiming to elucidate the mechanisms underlying such dysfunctional humoral response, we observed the progressive expansion of a splenic B cell population with the atypical CD21⁺CD27⁻ phenotype after infection. Also, the frequency of B cells with an activated memory phenotype (CD21⁺CD27⁺CD45RA⁻) correlated strongly with the production of parasite-specific IgG. By examining splenic sections of infected macaques we observed the formation of germinal centers during the acute phase of infection. However, at chronic infection their numbers were significantly decreased. Altogether, these observations incited us to explore the dynamics of T follicular helper cells (Tfh cells) in the spleen of rhesus macaques infected with *L. infantum*, as these play a crucial role in coordinating germinal center reactions that result in the production of high affinity antibodies. CD4 T cells expressing CXCR5 and PD-1 were observed in splenic B cell areas during the acute phase of infection, but mostly absent at the chronic phase. Flow cytometric analysis revealed that the frequency of a population of CD4 T cells expressing CXCR5 and the Tfh master transcription factor Bcl-6, strongly correlated with the percentage of activated memory B cells and the serum levels of *L. infantum*-specific IgG.

Our work demonstrates the feasibility of the rhesus macaque as a model for fundamental studies on the immune response during VL. We provided a phenotypic and functional analysis of the main adaptive immune populations over the course of infection and a mechanistic basis for the dysfunctional humoral response. Overall, our work may pave the way for a more frequent employment of the macaque model for studies in the immune response during VL.

Keywords

Visceral leishmaniasis, rhesus macaque, dysfunctional humoral response, T cell exhaustion

Resumo

Os eucariotas unicelulares do gênero *Leishmania* são coletivamente responsáveis por um grupo heterogêneo de doenças designadas por leishmaniose. A forma visceral da leishmaniose, causada por infecções por *L. donovani* ou *L. infantum*, é uma doença devastadora, retirando cerca de 50.000 vidas por ano, com especial incidência em algumas das regiões mais pobres do mundo. Embora alguns correlativos de proteção imunológica contra a leishmaniose visceral (LV) sejam conhecidos, ainda não foi desenvolvida qualquer vacina eficaz. Esta falta de sucesso resulta da dificuldade em traduzir para aplicação clínica humana, os resultados obtidos com modelos experimentais. Por exemplo, o modelo de ratinho tem sido instrumental na elucidação de inúmeros mecanismos imunológicos que operam durante a doença. No entanto, é incapaz de replicar a doença progressivamente fatal que se observa nos humanos. Neste contexto, os primatas não-humanos apresentam-se como modelos experimentais únicos das doenças humanas, devido à sua estreita relação filogenética e fisiologia semelhante. Apesar disso, os primatas não-humanos têm sido pouco explorados na investigação da LV, o que contrasta com a sua ampla utilização como modelos de outras doenças infecciosas.

Neste trabalho, realizaram-se infecções experimentais com *L. infantum* em macacos rhesus, tendo como objetivo o desenvolvimento de um modelo de primata não-humano para estudos imunológicos na LV. A estratégia experimental seguida consistiu na injeção intravenosa de promastigotas de *L. infantum*. A vários tempos após a infecção, subgrupos de animais foram eutanasiados e os órgãos recuperados para análise.

Durante o primeiro mês após infecção, os parasitas foram progressivamente eliminados de circulação, contidos no baço e no fígado e essencialmente ausentes de outros órgãos. Pelo contrário, na fase crónica (oitavo mês), a população parasitária visceral aumentou e o parasita disseminou-se para órgãos adicionais, como a medula óssea e os gânglios linfáticos.

Embora os macacos infetados não tenham desenvolvido os sintomas da LV de forma evidente, manifestaram ainda assim um conjunto de sinais clínicos remissivos da doença humana, incluindo hipergamaglobulinemia, anemia, elevação da alanina transaminase sérica e um aumento do número de células do baço. Em particular, este aumento da celularidade do baço resultou sobretudo da expansão das populações T, CD4 e CD8. Acompanhando o aumento de número, ambas as linhagens celulares T exibiram diferenciação para fenótipos efetores e efetores-memória. As células T CD4 esplénicas exibiram uma assinatura molecular Th1 durante o primeiro mês de infecção. No entanto, na fase crónica, a diminuição dos níveis de citocinas Th1 e o aumento de expressão de IL10

revela uma transição para um fenótipo regulador. As células T CD8 do baço, embora modestas na capacidade de expressar citocinas Th1 durante a fase aguda da infecção, exibiram níveis elevados de granzima B. Acompanhando esta indução de granzima B, as células T CD8 também aumentaram os seus níveis de expressão dos fatores de transcrição de T-bet e Eomesodermin. No entanto, na fase crónica, a função efetora das CD8 diminuiu, dada a drástica diminuição da expressão de citocinas Th1 e de granzima B. Este comprometimento funcional sugere a ocorrência de exaustão imunológica. De facto, a expressão do recetor inibitório LAG-3 nas células T CD8⁺ do baço aumentou a partir do final da fase aguda da infecção e persistiu elevada na fase crónica, revelando uma possível conexão com a exaustão de células T CD8.

Apesar da hipergamaglobulinemia persistente, os níveis séricos de IgG específico para o parasita aumentaram apenas ligeiramente durante a fase aguda, e diminuíram na fase crónica. Em suporte desta resposta humoral disfuncional, observamos a progressiva expansão, ao longo de todo o período experimental, de uma população atípica de células B do baço com o fenótipo CD21⁻CD27⁻. Além disso, a frequência de células B com um fenótipo de memória ativada (CD21⁻CD27⁺CD45RA⁻) apresentou uma forte correlação com os níveis de IgG específica séricos, diminuindo na fase crónica. Microscopia confocal em tecido esplénico de macacos infetados revelou a formação de centros germinais, durante a fase aguda da infecção, mas a sua diminuição na fase crónica. Estas observações levaram-nos a explorar a dinâmica das células T foliculares *helper* (Tfh), dado o seu papel importante na coordenação dos eventos no centro germinal. Células T CD4 exprimindo CXCR5 e PD-1 surgiram nas zonas B do baço durante a fase aguda da infecção, mas desapareceram maioritariamente na fase crónica. Em complemento, os nossos resultados mostraram que a frequência de uma população de células T CD4 exprimindo CXCR5 e Bcl-6, se correlaciona positivamente com a percentagem de células B de memória e os níveis séricos de IgG específico.

Concluindo, o trabalho aqui apresentado demonstra a utilidade de um modelo baseado no macaco rhesus para estudos na LV. Este trabalho apresenta uma detalhada análise fenotípica e funcional das principais populações imunológicas ao longo do curso da infecção e uma base mecanicista para a resposta humoral disfuncional. Em geral, este trabalho pode abrir novas perspetivas para uma utilização mais frequente do modelo de macaco para estudos na resposta imune durante a LV.

Palavras-chave

Leishmaniose visceral, macaco rhesus, resposta humoral disfuncional, exaustão de células T.

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List of Abbreviations

AID	Activation-Induced Deaminase
AIDS	Acquired Immunodeficiency Syndrome
AP-1	Activator Protein-1
APC	Antigen-Presenting Cell
BATF	Basic leucine zipper transcription factor, ATF-like
BCR	B Cell Receptor
BLIMP-1	B Lymphocyte-Induced Maturation Protein-1
C3b	Complement component 3b
CCL2	Chemokine C-C motif Ligand-2
CD95L	CD95 Ligand
CL	Cutaneous Leishmaniasis
CLR	C-Type Lectin Receptor
c-maf	cellular-musculoaponeurotic fibrosarcoma
CR3	Complement Receptor 3
CTL	Cytotoxic T Lymphocyte
CTLA-4	Cytotoxic T Lymphocyte Antigen-4
CXCR2	C-X-C chemokine Receptor type 2
DALY	Disability Adjusted Life Year
DAMP	Damage-Associated Molecular Patterns
DAT	Direct Agglutination Test
DC	Dendritic Cell
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DEC	Diethylcarbamazine Citrate
Dectin-1	DC-Associated C-Type Lectin-1
DICER-1	Double-Stranded RNA-Specific Ribonuclease-1
EBI-2	Epstein–Barr virus-induced G-protein-coupled receptor-2
EF-1	Elongation Factor-1
Eomes	Eomesodermin
ER	Endoplasmic Reticulum
ERK	Extracellular Signal-Regulated Kinase
FAST	Fast Agglutination Screening Test
FCγR	Fc-γ Receptor
FDA	Food and Drug Administration

FDC	Follicular Dendritic Cell
FML	Fucose Mannose Ligand
GC	Germinal Center
GIPL	Glycoinositol Phospholipid
gp63	Glycoprotein 63
gRNA	guide RNA
GTPase	Guanosine Triphosphatase
HAART	Highly Active Anti-Retroviral Therapy
HEV	High Endothelial Venule
HIV	Human immunodeficiency virus
HVEM	Herpes Virus Entry Mediator
ICOS	Inducible T cell Co-Stimulator
ICOSL	ICOS Ligand
IDO	Indoleamine 2,3-Dioxygenase
IFN- γ	Interferon- γ
Ig	Immunoglobulin
IL-2R β	Interleukin-2 Receptor Beta
IL-4R	IL-4 Receptor
IL-6	Interleukin-6
iNOS	Inducible Nitric Oxide Synthase
IRAK	Interleukin-1 Receptor Associated Kinase
IRF	Interferon Regulatory Factor
JAK-2	Janus Kinase-2
kbp	Kilobase Pairs
LAG-3	Lymphocyte-Activation Gene-3
LAMP	Lysosome-Associated Membrane Protein
LCMV	Lymphocytic Choriomeningitis Virus
LPG	Lipophosphoglycan
LPS	Lipopolysaccharide
LRR	Leucine-Rich Repeat
LRV	<i>Leishmania</i> RNA Virus
mAb	Monoclonal Antibody
MAC	Membrane Attack Complex
MAPK	Mitogen-Activated Protein Kinase
MBL	Mannose-Binding Lectin
MHC	Major Histocompatibility Complex

miR-122	Micro-RNA-122
ML	Mucocutaneous Leishmaniasis
MLEE	Multilocus Enzyme Electrophoresis
MMM	Marginal Metallophilic Macrophages
MR	Mannose Receptor
Myd88	Myeloid Differentiation Primary Response Gene 88
MZB	Marginal Zone B cell
MZM	Marginal Zone Macrophages
NADPH oxidase	Nicotinamide Adenine Dinucleotide Phosphate-Oxidase
NFAT-2	Nuclear Factor of Activated T cells-2
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NGO	Non-Governmental Organization
NK	Natural Killer
NLR	Nucleotide-binding Oligomerization domain-like Receptor
NO	Nitric Oxide
NRAMP1	Natural resistance-associated macrophage protein 1
NTD	Neglected Tropical Disease
NW	New World
OW	Old World
PALS	Periarteriolar Lymphoid Sheaths
PAMP	Pathogen-Associated Molecular Pattern
PBMC	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction
PD-1	Programed Death-1
PI3K	Phosphoinositide-3-Kinase
PKDL	Post Kala-azar Dermal Leishmaniasis
PM	Peritrophic Membrane
PPG	Proteophosphoglycan
PRR	Pattern Recognition Receptor
PSG	Promastigote Secretory Gel
PTP	Protein tyrosine phosphatase
PV	Parasitophorous Vacuole
qPCR	quantitative Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
ROS	Reactive Oxygen Species

SACP	Secreted Acidic Phosphatase
SHP-1	Src Homology region 2 domain-containing Phosphatase-1
SIRP- α	Signal Regulatory Protein- α
SIV	Simian Immunodeficiency Virus
SNARE	Soluble N-ethylmaleimide-sensitive factor Attachment protein Receptor
STAT-1	Signal Transducers and Activators of Transcription-1
TCPTP	T cell Protein Tyrosine Phosphatase
Tfh	T follicular helper
Tfr	T follicular regulatory
Th1	T helper-1
TIM-3	T cell Immunoglobulin and Mucin domain
Tip DC	TNF and iNOS-Producing Dendritic Cell
TLR	Toll-Like Receptor
TNF	Tumor necrosis factor
TRAF6	TNF-receptor-associated factor 6
Tregs	Regulatory T cells
TRIF	TIR Domain-Containing Adaptor-Inducing IFN- β
UNC93B1	Mammalian homolog B1 of Unc-93
VAMP8	Vesicle-Associated Membrane Protein-8
VL	Visceral Leishmaniasis
WHO	World Health Organization

Chapter I | Introduction

1. The neglected tropical diseases: a 21st century challenge

The neglected tropical diseases (NTDs) are a group of epidemic-prone chronic infections of helminthic, bacterial or protozoan etiology, which primarily occur in rural or poorly-urbanized areas of low-income countries (Molyneux 2004). The chronic nature of most NTDs imparts prolonged, frequently lifelong, suffering to patients as notoriously exemplified by the blindness that results from trachoma (Hotez *et al.* 2007). Besides the physical illness, the disfiguring features of some NTDs often lead to social stigma, which leads to progressive patient isolation, impacting on health-care seeking and treatment adherence (Weiss 2008).

The NTDs owe their designation to the low investment in drug development by pharmaceutical companies and insufficient research funding by governmental agencies. For instance, of the 1393 new chemical entities granted market authorization between 1975 and 1999, only 7 were registered for the treatment of NTDs. Even worse, between 2000 and 2011, no NCEs were approved to treat NTDs and, during this time lag, the anti-NTDs drug arsenal only benefited from the addition of 5 repurposed versions of existent medicines. Many of the compounds in clinical use to treat NTDs were developed during the first half of the 20th century, at the time fueled by colonial requirements. As the interests of Western nations in these locations waned during the second half of the past century, these conditions became progressively neglected (Trouiller *et al.* 2002, Pedrique *et al.* 2013).

These figures reveal a stark contrast between NTDs and the so-called “big three” infectious diseases: malaria, tuberculosis and acquired immunodeficiency syndrome (AIDS). As of 2009, there were 25 anti-retroviral compounds approved to treat AIDS, only 25 years after the discovery of the human immunodeficiency virus (HIV) as the causative agent of AIDS. Over the past two decades, the fight against the “big three” has benefited from major partnerships promoted by the G8 governments, non-governmental organizations (NGOs) and private foundations. Along with mass media campaigns, these initiatives succeeded in increasing social awareness and raised massive funding for research and interventions to tackle the “big three” (Hotez *et al.* 2006a).

If the death toll were the main criterion to evaluate the burden of a disease, the NTDs would lie considerably below any of the “big three” (**Table 1**). However, given their chronic nature, the impact of the NTDs is better expressed in terms of disability-adjusted life years (DALYs), a measure of the number of healthy life years lost due to premature death or disability. Using this metric, the burden associated with the ensemble of the NTDs is comparable to each of the “big three” (**Table 1**) and, presumably, worth-meriting equivalent attention and commitment (Hotez *et al.* 2007).

Table 1. Global burden for the major NTDs and the “big three” infectious diseases

Disease	Causative agent	Global Prevalence (millions) ¹	Annual deaths	DALYS (millions) ²
Helminthic infections				
Ascariasis	<i>Ascaris lumbricoides</i>	802	60.000	10.5
Trichuriasis	<i>Trichuris trichiura</i>	604	10.000	6.4
Hookworm infection	<i>Necator americanus</i>	576	65.000	22.1
Schistosomiasis	<i>Schistosoma mansoni</i>	207	280.000	4.5
Lymphatic filariasis	<i>Wuchereria bancrofti</i>	120	0	5.8
Onchocerciasis	<i>Onchocerca volvulus</i>	90	0	0.5
Dracunculiasis	<i>Dracunculus medinensis</i>	0.01	ND*	<0.1
Bacterial infections				
Trachoma	<i>Chlamydia trachomatis</i>	84	0	2.3
Leprosy	<i>Mycobacterium leprae</i>	0.4	6.000	0.2
Buruli ulcer	<i>M. ulcerans</i>	ND	ND	ND
Protozoan infections				
Leishmaniasis	<i>Leishmania</i> spp.	12	51.000	2.1
Chagas' disease	<i>Trypanosoma cruzi</i>	8-9	14.000	0.7
African trypanosomiasis	<i>Trypanosoma brucei</i>	0.3	48.000	1.5
NTD-associated total burden		2503	534.000	56.6
Burden associated with the “big three” infectious diseases				
AIDS	HIV	35.3 ³	1.654.400 ⁶	81.5 ⁷
Tuberculosis	<i>M. tuberculosis</i>	10.0 ⁴	1.196.000	49.4
Malaria	<i>Plasmodium</i> spp.	219 ⁵	1.169.500	82.7

¹The global prevalence data for the NTDs was obtained from (Hotez *et al.* 2007);

²Mortality and DALYs for the NTDs were obtained from (Hotez *et al.* 2006b)

³AIDS global prevalence from the UNAIDS Report on the Global AIDS Epidemic 2013 (UNAIDS 2013)

⁴Tuberculosis global prevalence obtained from the WHO Global Tuberculosis Report 2012 (WHO 2012a)

⁵Malaria global prevalence from the WHO World Malaria Report (WHO 2012b)

⁶Mortality data for the big three from the 2010 Global Burden of Disease Study (Lozano *et al.* 2012)

⁷DALYs for the big three obtained from the 2010 Global Burden of Disease Study (Murray *et al.* 2012)

*ND denotes not determined

It is perhaps perplexing to note that, when applied, simple and affordable measures can be highly effective in mitigating the burden of the NTDs (Hotez *et al.* 2007). By the mid-20th century, China was heavily afflicted by filarial infections. During the 1960's the Chinese government mobilized a set of measures intended to eradicate the disease, the most effective of which was the fortification of common table salt with diethylcarbamazine citrate (DEC), a low cost anti-helminthic drug. The sustained use of this treatment scheme, along with close surveillance, led to a complete eradication of filariasis in China, by the turn of the century (De-Jian *et al.* 2013).

Whereas filarial infections and the other NTDs still exert a heavy burden in the poorest regions of the globe, the perspectives for their control have substantially improved; particularly since the turn of the millennium (Hotez 2013). A landmark event occurred in 1987, when Merck committed to donate the worm-killing drug ivermectin “as much as needed, for as long as needed” in an effort to eliminate river blindness in sub-Saharan Africa. The philanthropic act was soon followed by similar donations by other

pharmaceutical companies (Hotez *et al.* 2007). Fuelled by the millennium development goals, established by the United Nations in 2000, a number of public-private partnerships were created to control or eliminate several NTDs in the following decades (Hotez 2013). These programs are, for the most part, targeted at helminthic and *Chlamydia* infections, highly prevalent in sub-Saharan Africa. Their goal is to achieve an integrated control of several NTDs, taking advantage of the extensive geographical overlap among endemic areas. Indeed, it is common to find multiparasitized individuals in many endemic regions. (Hotez *et al.* 2007). The rationale for these interventions relies on preventive chemotherapy, achieved by mass administration of combinations of drugs targeted at the NTDs affecting each intervened region (Molyneux *et al.* 2005). Given that most of the drugs are donated, costs essentially result from the logistics of distribution at the community level. The main advantage of these drug packages is to obtain a rapid impact by simultaneously targeting several diseases, through the use of low-cost drugs that are safe enough for mass drug administration (Hotez 2011). Potential drawbacks include the development of resistance and the lack of back-up drugs, as well as the interventions being hindered by fragile political contexts or conflicts in intervened regions (Hotez *et al.* 2007). The use of these integrated policies to manage some of the NTDs has enjoyed a remarkable success in the past few years. According to the latest reports, dracunculiasis (guinea worm) is on the verge of worldwide eradication (WHO 2013), and Togo is very close to become the first sub-Saharan nation to eliminate lymphatic filariasis (Sodahlon *et al.* 2013). However, these programs have failed, so far, to effectively tackle the NTDs associated with the highest death rates: the kinetoplastid infections. A fundamental premise for the implementation of a preventive chemotherapy program for NTDs is the existence of at least one drug that is simultaneously effective, safe and available at low cost (Hotez *et al.* 2007). For instance, until recently, African trypanosomiasis were treated with melarsoprol, a dangerous the organoarsenical drug that requires close medical surveillance during treatment (Babokhov *et al.* 2013). Quite likely, the best prospects to achieve a complete eradication of the NTDs relies on the development of effective human vaccines, as testified by the smallpox and polio examples. Unfortunately, to date, not a single NTD has an approved vaccine for human use (Hotez 2011). The reasons for this lack of success are not tied at the basic research level as numerous promising vaccine candidates emerged in the last years from experimental models (Hotez 2011). Rather, it is the cumbersome and expensive procedure required to convert a vaccine candidate into a safe and effective field product that hampers vaccine development for the neglected diseases (Bethony *et al.* 2011). Given the absence of a commercial market for vaccines against the NTDs, the best hopes rely on public-private partnerships, and some candidates are now entering the early stages of clinical development (Hotez 2013).

2. The biology of *Leishmania* protozoa

2.1. Morphology, life cycle, and cell biology

Leishmania is a genus of kinetoplastid eukaryotes whose life cycle relies on continuous shuttling between a mammalian host and an insect vector (**Figure 1**). These protozoa have a digenetic lifestyle, undergoing dramatic morphological changes to ensure adaptation and survival in either host. Within the gut of the sandfly vector, *Leishmania* endures as extracellular, flagellated and motile promastigotes. Conversely, in mammalian hosts, parasites thrive as non-motile, round and obligate intracellular amastigotes.

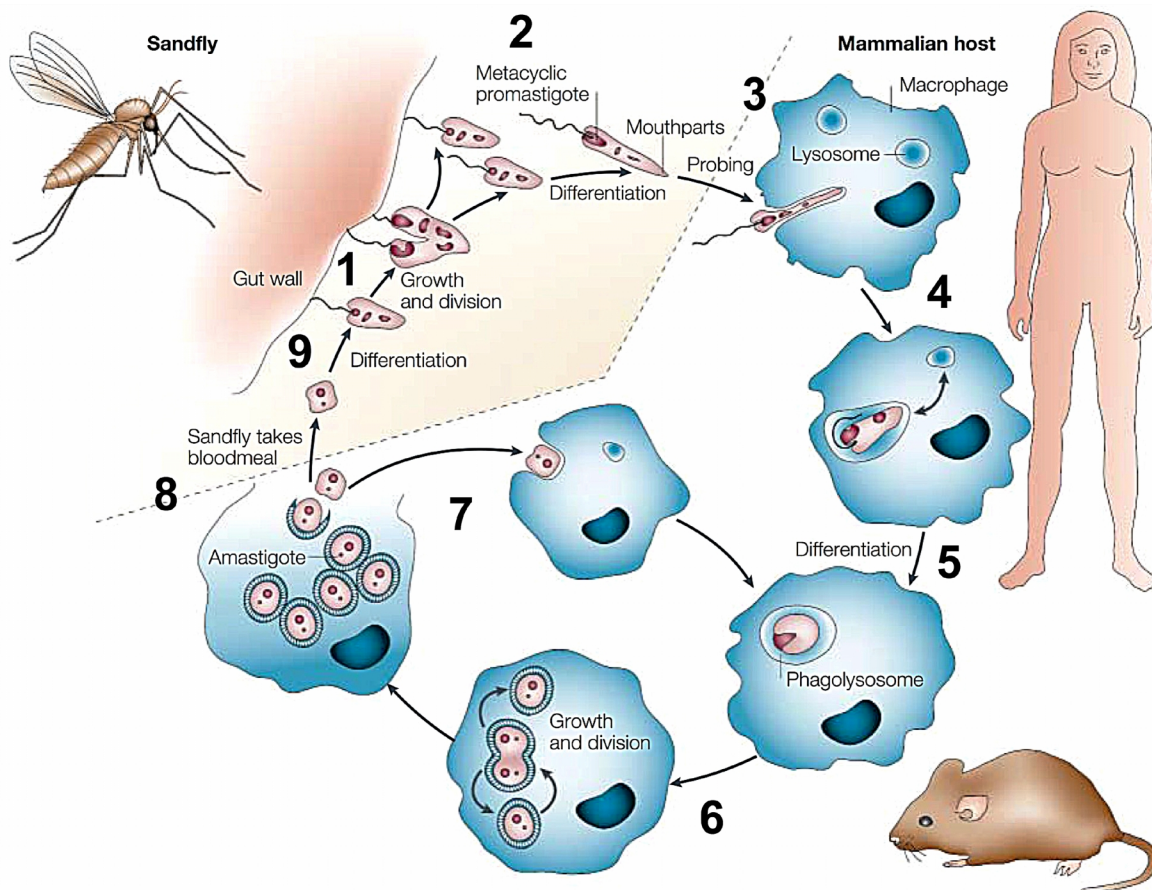


Figure 1. Life cycle of *Leishmania*. **1** Parasites grow in the midgut of a sandfly, as extracellular procyclic promastigotes. **2** Procyclics differentiate into metacyclic promastigotes, which do not divide and constitute the infective stage. **3** Metacyclics are transmitted to the skin of a mammal when a sandfly takes a blood-meal. **4** Promastigotes infect host phagocytes, residing in phagolysosomes. **5** The increased temperature and low pH trigger the differentiation into round, non-motile amastigotes. **6** Amastigotes are highly resistant forms and able to endure the harsh environment of the phagolysosome. **7** Amastigotes replicate and the cell eventually bursts, releasing parasites that infect new cells. **8** A sandfly feeding in an infected mammal transmits parasite to the vector. **9** The life cycle is completed when amastigotes revert back to the procyclic promastigote form. Adapted from (Sacks *et al.* 2002a).

Leishmania is a polarized cell defined by the presence of a flagellum at the anterior cell end. Whereas long in promastigotes, the flagellum is much shorter in amastigotes (**Figure 2**) (Landfear *et al.* 2001). The promastigote flagellum has a typical locomotive function, propelling the parasite forward through wave-like beats, but it also mediates parasite attachment to the gut of the sandfly. Its axoneme has the canonical “9+2” microtubule arrangement found throughout the *Eukarya* domain (Landfear *et al.* 2001). The central pair of microtubules is absent in amastigotes, and one or more of the peripheral pairs take a central position in the axoneme, in what is considered a 9v (variable) arrangement (Gluenz *et al.* 2010).

The flagellum inserts in the cell body at a deep invagination known as the flagellar pocket. The plasma membrane at the flagellar pocket is functionally specialized in the uptake of large nutrients, by receptor-mediated endocytosis, and it is also the site of protein secretion. Furthermore, newly-synthesized proteins destined for integration in the plasma membrane are sorted at the flagellar membrane (Field *et al.* 2009).

The most distinguishable morphological feature of *Leishmania* spp. is the presence of a single, large and tubular, mitochondrion containing a unique mass of mitochondrial DNA, known as the kinetoplast (Figure 2). The kinetoplast is composed of two forms of circular DNA; minicircles and maxicircles (Simpson 1986). There are thousands of minicircles per kinetoplast that range in length from 0.5 to 1 kilobase pairs (kbps) and are organized in complex catenated networks. A few dozen catenated maxicircles are present in the kinetoplast, with sizes ranging from 23 to 36 kbps (Simpson *et al.* 2000). The maxicircles encode genes required for mitochondrial function; however, the encoded information requires post-transcriptional editing either by insertion or deletion of uridine residues. Minicircles encode guide RNAs (gRNAs) that serve as complementary templates for the correct editing of the maxicircle-encoded genes (Simpson *et al.* 2000). This peculiar physical division of the mitochondrial genome is thought to be unique to the kinetoplastids and may provide potential drug targets (Lukes *et al.* 2002).

Kinetoplastids also contain glycosomes. These are membrane-enclosed organelles containing many enzymes involved in glycolysis and other metabolic pathways (Chandra *et al.* 2010). Finally, acidocalcisomes are unusual organelles present in kinetoplastids and other protists. These acidic, electron-dense structures function as intracellular reserves of several ions, including phosphorous, magnesium, calcium or zinc. They also play a role in osmoregulation and pH homeostasis (Moreno *et al.* 2009).

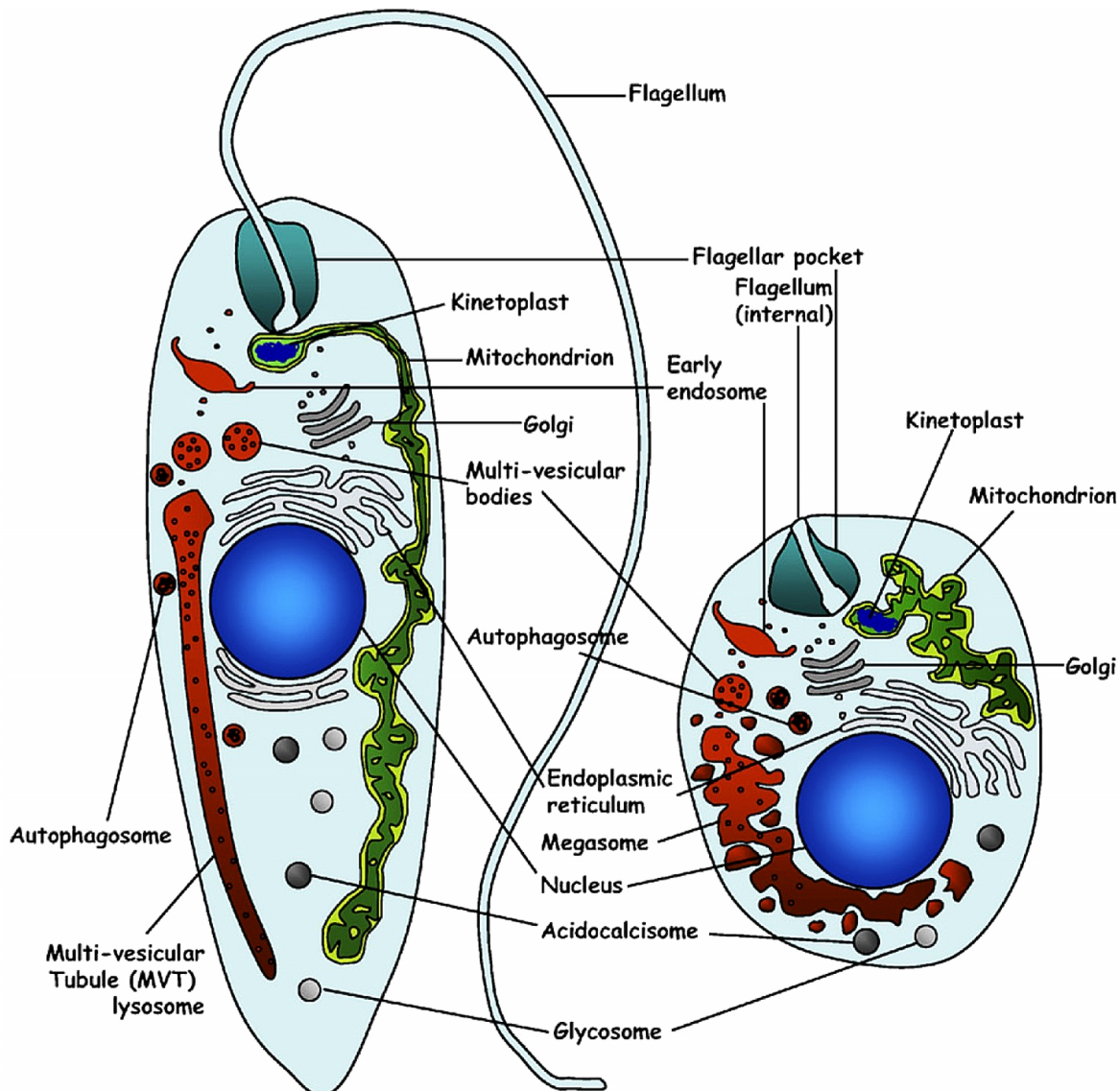


Figure 2. Illustrative representation of the ultrastructure of *Leishmania* promastigotes (left) and amastigotes (right). Adapted from (Besteiro *et al.* 2007).

2.2. Taxonomy and evolution

Modern phylogenetic analysis has helped to elucidate the taxonomy of the *Leishmania* genus (**Figure 3.**) (Cupolillo *et al.* 2000, Ponte-Sucre *et al.* 2013). These studies suggested an early branching of the genus in two major taxa; *Euleishmania* and *Paraleishmania*. The latter comprises several species that parasitize hystricomorphic rodents and sloths, and is collectively known as the *L. hertigi* complex. Some members of the *Leishmania* closely-related genus *Endotrypanum* are also placed within the *Paraleishmania* division.

The *Euleishmania* division encompasses all the species pathogenic to humans and is further subdivided into the *Viannia* (V.) and *Leishmania* (L.) subgenera. The latter contains

three species complexes with worldwide distribution, each predominantly associated with a clinical form of the disease (Ponte-Sucre *et al.* 2013). The *Viannia* subgenera is constituted by species of the *L. braziliensis* complex that cause cutaneous and mucocutaneous pathology in Americas (Stevens *et al.* 2001). Contrary to previous beliefs, the lizard-infecting group, known as *Sauroleishmania*, appears closely related with the *Leishmania* subgenera, suggesting that it may have derived from mammalian parasites (Croan *et al.* 1997). Recently-elaborated sequence-based phylogenetic trees support a Neotropical (South America) origin for the common ancestor of the *Leishmania* genus. Tough still a matter of dispute, it is speculated that parasites, carried either by hosts or vectors, arrived in Eurasia, by crossing Beringia (Ponte-Sucre *et al.* 2013).

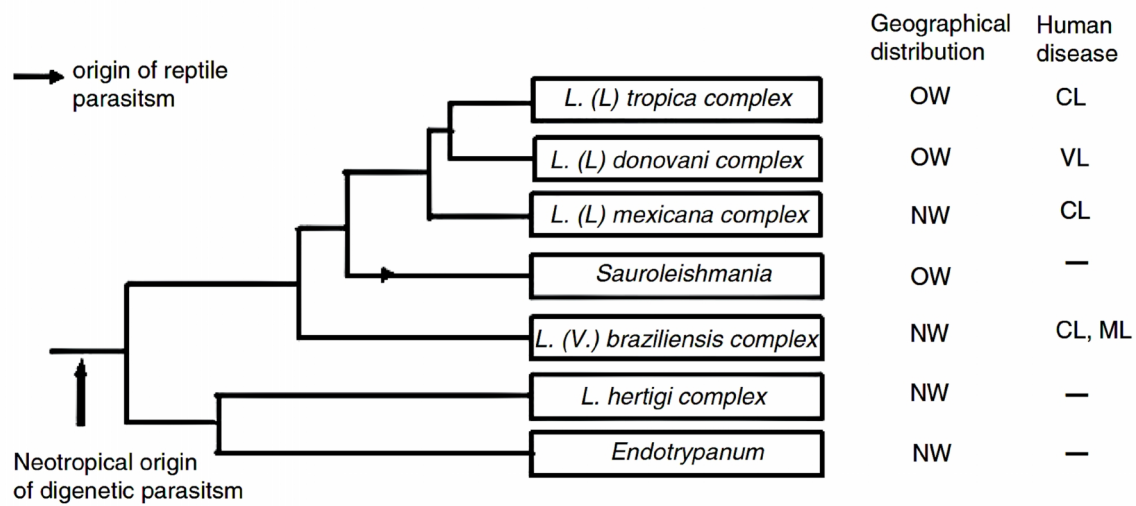


Figure 3. Phylogenetic tree depicting the evolution of the *Leishmania* genus as determined by DNA polymerase α sequence comparison. Alongside are shown general indications of the geographical distribution of each taxa (OW – Old World, NW – New World) and typical clinical manifestation (CL- Cutaneous leishmaniasis, VL- Visceral leishmaniasis, ML- Mucocutaneous leishmaniasis). Adapted from (Ponte-Sucre *et al.* 2013).

2.3. Development in the vector

Sandflies of the *Phlebotominae* sub-family (Order: *Diptera*; Family: *Psychodidae*) are the sole natural vectors of *Leishmania* spp., though vector requirement is bypassed in particular and rare circumstances such as needle sharing, transplacental or nosocomial transmission or laboratory accident (Murray *et al.* 2005).

Of the nearly 1000 phlebotomine species and subspecies known, about 70 are proven *Leishmania* vectors. In the Americas transmission is ensured by species of the *Lutzomyia* genus, whereas several *Phlebotomus* species serve as the propagating vector in the Old World (Killick-Kendrick 1999, Bates 2008). Sandflies are broadly classified into permissive

or specific vectors, according to their ability to allow the development of several or a single *Leishmania* species, respectively (Volf *et al.* 2007).

Only female sandflies are competent *Leishmania* vectors as they require periodic blood meals to obtain the essential nutrients for egg development (Myler *et al.* 2008). The complex development of the parasites in the vector initiates with the arrival of the amastigote-infected blood meal to the midgut. The engorgement of the gut caused by the bolus of blood induces epithelial cells to secrete a Type I peritrophic membrane (PM), composed of proteins and glycoproteins, held together by regularly-arranged chitinous microfibrils (Gemetchu 1974). Blood components and parasites become enveloped by the PM, which acts as a semipermeable barrier allowing the diffusion of digestive enzymes that breakdown the ingested contents (Secundino *et al.* 2005).

Within 6 to 12 hours post-feeding amastigotes differentiate to procyclic promastigotes due to decreased temperature and increased pH in the midgut as compared with the conditions in the mammalian host (Dostalova *et al.* 2012). Procyclic forms are small, ovoid and have short flagella, but are highly mitotic leading to a rapid increase in the parasite population. Procyclics express a surface glycoconjugate, termed proteophosphoglycan (PPG) that protects the parasites against proteolytic damage caused by digestive enzymes (Secundino *et al.* 2010).

Around two days after feeding, procyclics convert to the elongated and highly motile nectomonad form (Walters *et al.* 1989). Concomitantly, the PM starts to degrade, breaches and the partially-digested contents move posteriorly, towards the hindgut. Nectomonads escape from the PM, hence avoiding excretion with the blood remnants, and migrate anteriorly, to the stomodeal valve. Parasite migration is directed by chemotactic gradients, with sugars, taken by the flies in complementary sugar-meals, acting as attractive substances (Barros *et al.* 2006).

Nectomonads insert their flagella between the epithelial microvilli and attach to the thoracic midgut epithelia to effectively avoid defecation (Bates 2007). The promastigotes' surface glycolipid lipophosphoglycan (LPG) and lectins expressed by epithelial cells mediate parasite attachment to the thoracic midgut (Pimenta *et al.* 1994, Sacks *et al.* 1995). LPG is a highly polymorphic molecule and is a major determinant of the species-restricted vector competence (Bates 2007).

As the midgut empties, nectomonads differentiate into the shorter leptomonads that resume proliferation. Additionally, leptomonads produce a secretory form of the PPG that is the major constituent of a gel-like substance termed promastigote secretory gel (PSG). The continuous production of the PSG forms a packed matrix, known as the PSG plug, that extends throughout the thoracic midgut (Rogers *et al.* 2002). Some leptomonads also associate with the chitinous cuticle that lines the stomodeal valve and differentiate into

haptomonads. Attachment of the leaf-shaped haptomonads to the valve is mediated by foot-like enlargements at the tip of the flagella (Killick-Kendrick *et al.* 1974). Through the action of a secreted chitinase, haptomonads cause damage to the structure of the valve, corrupting its function (Rogers *et al.* 2008).

By day 5 after feeding, the gut is cleared of the blood meal, but the PSG plug occupies a substantial part of the anterior midgut. During production of the PSG, many leptomonads differentiate into metacyclic promastigotes that appear in large numbers behind the stomodeal valve (Rogers *et al.* 2002). These free and unencumbered forms constitute the final stage of the parasite in the sandfly, which, by this time is actively seeking another blood meal to initiate a new gonotrophic cycle.

The whole process of parasite development in the midgut that leads to accumulation of the mammal-infective metacyclic promastigotes is named metacyclogenesis (Sacks *et al.* 1984). Metacyclogenesis can be reproduced in culture flasks in the lab, which has allowed important studies on the biochemical changes occurring during the process (Sacks 1989). Metacyclics are smaller than nectomonads, and their flagella is longer than the cell body (Rogers *et al.* 2002). Furthermore, their lysosomal compartments have a lower pH and a higher lytic capacity, as compared to procyclics (Mullin *et al.* 2001). A functional autophagic pathway is critical for metacyclogenesis (Besteiro *et al.* 2006), presumably to allow survival in starvation conditions as nutrients are likely to be scarce at this stage (Bates 2008).

2.4. Transmission

Sandflies are telmophagic, meaning that they feed on a pool of blood that results from laceration of superficial vessels (Killick-Kendrick *et al.* 1981). The flies maintain the blood liquid during feeding by continuously secreting saliva and then ingest it through the proboscis. The blood reaches the pharyngeal chamber and, from there, arrives at the midgut by passing through the stomodeal valve (Rogers 2012).

The best available evidence currently suggests that the majority of the parasites transmitted to the mammalian host are regurgitated by the sandfly during blood feeding, in a hypothesis known as the “blocked fly” (Rogers *et al.* 2004, Rogers 2012). The two fundamental premises for the regurgitation theory are the existence of the PSG plug and the damage caused to the stomodeal valve by the parasite-secreted chitinase (Bates 2007). *Leishmania*-infected sandflies have long been known to behave anomalously while feeding. They bite more frequently than uninfected siblings, but take longer to feed and also have many incomplete blood meals (Shortt 1946, Killick-Kendrick 2013). When an infected fly attempts to feed, the presence of the PSG plug impedes the blood to enter the midgut. As

such, the fly must first regurgitate part of the plug to allow the passage of the blood. In this manner, the parasites embedded in the plug gain access to the mouth parts of the fly and eventually to the skin of the future host (Rogers *et al.* 2004). The damage to the stomodeal valve further contributes to this process, as the eroded valve remains open, promoting the “reflux” of the parasites from the midgut to the mouthparts (Rogers *et al.* 2008). The presence of the PSG plug and the occurrence of regurgitation have been documented in a large number of parasite/vector combinations (Rogers 2012) and appear the result of a sophisticated mechanism developed by the parasites to condition the sandfly for efficient transmission.

2.5. Establishment and survival in the mammalian host

Most sandflies deposit less than 1000 parasites in the skin of the mammalian host (Dostalova *et al.* 2012). Besides humans, mammals of several orders, either sylvatic or domestic, can serve as *Leishmania* hosts, including rodents, carnivores, edentates or marsupials (WHO 2010). An important distinction among hosts is whether they serve as reservoirs, helping to maintain the parasite population over time or are incidental hosts, not contributing to the ecological niche.

Once in the dermis, promastigotes remain relatively immotile until they are phagocytized by host cells (Beattie *et al.* 2011). The wounding of the microvasculature caused by the fly bite leads to prominent neutrophil recruitment from the blood and their swarming around the infection site (Peters *et al.* 2008). Neutrophils efficiently capture the parasite and become the predominant infected population during the first hours/days after the sandfly bite (Peters *et al.* 2008). Metacyclic promastigotes survive inside neutrophils, possibly by inhibiting the fusion of the phagolysosome with neutrophil granules that are necessary for acidification of the parasite-containing vacuole (Mollinedo *et al.* 2010). Apparently, parasites do not differentiate into the amastigote form inside neutrophils and, as the short-lived neutrophils become apoptotic, they are released again into the extracellular space (Peters *et al.* 2008). Alternatively, and according to the Trojan horse hypothesis, parasite-containing apoptotic neutrophils are phagocytized by macrophages hence allowing a silent entry of the parasite into the definitive host cell (**Figure 4**) (Laskay *et al.* 2003).

The role of neutrophils as temporary/intermediate parasite hosts has received ample experimental evidence (Peters *et al.* 2008, Ribeiro-Gomes *et al.* 2012a). Less clear is whether they help to contain the infection or instead serve as initial safe niches for parasite adaptation to subsequent infection of definitive host cells (**Figure 4**) (Ritter *et al.* 2009). Indeed, neutrophil-depletion experiments yielded conflicting results, depending on the

parasite or mouse strains employed, the experimental strategy employed to deplete neutrophils or the time of analysis (Ribeiro-Gomes *et al.* 2012b).

Following the neutrophil wave, a population of monocytes also extravasates from the blood and differentiates locally into macrophages and some dendritic cells (DCs) (Leon *et al.* 2007, Goncalves *et al.* 2011). As a consequence, by one week after inoculation, most parasites are found inside macrophages (Peters *et al.* 2008). Live cell imaging approaches shed light into the dynamic process of parasite uptake by macrophages (Lippuner *et al.* 2009, Forestier *et al.* 2011). Promastigotes initially interact with the macrophage via the flagellar tip and are engulfed in a polarized manner. After formation of the parasitophorous vacuole (PV) the parasite flagellum starts to beat rapidly, leading to reversal of the parasite orientation. The persistent flagellar oscillation also promotes migration of the phagolysosome to the cell periphery and causes host cell wounding. Retraction of the flagellum accompanies the differentiation to amastigote results in the final localization of the PV close to the nucleus and additional fusion with host lysosomes (Forestier *et al.* 2011). Once differentiated, the amastigote form is highly resistant to the harsh environment of the macrophage phagolysosome. As a result of continuous replication, amastigotes are eventually released and able to infect new cells and propagate the infection. A fraction of the invading parasites is instead internalized by DCs, which are critical for initiation of adaptive immunity (**Figure 4**) (Leon *et al.* 2007). The full scope of the interactions between the parasite and the mammalian host, including the mechanisms regulating immunity to *Leishmania* and the evasive strategies employed by the parasite to ensure persistence, are discussed in detail in sections 4 and 5 of this Introduction.

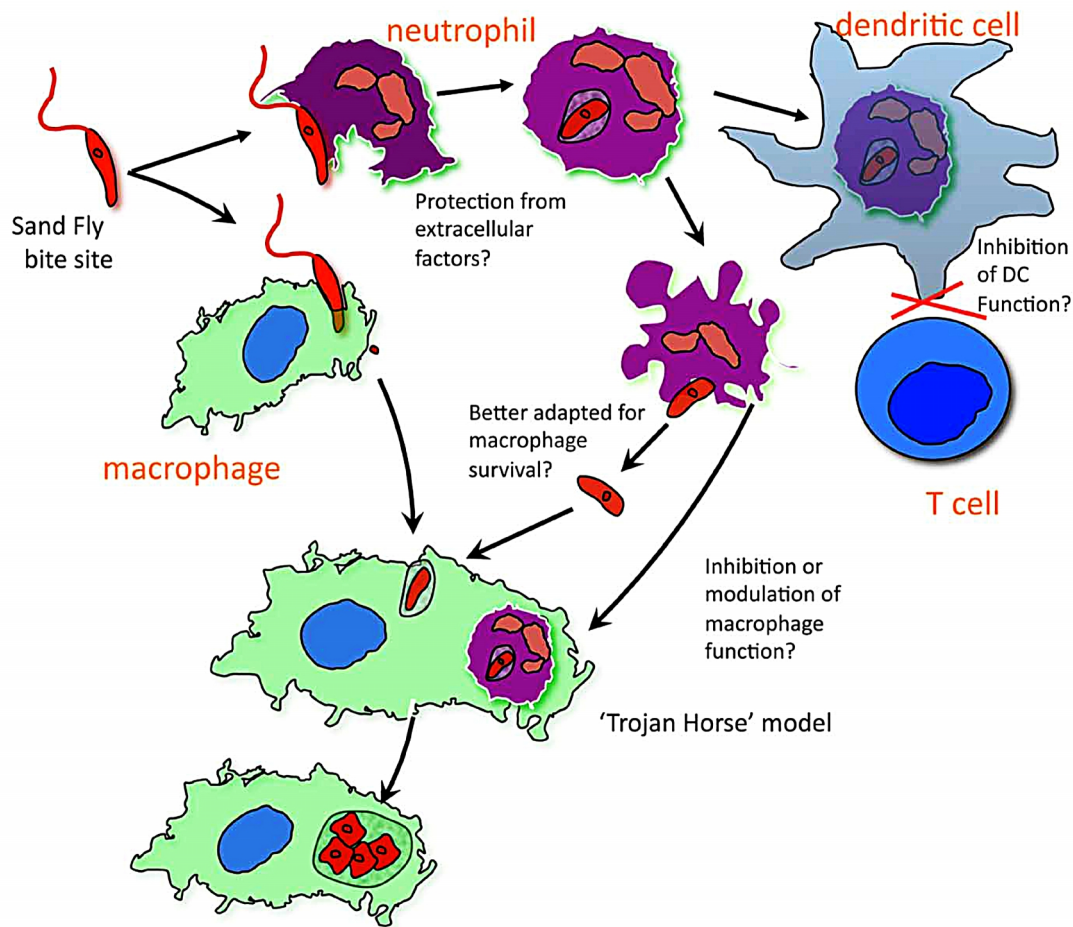


Figure 4. Establishment and survival of *Leishmania* in the skin of the mammalian host. The picture highlights the distinct cell populations that interact with the parasites at the early stages of infection. Certain open questions, concerning the impact of neutrophil infection to parasite fate in other cell types, are also shown. Adapted from (Ribeiro-Gomes *et al.* 2012b).

3. The leishmaniasis

Intimately linked with the ability to survive and proliferate inside the mammalian host, lies the propensity of *Leishmania* parasites to cause disease. More than 30 *Leishmania* species have been identified, of which about 20 are human pathogens. The leishmaniasis are broadly divided into three distinct medical conditions that involve either cutaneous, mucocutaneous or visceral pathology, with all forms considered neglected. A particular *Leishmania* species is typically associated with, but not limited to, a clinical form of leishmaniasis (Murray *et al.* 2005). Collectively, the leishmaniasis are prevalent in 98 countries distributed through five continents, accounting for 350 million people at risk of contracting the infection. About 700.000 new cases were reported annually in the past years. However, given that reporting is mandatory only in 33 of the 98 countries affected, the real burden is probably much higher and estimated at two million new cases per year (Alvar *et al.* 2012). These figures confer leishmaniasis the ninth largest disease burden among the infectious diseases. The estimated 50.000 annual deaths are only surpassed by malaria among the parasitic infections (WHO 2010, Alvar *et al.* 2012).

3.1. Cutaneous leishmaniasis

Several *Leishmania* species cause cutaneous leishmaniasis (CL). In the Old World, *L. tropica*, *L. major* and *L. aethiopica* predominate as the causative agents. In the Americas, CL can result from infection by species of both the *Leishmania* (ex. *L. amazonensis*), and *Viannia* (ex. *L. braziliensis*) subgenera (WHO 2010). Parasite species generally associated with visceral disease may also cause cutaneous lesions. For instance, *L. infantum* is the most frequent cause of CL in Europe (del Giudice *et al.* 1998). CL is mainly a zoonotic disease, though humans may serve as *L. tropica* reservoirs (WHO 2010). CL has worldwide distribution, being endemic in more than 70 countries (**Figure 5A**) (Reithinger *et al.* 2007b). Lesions are usually found on exposed areas of the skin. The typical disease course starts with the appearance of a small erythema at the site of sandfly bite, after a variable prepatent period. The erythema develops into a papule or a nodule that grows slowly, often taking several weeks to reach its final size. A crust appears centrally and eventually falls off, exposing the ulcerated lesion. Ulcers generally present raised and indurated margins and heal gradually over months or years, frequently leaving depressed scars (**Figure 5B**) (Reithinger *et al.* 2007b, WHO 2010).

The diversity of CL-causing species and the host status integrate to give rise to highly variable disease expression. For instance, the time required for lesions to self-heal ranges from 2-6 months for *L. major* to 2-5 years for *L. aethiopica*. While *L. tropica* infection produces dry ulcers, those caused by *L. major* are wet and severely inflamed. In contrast, *L. aethiopica* lesions do not ulcerate, but infection may spread locally, giving rise to disseminated cutaneous nodules in the face or limbs that resemble lepromatous leprosy (Reithinger *et al.* 2007b, WHO 2010).

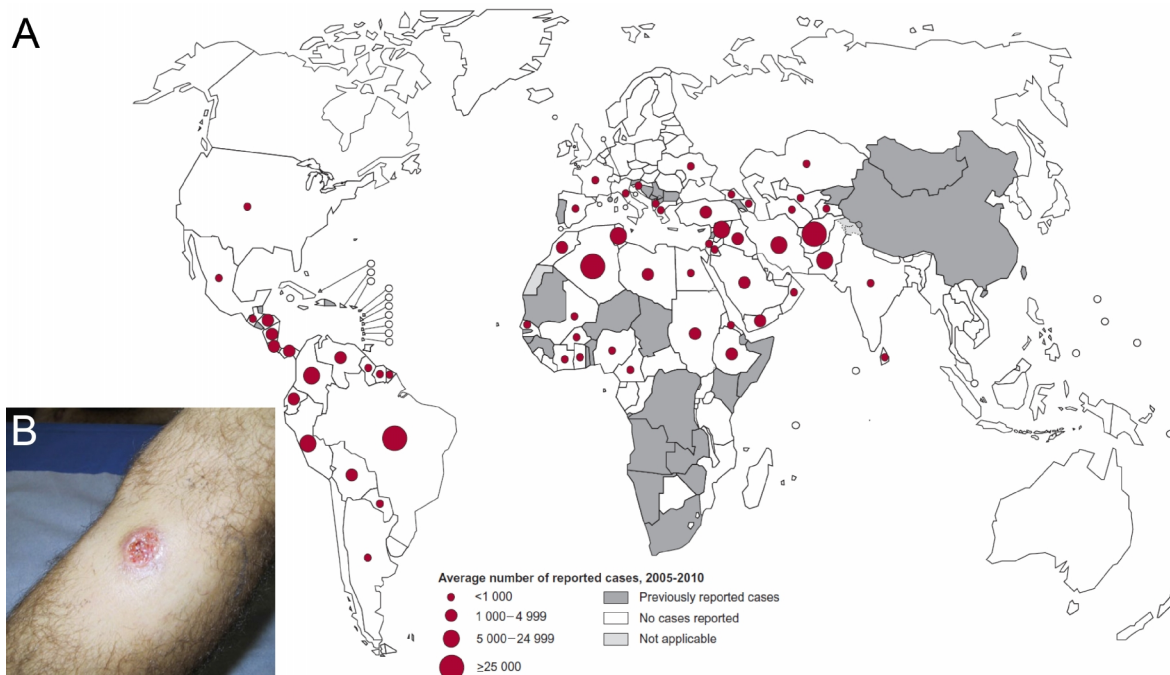


Figure 5. (A) Global distribution and average annual incidence of cutaneous leishmaniasis between 2005 and 2010. Obtained from (WHO 2013). (B) Cutaneous leishmaniasis due to *L. panamensis* infection in an Italian traveler. The raised and indurated edges of the ulcer confer it the typical “volcano-like” appearance. Adapted from (Antinori *et al.* 2012).

3.2. Mucocutaneous leishmaniasis

Mucocutaneous leishmaniasis (ML) is restricted to the Americas and caused by species of the *Viannia* subgenera, particularly *L. braziliensis*, *L. panamensis* and *L. guyanensis*. The majority of the cases occur in Bolivia, Brazil and Peru (Alvar *et al.* 2012), but epidemiological information is scarce. In Bolivia, which has the highest ML incidence, 4619 cases were reported between 1983 and 2006 (Tedesqui *et al.* 2012).

After resolution of a primary cutaneous lesion, ML-causing species are able to metastasize, either via blood or lymph, to the mucosal tissues around the mouth and the upper respiratory

tract. Mucocutaneous lesions appear in 5 to 10% of the patients, with a period of latency that ranges from a few months to more than 20 years (David *et al.* 2009, WHO 2010).

The symptomatology of ML starts with erythema and ulceration of the nares, followed by nodule appearance in the nasal septum and obstruction of the nostrils. Perforation and collapse of the septum occurs latter and leads to broadening of the nose. In many patients, other sites also appear involved including the pharynx, larynx and trachea. MCL rarely heals spontaneously and is potentially fatal, usually due to secondary bacterial infections (David *et al.* 2009, WHO 2010). The usual outcome of ML is facial disfigurement, which burdens the patient with lifelong social stigma; an aspect rarely taken into account in estimations of disease based on DALYs.

The extensive tissue destruction characteristic of ML is accompanied by massive immune cell infiltration and uncontrolled inflammation. Remarkably, relatively few parasites are present in the lesions (Ronet *et al.* 2011). These observations led to the view that ML results mainly from the genetic background of the host. In particular, polymorphisms in genes encoding for mediators of the inflammatory response, such as tumor necrosis factor (TNF), Interleukin-6 (IL-6) or chemokine C-C motif ligand-2 (CCL2) have been associated with MCL (Ronet *et al.* 2011). Nevertheless, recent evidence suggests that the hyper-inflammatory response in ML might be fostered by the presence of the *Leishmania* RNA Virus (LRV) (Ives *et al.* 2011). LRVs are double-stranded RNA viruses that parasitize some *Leishmania* species and were detected in many *Leishmania* (*Viannia*) isolates (Salinas *et al.* 1996, Zangger *et al.* 2013). While the occurrence of ML is primarily determined by the host genetics, the aggressiveness of the pathological inflammation might be tuned by parasite-derived factors, in this case, by the presence of a virus parasitizing the parasite (Hartley *et al.* 2012).

3.3. Visceral leishmaniasis

3.3.1. Epidemiology

Virtually all the mortality associated with the leishmaniases results from visceral disease. Due to the remote location of many VL endemic areas, the degree of under-reporting is severe. Conservative assumptions estimate the annual incidence at 400.000 cases, with about 50.000 associated deaths (Alvar *et al.* 2012). The primary VL endemic foci are located in the Indian sub-continent (India, Bangladesh and Nepal) and East Africa (Sudan, Ethiopia and Somalia), accounting for approximately 90% of all cases (Alvar *et al.* 2012). *L. donovani* causes VL in Asia and East Africa, while *L. infantum* accounts for visceral disease in the

Mediterranean basin and South America (**Figure 6**) (Murray *et al.* 2005). Dogs are the incriminated reservoirs in VL caused by *L. infantum* while *L. donovani*-mediated disease is mainly anthroponotic (Chappuis *et al.* 2007). Until recently, the age group most affected by VL caused by *L. infantum* were children of 1 to 4 years old. However, the emergence of HIV/ *L. infantum* co-infections has shifted the demographics of VL, with adults now representing nearly half of the cases in Europe. *L. donovani*, in contrast, appears capable of causing disease in people of all ages, though the highest incidence is found in children and young adults (WHO 2010).

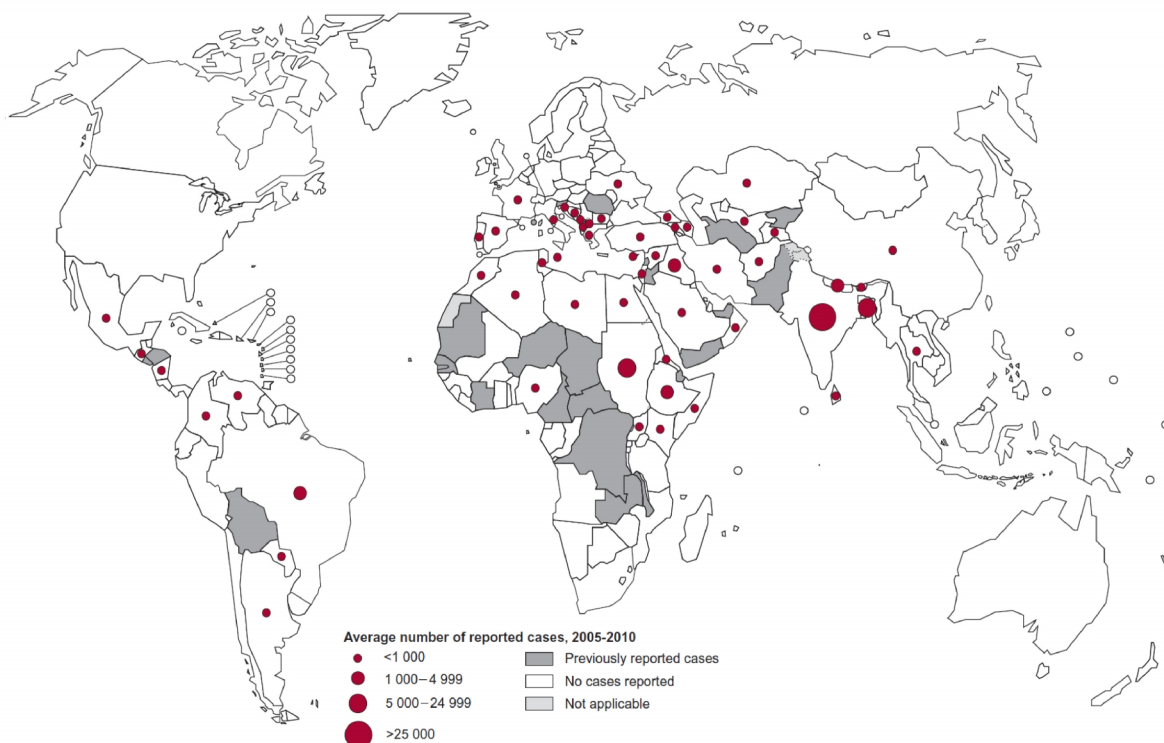


Figure 6. Global distribution and average annual incidence of visceral leishmaniasis between 2005 and 2010. Obtained from (WHO 2013).

3.3.2. Clinical features

The asymptomatic incubation period of VL is highly variable, ranging from a few weeks to one year or more. The onset of the disease is usually gradual, progressing over several months. Early symptoms include intermittent fever, malaise, weight loss, shivering and discomfort in the left hypochondrium. Overt disease manifests by striking splenomegaly, accompanied or not by hepatomegaly (**Figure 7A**). Lymphadenopathy is less frequent and occurs mainly in Sudan. The hyperplasia of the reticuloendothelial system is accompanied

by wasting and pallor of the mucous membranes (Murray *et al.* 2005, WHO 2010, van Griensven *et al.* 2012).

Mononuclear phagocytes in the spleen, liver, bone marrow and lymph nodes appear heavily parasitized, but lymphocyte infiltration is usually scarce. In the spleen, atrophy of the white pulp is common, with the loss of architectural organization of lymphoid structures. Plasma cells are abundant in the spleen, and probably associated with the occurrence of polyclonal hypergammaglobulinemia. Anemia, thrombocytopenia and neutropenia are frequent and reflect both splenic sequestration and suppression of bone marrow function. Complement activation further contributes to anemia. Immune complexes are present, sometimes associated with nephritis, proteinuria and microscopic hematuria. Liver function may be altered with moderate increases in liver enzymes commonly reported (Murray *et al.* 2005, Chappuis *et al.* 2007, WHO 2010, van Griensven *et al.* 2012).

Prothrombin depletion at advanced stages leads to severe mucosal hemorrhage. Intestinal parasitization results in diarrhea and enteritis. Jaundice and ascites also occur at advanced disease. Secondary infections, particularly tuberculosis and pneumonia, become common and are frequent causes of death (van Griensven *et al.* 2012).

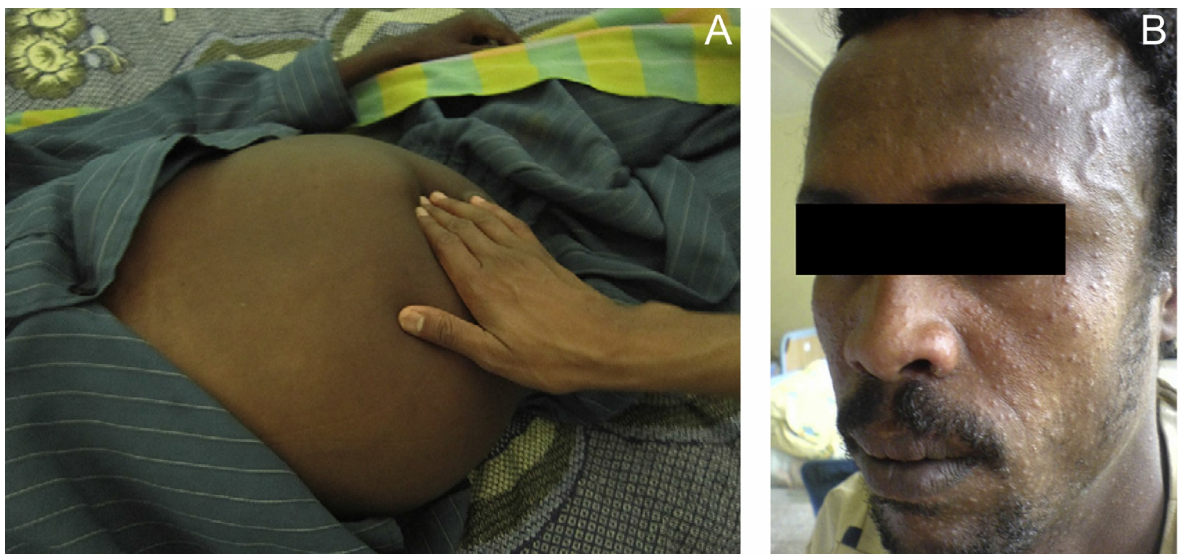


Figure 7. (A) A VL patient in Gondar (Ethiopia) manifesting prominent splenomegaly. (B) The characteristic facial rash of post kala-azar dermal leishmaniasis in a patient from Gondar. From (van Griensven *et al.* 2012)

In *L. donovani* endemic regions, a skin rash, termed post kala-azar dermal leishmaniasis (PKDL), may occur in 10 to 50% of cured patients (**Figure 7B**) (Singh *et al.* 2011). PKDL usually appears within six months to one year after VL resolution and is characterized by erythematous macules and papules that may evolve to nodules. The lesions may appear in any part of the body, but are especially frequent in the face, trunk and extremities. PKDL is apparently the consequence of the restoration of cell-mediated immunity after successful

drug treatment. As parasites can be detected in the lesions, PKDL may play a role in the maintenance of the parasite life cycle (WHO 2010, Singh *et al.* 2011).

3.3.3. Determinants for visceralization

The ability of some species to disseminate to visceral organs, while others remain confined to the skin has long been a fascinating research topic in leishmaniasis. It is not clear how parasites reach visceral organs. The sandfly may directly inject promastigotes into the bloodstream, which are subsequently conveyed to blood-filtering organs (Moreno *et al.* 2010). Alternatively, parasites may attain the viscera via infected cell migration, likely through the lymphatics (McCall *et al.* 2013). Some differences between visceral and cutaneous species have been reported with respect to the initial events occurring at the inoculation site. For example, the infected DC population in the dermis differs between *L. donovani* and *L. major* (Thalhofer *et al.* 2011), and infected cells leave the inoculation site in higher numbers after infection with a viscerotropic species (Zhang *et al.* 2003). These observations suggest that the early response in the skin may play a role in determining visceral disease. However, after intravenous injection, which bypasses the early events in the skin, cutaneous species have a limited ability to colonize the viscera (Zhang *et al.* 2001). As such, regardless of the initial interactions at the bite site, it is the ability to survive in visceral organs that defines the tropism of a *Leishmania* species.

The completion of the genome sequences of several *Leishmania* species allowed the identification of genes involved in visceralization (Peacock *et al.* 2007, Rogers *et al.* 2011). To date, only the A2 gene family has received convincing evidence to support a role as a factor required for visceral colonization. These proteins are present in both *L. donovani* and *L. infantum*, but are pseudogenes in several cutaneous species (Charest *et al.* 1994, Ghedin *et al.* 1997). Transgenic expression of A2 genes is sufficient to confer viscerotropism to a cutaneous species (Zhang *et al.* 2003). A2 gene expression is induced during amastigote differentiation and plays a role in the protection against oxidative stress and heat shock (McCall *et al.* 2012). Indeed, visceral species are able to withstand higher temperatures than cutaneous ones (Callahan *et al.* 1996); a property probably related with the fact that the temperature in the febrile spleen during VL may reach 40°C, hence much higher than the 28-32°C observed in cutaneous lesions (Scott 1985).

Additional parasite genes and gene families should also play roles in mediating parasite survival in the viscera. However, it is frequently hard to discern whether a particular gene is a visceralization-promoting factor or instead a general virulence factor (McCall *et al.* 2013). Furthermore, given the genomic plasticity of the kinetoplastids, many genes are present in

multiple copies and their graded expression may account for the differences in tissue tropism between cutaneous and visceral species (Rogers *et al.* 2011, McCall *et al.* 2013). An intriguing observation is the apparent switch from a visceral to a cutaneous parasite underwent by *L. donovani* when causing the PKDL sequelae of VL. It is interesting to note that the A2 genes are downregulated in *L. donovani* isolates from PKDL patients (Peacock *et al.* 2007). Nevertheless, additional parasite factors and, likely, increased host pressure should also influence the cutaneous localization of *L. donovani* in PKDL.

Future studies aiming to dissect the determinants for visceralization may take advantage of the sexual crossing between *Leishmania* species (Akopyants *et al.* 2009). With these approaches, hybrids of cutaneous and visceral species may be generated to allow mapping of genes or genomic regions that favor visceral tropism (McCall *et al.* 2013).

3.3.4. Asymptomatic infections and genetic control of visceral leishmaniasis

The majority of infected individuals in VL endemic areas do not manifest clinical disease, but instead remain asymptomatic carriers, which is indicative of a capacity to mount an effective immune response against the parasite (Blackwell *et al.* 2009). In India and Nepal, 8.9 asymptomatic infections occur per case of clinical disease (Ostyn *et al.* 2011), while the ratio is of 5.6 to 1 in Ethiopia (Ali *et al.* 1994). Identification of asymptomatic carriers is based on the absence of clinical disease combined with a positive test for parasite DNA or serological demonstration of anti-*Leishmania* antibodies (Singh *et al.* 2014). Management of these sub-clinical infections is of the highest importance, as it is theorized that asymptomatic carriers constitute a reservoir capable sustaining transmission (Stauch *et al.* 2011, Singh *et al.* 2014).

Epidemiological studies have identified some of environmental factors associated with progression to clinical disease. Malnutrition, for instance, has long been known as a factor promoting disease severity (Cerf *et al.* 1987), due to its deleterious effects on the capacity of the immune system to control the parasite (Dye *et al.* 1993, Carrillo *et al.* 2014). Animal ownership has also been proposed as a risk factor for leishmaniasis, though conflicting results were obtained in distinct studies (Singh *et al.* 2014).

The fact that individuals differ markedly in their progression to clinical disease indicates that the susceptibility to VL is, at least partially, under genetic control (Blackwell *et al.* 2009). Candidate gene association studies identified a number of genes linked with susceptibility to VL (Blackwell *et al.* 2009). Of particular interest is the *SL11A1* gene which encodes for a phagosomal proton-coupled divalent metal transporter named Natural Resistance-Associated Macrophage Protein 1 (NRAMP1) that limits iron availability to the parasite

(Blackwell *et al.* 2001). Polymorphisms at *SLC11A1* were linked with susceptibility to human VL in Sudan (Bucheton *et al.* 2003, Mohamed *et al.* 2004), but no association was found in India (Mehrotra *et al.* 2011b). Additional gene candidate studies found important associations with symptomatic disease for polymorphisms in a number of cytokines, chemokines and their receptors, including TNF, the IL-2 receptor beta (IL-2R β), transforming growth factor- β (TGF- β) or C-X-C chemokine Receptor type 2 (CXCR2) (Karplus *et al.* 2002, Bucheton *et al.* 2007, Frade *et al.* 2011, Mehrotra *et al.* 2011a). Unfortunately, most of these studies were underpowered due to limited sample size, which precludes definitive conclusions (Blackwell *et al.* 2009).

A recent genome-wide association study employed large cohorts of both Indian and Brazilian populations to identify susceptible loci for VL. The study mapped a single genomic region, corresponding to the *HLA-DRB1-HLA-DQA1* segment of the major histocompatibility complex (MHC) class II region, as showing strong association with VL in both populations (LeishGEN Consortium *et al.* 2013). This study demonstrates that genetic risk factors for VL are shared across different parasite species and geographically-separated endemic regions.

3.3.5. *Leishmania* / HIV co-infection

The AIDS pandemic has changed the natural course of the leishmaniasis, both at the epidemiological and clinical levels. Since the mid-1980s, cases of *Leishmania* / HIV co-infection have been reported in 35 countries distributed across all the major endemic foci of leishmaniasis. All the clinical manifestations of leishmaniasis have been found among HIV patients; nevertheless, VL prevails in most endemic areas (Alvar *et al.* 2008).

In HIV-infected individuals *Leishmania* acts as an opportunistic parasite taking advantage of the suppressed state of the host, particularly when peripheral CD4 T cell counts fall below 200 per μ L (Alvar *et al.* 2008). It is estimated that HIV infection increases the risk of developing VL by 100 to 2320 times (Pintado *et al.* 2001). The widespread use of highly active anti-retroviral therapy (HAART) has contributed to a steady decrease in the number of *Leishmania* / HIV co-infections in Southern Europe (Lopez-Velez 2003, Russo *et al.* 2003). In sub-Saharan Africa, where access to HAART therapy was limited in some regions until very recently, the incidence of co-infection remains high in co-endemic areas (Alvar *et al.* 2008).

At the cellular level, *Leishmania* and HIV appear to cooperate in a synergy that is highly deleterious for the host. Parasite infection of the macrophage results in the production of pro-inflammatory cytokines, such as IL-1 β or TNF. These soluble factors induce immune

activation that fuels viral replication (Bentwich 2003, Zhao *et al.* 2004). Conversely, the marked immunosuppression that characterizes HIV infection impairs the control of intracellular parasite proliferation (Wolday *et al.* 1998, Zhao *et al.* 2006).

At the clinical level, the synergy between parasite and virus results in unusual disease manifestations. In VL co-infected patients, it is common to find parasites in otherwise atypical locations such as the gastrointestinal tract, lungs or pleural space (Alvar *et al.* 2008, WHO 2010). Due to severe immunosuppression, many co-infected individuals are unable to produce parasite-specific antibodies, rendering serological diagnosis inappropriate and prone to false negatives (Desjeux *et al.* 2003). Furthermore, the therapeutic response to routine anti-*Leishmania* drugs is frequently reduced in a co-infection context (Rosenthal *et al.* 1995, Pintado *et al.* 2001). *Leishmania* / HIV co-infection leads to aggravated clinical states, thus meriting particular attention from health authorities. In areas of co-endemicity, a coordinated approach involving both HIV and VL programs should produce the best results (Alvar *et al.* 2008).

3.4. Control of the leishmaniasis

In 2007, the World Health Assembly defined an ambitious plan to reduce the burden associated with the leishmaniasis drastically in the following years (WHO 2013). By 2020, it is expected to identify and treat all cases of VL in the endemic regions of Africa, Europe and Americas and to reduce the prevalence below 1/10.000 in the endemic districts of the Indian sub-continent. The plan further aims to manage 85% of all cases of cutaneous disease (WHO 2013). Fulfilling these goals will require active search for patients in remote villages of endemic areas (Matlashewski *et al.* 2011). Specific and sensitive diagnostic methods are necessary to identify these patients as well as to manage asymptomatic carriers. Control of vectors is important to block transmission. Furthermore, in areas of zoonotic disease, reservoir control should be taken in consideration. Importantly, the plan depends on affordable drugs. The WHO has already negotiated special prices for several anti-*Leishmania* drugs for low-income countries (WHO 2010). Ultimately, eradication of the leishmaniasis relies on the development of effective human vaccines. Given its deadly and poverty-promoting features, control of the leishmaniasis should be a high priority by policy makers of public health worldwide (Matlashewski *et al.* 2011).

3.4.1. Diagnosis

An accurate diagnosis is an essential premise to attain the leishmaniasis elimination goal. However, diagnosis of the leishmaniases is often complicated due to the lack of pathognomonic signs of the disease. Indeed, the clinical features of VL are easily mistaken with other co-endemic diseases, such as malaria, brucellosis or African trypanosomiasis (Srividya *et al.* 2012, Ejazi *et al.* 2013). Cutaneous *Leishmania* lesions may be confounded with many skin disorders (Reithinger *et al.* 2007b). As such, diagnostic tests are required to complement the clinical evaluation. Ideally, these tests should be fast, simple and affordable to allow field implementation (Kobets *et al.* 2012).

3.4.1.1. Parasitological diagnosis

Although a century-old, parasitological demonstration remains the gold standard for leishmaniasis diagnosis and is the classical confirmatory test for all forms of the disease. Direct demonstration of amastigotes can be made from biopsies or aspirates from the spleen, bone marrow, lymph node or skin (Kobets *et al.* 2012, Srividya *et al.* 2012). Samples are stained with routine colorations (Hematoxylin / Eosin, Giemsa, etc.) and examined for the presence of intracellular amastigotes. Biopsies may give additional information concerning the infiltration of immune cells, formation of granulomas or other pathological occurrences (Saleem *et al.* 2007). Parasitological methods are highly specific, though sensitivity varies with the tissue used. In VL, splenic aspirates provide the most reliable results; however, the risk of internal bleeding discourages its use in facilities lacking blood transfusion services (WHO 2010). Furthermore, microscopic demonstration of the parasite is time-consuming and requires technical expertise.

Culture of parasites recovered from infected tissue may provide information on the burden of the infection, through the use of the limiting-dilution assay (Lima *et al.* 1997). Parasite culture also provides starting material for downstream methods, such as multilocus enzyme electrophoresis (MLEE), that allow detailed molecular and biochemical characterization of the isolate (Mauricio *et al.* 2006). However, culture is not suited for field application as it is laborious, time-consuming and requires expensive lab equipment (Srividya *et al.* 2012). Recent developments in micro-culture methods employing capillary tubes reduced the time necessary for parasite recovery while retaining high sensitivity for the detection of promastigotes (Allahverdiyev *et al.* 2005, Boggild *et al.* 2008).

3.4.1.2. Immune-based diagnosis

Methods based on the detection of antibodies against the parasite are widely used for the diagnostic of VL. The direct agglutination test (DAT) has been routinely employed in some endemic areas in the past two decades and is based on the crosslink of killed parasites when in contact with antibodies from the patient's serum (Harith *et al.* 1986, Harith *et al.* 1987). The DAT can be adapted to microtiter plates to give semi-quantitative information and has demonstrated high sensitivity and specificity (Jacquet *et al.* 2006). A simplified version of the DAT, termed the fast agglutination screening test (FAST), uses a single serum dilution to provide results in a few hours with comparable accuracy (Schallig *et al.* 2002). A number of *Leishmania* antigens induce the production of specific antibodies, hence having diagnostic potential (Kobets *et al.* 2012). Among these, the presence of serum antibodies raised against the kinesin-related antigen rK39 has proven to be a faithful marker of VL (Burns *et al.* 1993, Chappuis *et al.* 2006). A dipstick-based immune-chromatographic qualitative test for rK39 has been developed and requires only a few drops of blood and 10 to 20 minutes to give a definitive result (Chappuis *et al.* 2007). The rK39 test is currently the best diagnostic tool for VL in endemic rural areas and is an integral part of the VL elimination program in the Indian sub-continent (Chappuis *et al.* 2007, WHO 2010). The fact that anti-*Leishmania* antibodies persist in the serum after drug cure is a major limitation of serological diagnosis, as these tests become unable to distinguish between present and past infections (De Almeida Silva *et al.* 2006). Also, the heterogeneity observed in the immune response during VL suggests that the development of a serodiagnostic test based on an array of parasite antigens may prove superior to the mono-specific tests currently employed (Srividya *et al.* 2012).

Direct detection of parasite antigen in body fluids should be advantageous over serological tests as it avoids cross-reactivity and can distinguish between present and past infections. A latex agglutination test (KAtex) detecting a parasite carbohydrate antigen in the urine has been developed (Hommel *et al.* 2001). The test demonstrates high sensitivity but is prone to false positives (Sundar *et al.* 2005), and work to improve its specificity and reproducibility is ongoing (Chappuis *et al.* 2007, Hatam *et al.* 2009).

A different type of immunological diagnosis is based on the development of cell-mediated immunity against *Leishmania*. In 1926, Montenegro developed the leishmanin skin test, which consists in an intradermal injection of parasite antigen followed by measurement of the skin induration that results from delayed type hypersensitivity (Montenegro 1926). A positive leishmanin skin test is an indicator of previous exposure to the parasite, asymptomatic infection or successful cure (Sassi *et al.* 1999, Sadeghian *et al.* 2013). In contrast, patients with active VL are generally negative for the leishmanin skin test, which

is reminiscent of the anergic state of their immune system (Gidwani *et al.* 2009). Nevertheless, the test has significant limitations associated with poor specificity and sensitivity (Sadeghian *et al.* 2006).

3.4.1.3. Nucleic acid-based tests

Diagnosis based on methods that detect parasite DNA, through the use of the polymerase chain reaction (PCR), have received ample interest in the past years (Reithinger *et al.* 2007a). These methods can be applied to a wide variety of organs, tissues or body fluids (Srividya *et al.* 2012). Additionally, PCR-based methods are highly specific and sensitive and consistently prove superior to microscopy, particularly in samples with few parasites (Garcia *et al.* 2005). Through the use of quantitative PCR (qPCR), is it possible to monitor disease progression (Nicolas *et al.* 2002). Additionally, PCR can easily be coupled to downstream methods such as restriction fragment length polymorphism (RFLP) or sequencing. These allow species' identification for epidemiological studies and may also provide complementary information for treatment strategy decisions (Cupolillo *et al.* 1995, Oshaghi *et al.* 2009). Numerous multi-copy parasite sequences have been tested for the diagnosis of the leishmaniasis (Srividya *et al.* 2012). Among these, the conserved region of the minicircle kinetoplast DNA provides the most sensitive PCR approach for *Leishmania* detection and quantification (Lachaud *et al.* 2002, Bensoussan *et al.* 2006). Additional reliable target sequences include several ribosomal genes (Lachaud *et al.* 2000), the internal transcribed spacer (Mauricio *et al.* 2004) or the glycoprotein-63 (gp63) (Quispe Tintaya *et al.* 2004). The major drawbacks for a widespread implementation of PCR-based methods in the field are the technical complexity of the procedure and the requirements for expensive equipment. These issues are being tackled by recent efforts aimed at simplifying the technique (Srividya *et al.* 2012). A *Leishmania*-specific oligo-chromatographic PCR test (OligoC-Test) was developed and is based on PCR amplification of the 18S RNA gene followed by detection of the PCR product in a dipstick format by hybridization with a gold-conjugated probe (Deborggraeve *et al.* 2008).

3.4.2. Chemotherapy

The current drug arsenal to treat the leishmaniasis is relatively short, when compared, for instance, with malaria (Kar *et al.* 2010, Singh *et al.* 2012b). Even though the available drugs are associated with adverse effects (Sundar *et al.* 2006a), they represent, in the absence

of a human vaccine, the major weapon to tackle *Leishmania* infections (Matlashewski *et al.* 2011).

3.4.2.1. Available anti-*Leishmania* medicines

Pentavalent Antimonials

In 1921, mainly inspired by the work of Paul Ehrlich, Brahmachari synthesized the first organic pentavalent antimonial, urea stibamine (Brahmachari 1940, Marsden 1986). The compound demonstrated high leishmanicidal activity and saved countless lives in the region of the Brahmaputra valley in India. Soon after, urea stibamine was replaced by safer pentavalent antimonials that remain the first choice for the treatment of leishmaniasis in many endemic areas (Singh *et al.* 2004, Haldar *et al.* 2011). Sodium stibogluconate and meglumine antimoniate are the two antimonial formulations marketed nowadays. Intravenous daily injection for one month is the standard regimen for VL (Sundar *et al.* 2013). Antimonials are associated with a myriad of adverse effects that, at times, may be life-threatening including several cardiac dysrhythmias, hepatitis, nephrotoxicity and pancreatitis (Kobets *et al.* 2012).

The mechanism of action of pentavalent antimonials remains poorly understood (Haldar *et al.* 2011). Some studies propose that these compounds are prodrugs that require reduction to trivalent forms to gain leishmanicidal activity (Shaked-Mishan *et al.* 2001). Trivalent forms are thought to disrupt the oxidoreductive balance inside the parasite by targeting trypanothione reductase (Wyllie *et al.* 2004). Nevertheless, pentavalent forms showed intrinsic anti-leishmanial activity; for instance, by targeting the DNA topoisomerase (Chakraborty *et al.* 1988). Antimonials also impact on the parasite's energy metabolism by inhibiting glycolysis and β -oxidation, though the specific targets have not been identified (Berman *et al.* 1987).

Antimonials became obsolete in the Indian region of Bihar due to widespread resistance resulting from drug misuse (Sundar *et al.* 2001). Resistance mechanisms are multi-factorial and include expression of γ -glutamylcysteine that counteracts the oxidative stress posed by antimonials or decreased drug uptake (Carter *et al.* 2006).

Amphotericin B

In Indian regions where VL is refractory to antimonials, amphotericin B is the agent of choice. This polyene antimycotic targets ergosterol on the parasites' plasma membrane, creating aqueous pores that lead to cell depolarization (Sundar *et al.* 2006b). The cytotoxic effect of amphotericin B has also been linked to the production of free radicals (Lamy-

Freund *et al.* 1985). Conventional amphotericin B is formulated with deoxycholate and is associated with many adverse effects, including hypokalemia, nephrotoxicity or myocarditis. To minimize side reactions the drug has to be administered by slow infusion, hence requiring hospitalization and close monitoring that escalates the cost of the therapy (Sundar *et al.* 2013).

The development of newer lipid formulations improved the therapeutic window of amphotericin B due to preferential uptake by phagocytes, hence concentrating the drug in infected organs where it remains for long periods. Particularly, the liposomal formulation of amphotericin B (AmBisome®) is considered the best existing medicine to treat VL (Chappuis *et al.* 2007).

Until recently, a major obstacle for the widespread use of AmBisome was its prohibitive price. Following efforts by the WHO, the drug is now sold at a particular price for low-income countries with the estimated regimen for one patient reduced to 200\$ (Chappuis *et al.* 2007, Sundar *et al.* 2013). While advantageous, the cost is still considered elevated. Some calculations indicate that the price should be further halved to make its cost-effectiveness comparable to other drugs in India (Meheus *et al.* 2010). For sub-Saharan nations, the ideal scenario would be compassionate donation of AmBisome for use in endemic areas (den Boer *et al.* 2011).

Though still rare, resistance to amphotericin B has been reported in a few foci (Srivastava *et al.* 2011, Purkait *et al.* 2012) and appears to result from the substitution of ergosterol in the membrane by one of its precursors in the biosynthetic pathway (Purkait *et al.* 2012).

Miltefosine

Introduced in India in 2002, the phosphatidylcholine (PC) analogue miltefosine is the first orally-active antileishmanial drug (Sundar *et al.* 2013). The mechanism of action of miltefosine is not fully understood and appears to be manifold. Miltefosine halts intracellular ATP production by inhibiting cytochrome c oxidase (Luque-Ortega *et al.* 2007). Additionally, it blocks the biosynthesis of alkyl anchored molecules such as LPG and gp63 by inhibiting the alkyl-specific acyl coenzyme A acyltransferase (Lux *et al.* 2000). The main advantage of miltefosine is its oral availability, which facilitates administration. The standard regimen for VL implies one dose per day for 28 days, which makes adherence challenging. Some side-effects have been reported after miltefosine use, the most important of which is its teratogenicity, aggravated by drug's long half-life in the organism (WHO 2010). Initial phase IV studies conducted in India revealed a cure rate of 95% for VL (Bhattacharya *et al.* 2007). However, recent data suggests that the efficacy of miltefosine is already decreasing (Sundar *et al.* 2012), but it is not yet clear whether relapses occurred due to resistance, re-infection or noncompliance (Rijal *et al.* 2013). While considerably more affordable than

AmBisome, the cost of miltefosine has nevertheless hampered its choice over antimonials for VL in many endemic areas (den Boer *et al.* 2011).

Paromomycin

The aminoglycoside antibiotic paromomycin was the latest addition to the antileishmanial drug arsenal, following a phase III trial demonstrating cure rates of 95% for the treatment of VL (Sundar *et al.* 2007). Paromomycin is internalized by the parasite following interactions with the glycocalyx (Chawla *et al.* 2011), and it affects a range of biological processes including mitochondrial respiration, protein synthesis, membrane composition and fluidity, though the exact molecular targets are yet to be identified (Maarouf *et al.* 1997). The standard treatment course for VL involves daily intramuscular injection for three weeks, which frequently causes pain at the injection site. Reversible ototoxicity was also reported and some patients exhibited elevated transaminases during the period of treatment (Sundar *et al.* 2013). Paromomycin is the cheapest drug for VL, with the typical regimen costing about 10\$ per patient (Sundar *et al.* 2013). Its low cost makes paromomycin attractive for the treatment of CL. Topical formulations have been developed and demonstrated varying results compared to intralesional injection of antimonials, depending either on the region or the parasite species (Ben Salah *et al.* 2013, Sundar *et al.* 2013). As an aminoglycoside, resistance to paromomycin is likely to occur, though not yet reported in the field (Mingeot-Leclercq *et al.* 1999, Bhandari *et al.* 2014).

3.4.2.2. Drug development for the leishmaniases

All the major antileishmanial agents are associated with at least one drawback, including toxic effects, high cost, resistance, inadequate route of administration or treatment duration (Freitas-Junior *et al.* 2012). Thus, efforts to develop new drugs should continue. Due to the lack of commercial incentive, private companies are unlikely to support the development of new drugs on their own. As such, the discovery and development of new antileishmanial agents rely on public-private partnerships. A common strategy is to test the leishmanicidal potential of drugs already on the market for different therapeutic purposes. This approach substantially decreases the time and cost necessary to meet the regulatory requirements for drug approval as most of the toxicological data is already available (Nwaka *et al.* 2009). Amphotericin B, miltefosine and more recently paromomycin are successful examples of the application of this strategy to VL. Nevertheless, an approach based on drug repurposing is limiting and only takes into account a fraction of the chemical space (Nwaka *et al.* 2009).

The process of *de novo* drug discovery against leishmaniasis may follow a phenotype-based approach, which does not depend on a particular molecular target, but instead relies on systematic screening of compound libraries for their leishmanicidal activity (Freitas-Junior *et al.* 2012). Ideally, candidates should be screened against intracellular amastigotes as drug activity against axenic parasites rarely translates to the relevant physiological form (Freitas-Junior *et al.* 2012). Screening methodologies include the classical microscopic or biochemical methods, such as counting of intracellular amastigotes after Giemsa staining or the MTT assay. Other approaches employ transgenic parasites expressing reporter genes (Sereno *et al.* 2007).

Drug discovery based on the target is more cumbersome as it requires detailed knowledge on the biochemical pathways of the parasite, a task partially facilitated by the completion of the genome sequences of several *Leishmania* spp. (Ivens *et al.* 2005, Peacock *et al.* 2007, Real *et al.* 2013). Genetic invalidation of the target is required to demonstrate its essentiality for the parasite (Croft *et al.* 2003). Design of potential inhibitors requires detailed knowledge of the target structure, usually obtained from crystal structures (Blundell *et al.* 2002).

Both phenotype- or target-based approaches generate lead compounds that progress to animal testing for *in vivo* efficacy, assessment of toxic effects and determination of pharmacodynamic profiles (Nwaka *et al.* 2009). Whereas more complex and laborious, target-based drug discovery is also a more controlled process (Werbovetz 2000). Careful target choice also minimizes the occurrence of resistance in the field (Gilbert *et al.* 2011).

3.4.3. Preventive measures for leishmaniasis

Epidemiological trends demonstrate that the incidence of the leishmaniasis is not decreasing, despite the existence of better drugs and improved diagnosis (Desjeux 2004). A number of risk factors have been described in the last years that account for the observed increase in infected individuals in some endemic areas (Desjeux 2001, Alvar *et al.* 2006). These are mainly related with migratory movements from rural to urban areas in sub-Saharan Africa and Latin America countries. The continuous expansion of poorly-sanitized suburbs leads to the occupation of forest zones of *Leishmania* transmission by urban settlements (Ashford 2000, Desjeux 2001).

Preventive measures for leishmaniasis can be directed at human beings, vectors or, in areas of zoonotic disease, reservoirs (Stockdale *et al.* 2013). Vector control aims to reduce or interrupt transmission; particularly in domestic or peridomestic habitats (WHO 2010). Typical interventions for vector control include the use of insecticides in houses, destruction the sandfly breeding habitats or simply plastering the cracks in the walls, to prevent the

sandflies from laying eggs inside houses (Sharma *et al.* 2008, Stockdale *et al.* 2013). Reservoir control can range from compulsory elimination of infected animals, which demonstrated limited efficacy (Costa 2011), to the use of insecticide-impregnated collars (Gavani *et al.* 2002), or the development of vaccines for the animal reservoir (Palatnik-de-Sousa *et al.* 2009). Finally, preventive measures targeted at the human being include the use of bed-nets impregnated with insecticides (Ritmeijer *et al.* 2007) and the ultimate goal of developing a prophylactic vaccine for human use (Kedzierski 2010). A recent systematic review highlighted some important problems in assessing the benefit of preventive interventions (Stockdale *et al.* 2013). Most reports do not measure the impact of the intervention on human infection, which is the outcome of interest. Furthermore, those who do so, frequently have reduced sample sizes. As such, it is risky to generalize measures that proven effective in a single study and further studies are required, particularly those that measure the impact of a given intervention on the incidence of human infection (Stockdale *et al.* 2013).

4. *Leishmania* interactions with the host cell

The developmental program of *Leishmania* spp. in its vertebrate host is relatively simple, if compared with other parasitic protozoa, such as the *Apicomplexa*. Following establishment in the host, the parasite has only one developmental stage throughout its stay. Additionally, it infects and resides specifically within phagocytes, without undergoing complex processes such as gametogenesis. However, this apparently simple biology underlies an intricate host-parasite co-existence. Given its low transmissibility, *Leishmania* had to develop strategies to prolong survival and amplify the parasite population in the host, thus maximizing the chances of being transmitted back to the vector (Yazdanbakhsh *et al.* 2010). The host, on the other hand, counteracts parasite dissemination through sophisticated immune mechanisms that allow parasite detection, tracking and destruction (Kaye *et al.* 2011a). This “arms race” between parasite and host has consequences at several levels. In an infected cell, the parasite ensures survival by deactivating the leishmanicidal functions of the host cell as well as its ability to “communicate” with helper cells that are necessary to induce maximum phagocyte activation (Sacks *et al.* 2002b). At the local tissue level, the parasite/host interaction is revealed by the formation of microscopic structures, such as granulomas that serve as platforms for parasite elimination (Kaye *et al.* 2004). At the organism level, disturbance in the function of infected organs leads to clinical disease. A detailed comprehension of the complex interaction between *Leishmania* and the host cell should provide valuable hints for novel therapeutic strategies.

4.1. Evading innate humoral effectors

Leishmania promastigotes are inoculated in a pool of blood that results from laceration of superficial blood vessels by the sandfly (Killick-Kendrick *et al.* 1981). A few seconds after deposition, promastigotes are opsonized by complement proteins (Dominguez *et al.* 1999, Moreno *et al.* 2010). The complement system provides a fast-response mechanism that targets pathogens as soon as they gain access to the host. The complement mediates direct lysis of invading microbes by formation of the membrane attack complex (MAC), produces anaphylatoxins that cause inflammation, and favors microbial clearance by phagocytes through the action of the opsonins (Carroll 1998). However, there is sustained evidence to support the view that the complement system fails to eliminate *Leishmania* promastigotes when they first come into contact with the host (Goto *et al.* 2013).

Leishmania activates all the three complement pathways (Goto *et al.* 2013). Natural antibodies recognize the parasite and activate the classic pathway (Dominguez *et al.* 2002, Dominguez *et al.* 2003). Recognition of the parasite's LPG by the mannose-binding lectin (MBL) activates the lectin pathway (Green *et al.* 1994, Ambrosio *et al.* 2005). Spontaneous deposition of the complement component 3b (C3b) over the parasite's surface, as a result of binding to LPG and the parasite's major surface protease, gp63, results in engagement of the alternative pathway (Mosser *et al.* 1984, Mosser *et al.* 1986, Brittingham *et al.* 1995). Complement activation pathways converge at the level of formation of the C5 convertases that cleave C5 generating C5b, which leads to the assembly of the MAC (Carroll 1998). However, *Leishmania* metacyclic promastigotes are capable of blocking MAC deposition on the parasites' membrane (Franke *et al.* 1985). Resistance to complement-mediated lysis is conferred by LPG, which is longer and highly branched at the metacyclic stage, hence preventing MAC insertion in the membrane (Puentes *et al.* 1988, Puentes *et al.* 1990). Deposited C3b is eventually cleaved to iC3b that remains on the parasite's surface but is incapable of driving further complement activation (Goto *et al.* 2013). Formation of iC3b is mediated mainly by the concerted action of serum factor H and factor I, but gp63 also contributes to the process (Brittingham *et al.* 1995).

4.2. Parasite establishment in the host cell

Phagocytes are the host cells within which *Leishmania* survives and thrives. These are specialized cells, strategically placed to serve as a first-line of defense against intruder pathogens (Taylor *et al.* 2005). They are equipped with a range of receptors that detect pathogens and mediate their internalization by phagocytosis. Internalized microbes are then sent to lytic compartments where numerous microbicidal mechanisms come into action to destroy the pathogens (Flannagan *et al.* 2012).

4.2.1. Receptor-mediated internalization of *Leishmania* parasites

Phagocytosis of large particles, such as unicellular parasites, is a complex and coordinated process requiring the contribution of several surface receptors. Receptor ligation initiates intracellular signaling cascades leading to actin polymerization and extension of pseudopods around the particle (Flannagan *et al.* 2012). Several phagocytic receptors are involved in the internalization of *Leishmania* promastigotes with substantial variation across parasite species and developmental stages (**Figure 8A**) (Ueno *et al.* 2012). The

complement receptor 3 (CR3) is most important phagocytic receptor for *Leishmania* promastigotes and binds iC3b deposited on the parasite's surface (**Figure 8B**) (Wilson *et al.* 1988b, Mosser *et al.* 1992, Laufs *et al.* 2002). The complement receptor 1 (CR1) contributes to parasite uptake, either directly, by mediating parasite internalization via binding to deposited C3b or indirectly, by promoting the conversion of C3b to iC3b (**Figure 8A**) (Da Silva *et al.* 1989, Rosenthal *et al.* 1996). Early work also implicated the mannose receptor (MR) in *Leishmania* internalization by macrophages (Wilson *et al.* 1986), through its ability to bind gp63 at the parasite surface (Wilson *et al.* 1988a). Recent studies, however, indicate that metacyclic promastigotes avoid MR usage for internalization, while non-metacyclic forms are found within phagosomes containing MR (Ueno *et al.* 2009). It has been proposed that in pre-metacyclic promastigotes, gp63 is accessible on the surface for MR binding, while in metacyclic forms it is masked by the longer LPG chains hence unable to bind MR (Ueno *et al.* 2012). There are additional receptors shown to be implicated in macrophage internalization of *Leishmania* promastigotes (**Figure 8A-B**). These include the DC-associated C-type lectin-1 (Dectin-1), which recognizes β -1,3 glucans that are present in the LPG side chains of several *Leishmania* species (Brown *et al.* 2003, Coelho-Finamore *et al.* 2011, Lefevre *et al.* 2013). Fibronectin receptors also participate in parasite internalization due to their ability to bind gp63 (Rizvi *et al.* 1988, Brittingham *et al.* 1999, Kulkarni *et al.* 2008). Finally, the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) also mediates *Leishmania* internalization by macrophages but is particularly important for parasite entry in DCs (**Figure 8B**) (Colmenares *et al.* 2004, Caparros *et al.* 2005, Lefevre *et al.* 2013).

Leishmania amastigotes must undergo successive rounds of phagocytosis as they egress from heavily infected cells to enter new ones. Evidence shows that amastigotes employ separate routes of internalization from promastigotes (Ueno *et al.* 2012). Indeed, the amastigote surface differs markedly from the surface of the promastigote, which implies distinct receptor usage for phagocytosis. LPG expression is drastically reduced in amastigotes, and its structure is much shorter and simple (Glaser *et al.* 1991, McConville *et al.* 1991). Expression of gp63 is also substantially reduced (Medina-Acosta *et al.* 1989). As such, the current evidence points to a model in which amastigotes are coated by antibodies as they exit dying macrophages and their entrance into new host cells is mediated mainly by Fc- γ receptors (Fc γ Rs) (Guy *et al.* 1993, Kima *et al.* 2000). Nevertheless, and as with promastigotes, additional receptors have also been implicated in the internalization of *Leishmania* amastigotes (Ueno *et al.* 2012) (**Figure 8C**). The entry of *L. amazonensis* amastigotes into macrophages also involves fibronectin receptors (Bosetto *et al.* 2007). Also, *L. pifanoi* and *L. infantum* amastigote internalization was reduced when

DC-SIGN was blocked, implying a role in amastigote internalization for these species. The ligand for DC-SIGN has not been identified (**Figure 8B**) (Colmenares *et al.* 2004).

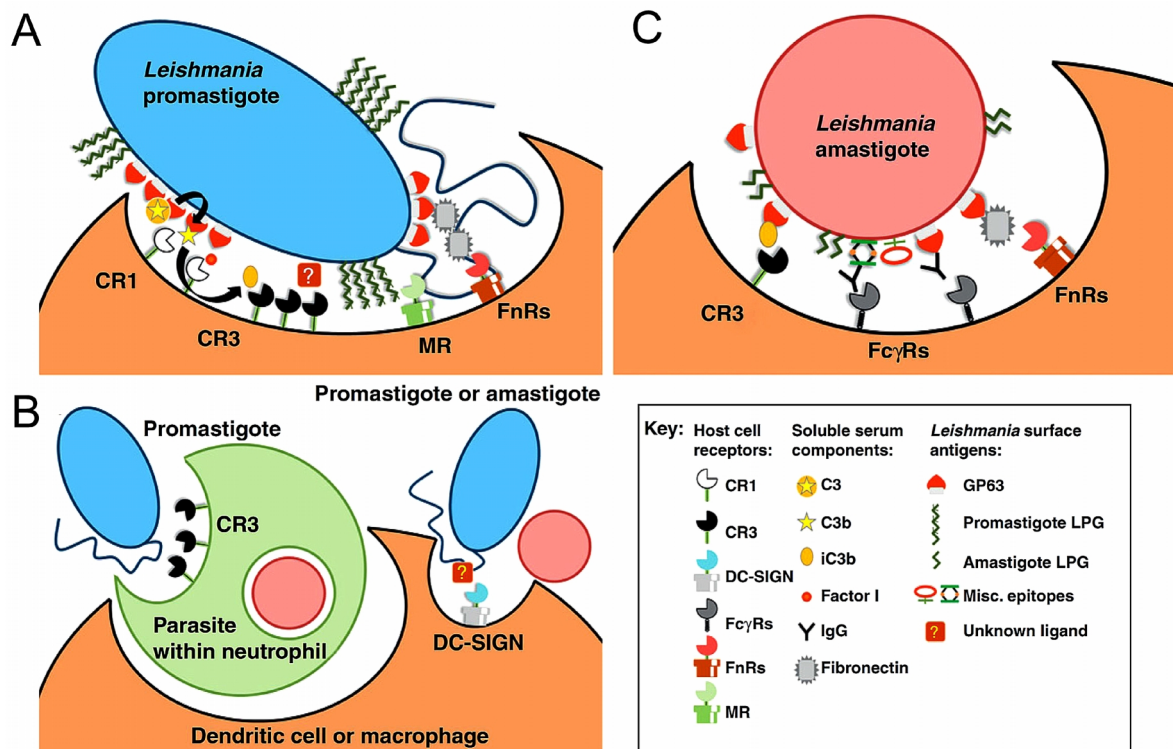


Figure 8. Receptor-mediated phagocytosis of *Leishmania*. (A) Phagocytic receptors involved in promastigote internalization in macrophages. CR1 can serve as a phagocytic receptor for C3b or facilitate its conversion in iC3b (B) CR3 is the main phagocytic receptor mediating parasite entry into neutrophils, while DC-SIGN plays a major role during parasite entry in dendritic cells. (C) Receptors important for the phagocytosis of *Leishmania* amastigotes. Promastigotes are depicted in blue, amastigotes in red, neutrophils in green and macrophages/DCs in orange/brown Adapted from (Ueno *et al.* 2012).

It is usually assumed that given the multiplicity of macrophage receptors interacting with *Leishmania*, internalization routes are redundant. However, as detailed below, ligation of each particular receptor is associated with specific downstream cellular responses, which play important roles in the outcome of the infection (Ueno *et al.* 2012).

4.2.2. Establishing a safe niche in the host cell

Once formed, the nascent phagosome is a relatively benign environment for internalized pathogens. In the canonical phagocytic pathway, soon after internalization, a series of biochemical events convert the phagosome into a potent microbicidal organelle (Fairn *et al.* 2012). Maturation of the phagosome is coordinated by a family of molecular switches, the Rab guanosine triphosphatases (GTPases) (Flannagan *et al.* 2012). Early phagosomes

express the Rab5 GTPase that promotes phagosome fusion with early endosomes by recruiting the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, which act as mediators of membrane fusion (Collins *et al.* 2002, Stenmark 2009). Rab5 is eventually replaced by the late phagosomal marker Rab7, through mechanisms that are not yet clear. Additional markers, such as the lysosome-associated membrane protein (LAMP)-1 and LAMP-2 are also recruited to the late phagosome (Flannagan *et al.* 2012). At this stage the phagosome lumen also becomes more acidic (pH 5.5-6.0) due to the proton-pumping action of the vacuolar ATPase (V-ATPase) (Marshansky *et al.* 2008). The ultimate microbicidal organelle is the phagolysosome that results from the fusion of the late phagosomes with pre-formed lysosomes (Ueno *et al.* 2012). The phagolysosome has a marked acidic pH (4.5-5.0), which is necessary for the optimal function of the lysosomal hydrolases. The phagolysosome also contains antimicrobial proteins, such as lysozyme and cationic peptides (Flannagan *et al.* 2009). In the context of *Leishmania* infections the most important microbicidal mechanisms are the production of reactive oxygen species (ROS) and nitric oxide (NO) (Murray 1982). In the phagolysosome, ROS is produced by the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase), a multimeric protein complex composed of membrane and cytosolic subunits that assemble together in the phagolysosomal membrane. The NADPH oxidase generates superoxide (O_2^-), which can be further dismutated to highly reactive H_2O_2 (Flannagan *et al.* 2012). NO is produced by the inducible nitric oxide synthase (iNOS), whose expression is relatively low in resting cells (Davis *et al.* 2013). High expression is induced by signaling cascades originating from pattern recognition receptors (PRRs) and inflammatory cytokines, which are triggered concomitant with pathogen internalization (Bogdan 2001). Through the action of iNOS, L-arginine is converted to $NO\cdot$ and L-citrulline (Aktan 2004). Nitric oxide is produced at the cytosolic face of the phagolysosome membrane and diffuses to the lumen where it combines with ROS originating highly reactive species such as peroxynitrite ($ONOO^-$). Acting in concert, ROS and NO bind to several proteins and DNA and efficiently destroy invading pathogens (Flannagan *et al.* 2012).

Leishmania induces several deviations from this canonical phagocytic pathway (Moradin *et al.* 2012). Part of these subversive strategies result from preferential use of some phagocytic receptors by the parasite and avoidance of others (Ueno *et al.* 2012). Phagocytic receptors may or may not induce the respiratory burst depending on which members of the Rho family of small GTPases are employed for signaling (Hordijk 2006, Hall 2012). CR3, for instance, signals through Rho, which does not induce ROS production (Ehlers 2000), possibly explaining the marked preference displayed by promastigotes for CR3 (Wright *et al.* 1983, Marth *et al.* 1997, Ehlers 2000). In contrast, some FcγRs employ the GTPases Cdc42, Rac1 and Rac2, which lead to assembly of the NADPH oxidase complex in the

phagosomal membrane and ROS production (Hordijk 2006, Tian *et al.* 2008, Flannagan *et al.* 2012). A recent study has provided mechanistic insight into the contribution of three phagocytic C-type lectin receptors (CLRs) in the internalization of *L. infantum* promastigotes by peritoneal macrophages (Lefevre *et al.* 2013). It was shown that Dectin-1 and MR are important for parasite control by inducing ROS production. Dectin-1 signaling led to the phosphorylation of the cytosolic p47 subunit of the NADPH-oxidase while activation of MR promoted the mobilization of arachidonic acid from the plasma membrane. Both events are crucial for the assembly of a functional NADPH oxidase (Osorio *et al.* 2011). Contrasting with MR and Dectin-1, DC-SIGN ligation did not induce ROS production but instead favored parasite survival (Lefevre *et al.* 2013).

Given that phagocytosis of *Leishmania* is mediated by multiple macrophage-parasite interactions, preferential receptor usage is not sufficient to completely circumvent ROS induction. Promastigotes are highly susceptible to ROS; as such, to escape phagosomal degradation, the parasite relies on additional evasive strategies (Moradin *et al.* 2012).

Early phagosomal markers are readily detectable in *Leishmania*-containing vacuoles (Duclos *et al.* 2000a); however, shedding of Rab5 and acquisition of late phagosomal markers such as Rab7 and Lamp-1 is delayed by 24-48h, presumably to allow differentiation to the intracellular resistant amastigote form (Ueno *et al.* 2009). The maturation arrest induced by promastigotes is dependent on LPG, as parasite mutants lacking either LPG or all surface phosphoglycans are rapidly destroyed in phagolysosomes (Desjardins *et al.* 1997, Scianimanico *et al.* 1999). The small GTPases of the Rho family play a central role in directing actin polymerization during pseudopodia extension in phagocytosis (Flannagan *et al.* 2012). Through mechanisms that are not yet clear, LPG is capable to retain the small GTPase Cdc42 at the phagosome membrane leading to F-actin accumulation around the phagosome, which presumably interferes with vesicle trafficking and phagosome maturation (Holm *et al.* 2001, Lodge *et al.* 2005). Additionally, secreted LPG inserts into lipid microdomains at the phagosome membrane disturbing the assembly of the NADPH-oxidase complex and impairing the recruitment of late phagosomal markers such as V-ATPase or cathepsin D (Vinet *et al.* 2008, Winberg *et al.* 2009). A distinct strategy is employed by parasites of the *L. mexicana* complex, which reside within large vacuoles, each containing many amastigotes, which is thought to dilute the hydrolytic content of the vacuole, resulting in a milder environment (Duclos *et al.* 2000b, Real *et al.* 2012).

The concerted action of these subversive mechanisms allows the differentiation of promastigotes into amastigotes in a relatively mild environment (Moradin *et al.* 2012). Once differentiated, amastigotes possess an array of mechanisms that allow survival in the harsh environment of the phagolysosome. These include heme degradation, which is essential for NADPH oxidase functioning (Pham *et al.* 2005); expression of a superoxide dismutase

that detoxifies ROS (Ghosh *et al.* 2003); and expression of peroxiredoxins that provide further protection against oxidative stress (Barr *et al.* 2003).

4.3. Long-term survival: subversion of the host cell functions

Establishment of an intracellular niche is not, by itself, sufficient to ensure the long-term survival of *Leishmania* in the macrophage (Olivier *et al.* 2005). Indeed, parasite products ligate macrophage PRRs, triggering signaling cascades that induce the expression of pro-inflammatory mediators. These will have multiple effects in a range of immune cells that, in turn, will promote the full expression of the macrophage leishmanicidal potential (Shio *et al.* 2012). In particular, interferon- γ (IFN- γ), produced by T cells and natural killer (NK) cells is a strong inducer of iNOS expression (Kaye *et al.* 2011a). The resulting production of NO is very effective in destroying intracellular amastigotes (Bogdan 2001). As such, *Leishmania* evolved strategies to interfere with macrophage signaling and ensure long-term survival.

4.3.1. Recognition of *Leishmania* by innate immunity and mechanisms of evasion

4.3.1.1. Toll-like receptors

The Toll-like receptor family

Toll-like receptors (TLRs) were the first PRRs demonstrating an involvement in *Leishmania* recognition (Faria *et al.* 2012). There are ten functional TLRs in humans (twelve in mice) that recognize a wide range of molecular patterns commonly expressed by pathogens, but usually absent in mammals. For example, lipopolysaccharide (LPS) is recognized by TLR4, double-stranded RNA (dsRNA) by TLR3 and flagellin by TLR5 (Kawai *et al.* 2011). TLRs are type I transmembrane glycoproteins comprising an ectodomain responsible for recognition of pathogen-associated molecular patterns (PAMPs) that consists in tandem copies of leucine-rich repeats (LRRs). The ectodomain is connected to the intracellular portion by a small transmembrane region. The cytosolic region consists of a Toll/interleukin-1 receptor (TIR) domain, capable of recruiting adaptor proteins, such as the myeloid differentiation primary response gene 88 (Myd88) or the TIR domain-containing adaptor-inducing IFN-beta (TRIF) and initiate intracellular signaling cascades that lead to nuclear translocation of several transcription factors (Akira *et al.* 2004). These include the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) or the activator protein-1 (AP-1) that associate with promoters of genes for inflammatory cytokines and chemokines and

induce their transcription. TLRs may also promote the activation of the interferon regulatory factors (IRFs) -3 and -7 that induce copious expression of type I IFNs (Kawai *et al.* 2011). TLRs that recognize PAMPs displayed on the surface of pathogens are usually located at the cell surface (TLR-1, TLR-2, TLR-4, TLR-5, and TLR-6), while those binding nucleic acids tend to be located in endosomal/phagosomal compartments (TLR3, TLR7, TLR8 and TLR9), presumably to avoid contact with self-nucleic acids (Blasius *et al.* 2010). The distinct molecular patterns, cellular localization and signaling cascades associated with each TLR allow the development of cellular responses tailored to each particular invading pathogen (Kawai *et al.* 2011).

TLRs implicated in Leishmania detection

The first hint that TLRs could be involved in the recognition and protective response against *Leishmania* spp. came when otherwise resistant C57Bl/6 mice became highly susceptible to *L. major* infection after genetic deletion of the adaptor protein Myd88 (Muraille *et al.* 2003, Vargas-Inchaustegui *et al.* 2009). This phenotype probably results from the fact that Myd88 is an adaptor protein common to all TLRs except TLR3 (O'Neill *et al.* 2007). Further work has defined the contribution of individual TLRs in the recognition of parasite molecules and disease progression. TLR2 appears to recognize LPG from several *Leishmania* species (de Veer *et al.* 2003, Kavosi *et al.* 2009). Additional TLR2 potential ligands include the silent information regulator 2-related protein 1 (SIR2RP1) (Silvestre *et al.* 2009b) and two antigens of 65 and 98 kDa isolated from *L. donovani* amastigotes (Srivastava *et al.* 2012). Yet, the contribution of TLR2 to the ensuing response is not clear from the studies available. Indeed, protective, detrimental and irrelevant consequences of TLR2 deletion for parasite survival were found, depending on the experimental model (Flandin *et al.* 2006, Silvestre *et al.* 2009a, Vargas-Inchaustegui *et al.* 2009). In contrast, sustained evidence supports a host-protective role for TLR4, as its genetic ablation resulted in increased susceptibility to infection (Kropf *et al.* 2004a, Kropf *et al.* 2004b). Furthermore, a recent genetic association study found that polymorphisms in the *TLR4* gene are associated with increased severity of cutaneous lesions in patients infected with *L. major* (Ajdary *et al.* 2011). However, a parasite ligand for TLR4 remains elusive.

Endosomal TLRs are also implicated in *Leishmania* infections. TLR9 is capable of discriminating parasite DNA and contributes to a protective type-1 immune response (Schleicher *et al.* 2007, Abou Fakher *et al.* 2009). Interestingly, endosomal TLRs appear to have additive contributions during *Leishmania* infections. A recent study evaluated the outcome of *L. major* infection in mice deficient for the gene encoding the mammalian homolog B1 of the *Caenorhabditis elegans* protein Unc-93 (UNC93B1) (Schamber-Reis *et al.* 2013). UNC93B1 is a chaperone protein that mediates the translocation of nucleic acid-

sensing TLRs from the endoplasmic reticulum to the phagosome (Casrouge *et al.* 2006). These mice were highly susceptible to *L. major* infection; a phenotype that could only be mimicked by a TLR3/TLR7/TLR9 triple knock-out mice, but not by the individual single knock-outs (Schamber-Reis *et al.* 2013).

Importantly, activation of TLRs during *Leishmania* infections may have a clear detrimental consequences to the host. The presence of the *Leishmania* RNA virus (LRV) parasitizing some strains of *L. guyanensis* leads to activation of TLR3 and, to a minor extent, TLR7 by viral RNA. Activation of endosomal TLRs favors the ability of the parasite to cause secondary metastatic lesions (Ives *et al.* 2011). As discussed in section 3.2, there is increasing evidence suggesting that the presence of LRVs may account for the metastatic potential of *Leishmania* strains causing MCL (Zangger *et al.* 2013).

Parasite-mediated interference with TLR signaling

Activation of TLRs is, in general, deleterious for the parasite. As such, it is hardly surprising to find that *Leishmania* developed efficient mechanisms to interfere with TLR signaling and inhibit the inflammatory response (**Figure 9**) (Faria *et al.* 2012).

Protein tyrosine phosphatases (PTPs) play important roles in regulating intracellular signaling cascades, by selectively removing activatory phosphate groups, hence avoiding excessive cellular responses (Zhang 2002). However, *Leishmania* is adept at exploiting the regulatory role of host phosphatases to promote its own survival (Olivier *et al.* 2005). It has long been known that *Leishmania* infection leads to activation of several host PTPs (Olivier *et al.* 1998). More recently, it was shown that the major *Leishmania* surface protease, gp63, localizes to the cytoplasm of the host cell, where it processes and activates host PTPs, including the Src homology region 2 domain-containing phosphatase-1 (SHP-1), PTP1B and the T cell PTP (TCPTP) (Gomez *et al.* 2009). Activation of SHP-1 is also mediated by binding of the parasite-derived elongation factor-1 α (EF-1 α) and fructose-1,6-biphosphate aldolase, highlighting the importance of targeting this phosphatase for the parasite (Nandan *et al.* 2002, Nandan *et al.* 2007). Once activated, PTPs decrease the efficiency of TLR signaling and expression of pro-inflammatory genes. SHP-1 dephosphorylates and inactivates members of the mitogen-activated protein kinases (MAPKs), which are activated downstream to most TLRs and lead to nuclear translocation of the transcription factor AP-1 (Prive *et al.* 2000, Forget *et al.* 2006, Halle *et al.* 2009, Kawai *et al.* 2011). Furthermore, gp63 directly binds, cleaves and inactivates c-Fos, the central component of AP-1, hence ensuring an efficient shut-down of this pathway by the parasite (Contreras *et al.* 2010). Additionally, SHP-1 is capable of removing phosphate groups from the interleukin-1 receptor-associated kinase-1 (IRAK1) (**Figure 9**) (Abu-Dayyeh *et al.* 2008), a central kinase in TLR pathways (O'Neill *et al.* 2007). Gp63 also cleaves the p65RelA subunit of the

transcription factor NF- κ B (Gregory *et al.* 2008). Interestingly, cleavage does not inactivate NF- κ B function. Instead, it produces a novel truncated form termed p35RelA that associates with the p50 subunit and directs the transcription of several chemokines (**Figure 9**) (Gregory *et al.* 2008, Neves *et al.* 2010). These findings led to an appealing model in which *Leishmania* hijacks NF- κ B signaling to induce a transcriptional program more favorable to the parasite. In this manner, *Leishmania* induces production of chemokines that attract potential new host cells, while simultaneously blocking the NF- κ B-mediated production of IL-12 and factors detrimental for parasite survival (Gregory *et al.* 2008, Olivier *et al.* 2012). Activation of the macrophage by IFN- γ is highly deleterious for the parasite by inducing the production of anti-microbial factors (Kima *et al.* 2013). *Leishmania* renders the host cell unresponsive to IFN- γ via SHP-1, which, activated by gp63, binds to and dephosphorylates the Janus Kinase-2 (JAK2) (Blanchette *et al.* 1999, Matte *et al.* 2010), a crucial kinase in the IFN- γ signaling pathway (**Figure 9**) (Schroder *et al.* 2004).

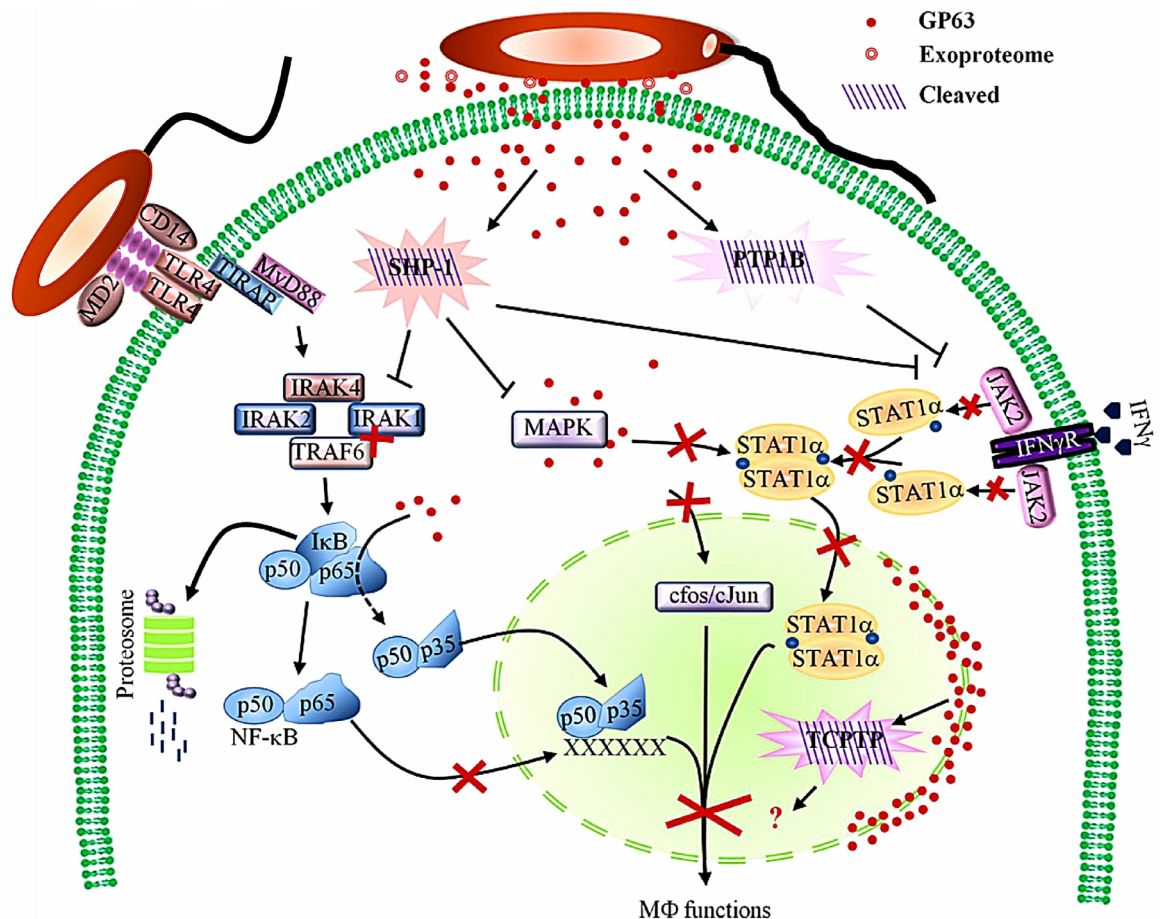


Figure 9. *Leishmania* employs multiple mechanisms to downregulate TLR and cytokine signaling cascades. The picture highlights the contribution of the metalloprotease gp63 in this process. Gp63 can directly cleave and inactivate important mediators of the pro-inflammatory response or, alternatively, induce the activation of protein phosphatases that efficiently shut-down signaling cascades. Adapted from (Olivier *et al.* 2012).

4.3.1.2. Additional PRRs involved in *Leishmania* infection

Nod-like receptors

TLRs are not the sole innate immune receptors involved in the recognition of *Leishmania*. Nucleotide-binding, oligomerization domain-like receptors (NLRs) are cytosolic sensors of PAMPs and damage-associated molecular patterns (DAMPs) (Lamkanfi *et al.* 2012). Some NLRs, such as NOD1 and NOD2, recognize PAMPs and initiate intracellular signaling cascades such as the MAPK or the NF- κ B pathways (Elinav *et al.* 2011). In contrast, activation of several NLRs leads to the assembly of multimeric complexes known as the inflammasomes, which function as platforms for the auto-proteolytic activation of the inflammatory caspase-1. Activated caspase-1 processes the precursor forms of the pro-inflammatory cytokines IL-1 β and IL-18. In this manner, the production of biologically active IL-1 β and IL-18 requires two signals. The first signal is provided by binding of a microbial molecule to a PRR (usually a TLR), which induces pro-IL-1 β and pro-IL18 genes. The second signal is usually provided by a danger-associated molecule that induces inflammasome assembly and caspase-1 activation (Lamkanfi *et al.* 2012). Several inflammasomes have been described that differ in their NLR or adaptor components as well as in the upstream signals leading to their activation (Chen *et al.* 2009). The best characterized inflammasome is NLRP3 which is thought to be activated in response to disturbance of cellular homeostasis, such as changes in the redox status or intracellular ion concentration, rather than by a particular DAMP or PAMP (Wen *et al.* 2013). With respect to *Leishmania* infection, a recent study has shown that activation of the NLRP3 inflammasome and the ensuing production of IL-1 β could restrict *L. amazonensis* growth in cultured primary macrophages and upon infection in mice (Lima-Junior *et al.* 2013). The activation of NLRP3 in *Leishmania*-infected cells depends on potassium efflux and cathepsin activity. The protective effect of NLRP3 activation was due to an increase in IL-1 β production that led to augmented production of NO (Lima-Junior *et al.* 2013). Nevertheless, previous studies reported that IL-1 β mediates immunopathology in susceptible mice (Voronov *et al.* 2010) or is dispensable for protection in resistant C57Bl/6 mice (Kautz-Neu *et al.* 2011). Interestingly, polymorphisms in the *IL1B* gene have been associated with increased severity in both cutaneous and visceral leishmaniasis (Fernandez-Figueroa *et al.* 2012, Moravej *et al.* 2012) suggesting a role for inflammasome activation and NLRs in humans.

C-type lectin receptors

CLRs were already referred in the context of parasite phagocytosis. Besides coordinating the phagocytic machinery, these receptors also recognize carbohydrate structures unique

to pathogens and initiate intracellular cascades leading to microbicidal effectors (Osorio *et al.* 2011). As discussed, three CLRs are implicated in *Leishmania* infection. Whereas MR and Dectin-1 contribute to protection against the parasite by inducing ROS; DC-SIGN ligation was detrimental to the host (Lefevre *et al.* 2013). Further analysis revealed a cross-talk between CLRs and NLRs in the context of *Leishmania* infection. Indeed, induction of ROS by MR and dectin-1 acts as a stress signal leading to NLRP3 inflammasome activation, IL-1 β production and parasite control (Lefevre *et al.* 2013). Furthermore, MR induced the production of leukotriene B₄, an eicosanoid previously linked to the activation of NLRP3 (Amaral *et al.* 2012). In contrast, DC-SIGN activation, diverts the metabolism of arachidonic acid towards the production of lipoxin A₄ (Lefevre *et al.* 2013), an anti-inflammatory molecule known to inhibit IL-1 β and to promote progression of leishmaniasis (Sodin-Semrl *et al.* 2000, Wenzel *et al.* 2009). These recent findings highlight the intricate signaling networks that characterize the macrophage response to *Leishmania*, and future studies will probably add further complexity to this relationship.

4.3.1.3. Mechanisms of secretion in *Leishmania*

Leishmania has to deliver virulence factors across the host cell/phagosome membranes for efficient modulation of host cell functions. Proteomic studies of the *Leishmania* secretome have highlighted the fact that many proteins delivered extracellularly by the parasite do not contain the typical N-terminal signals required for the classical secretory pathway mediated by the endoplasmic reticulum (ER) / Golgi (Revest *et al.* 2008, Silverman *et al.* 2008, Kima *et al.* 2010). These observations imply that the parasite uses alternative mechanisms for protein secretion. Recently, much attention has been devoted to the content and functional significance of membrane-delimited extracellular vesicles produced by *Leishmania* and capable of fusing with host's membranes (Silverman *et al.* 2011, Lambertz *et al.* 2012). These vesicles can either originate from plasma membrane blebs or intracellular multivesicular bodies, which upon fusion with the parasite's membrane, release 30-100 nm vesicles called exosomes (Lambertz *et al.* 2012). *Leishmania* extracellular vesicles were shown to contain many well-known *Leishmania* virulence factors, including gp63, EF-1 α , fructose-1,6-bisphosphate aldolase or the secreted acidic phosphatase (SACP) (Silverman *et al.* 2010a, Santarem *et al.* 2013, Hassani *et al.* 2014). Furthermore, the content of *Leishmania* exosomes matures during metacyclogenesis (Santarem *et al.* 2013), and vesicles are rapidly released by the parasite upon a 25 to 37°C temperature shift (Hassani *et al.* 2011). As such, exosomes may constitute a weaponry, build up by the parasite during development in the vector and ready to be deployed upon entry into the mammalian host.

Exosome-based secretion provides an efficient mechanism for virulence factor delivery across host's membranes and efficient modulation of host cell functions (Silverman *et al.* 2010b).

4.3.2. Modulation of macrophage polarization, metabolism and apoptosis

Early work on macrophage activation has defined two major subsets of these phagocytic cells. Classically-activated macrophages (M1) was a term given to a population that demonstrated enhanced microbicidal activity in response to IFN- γ produced by antigen-activated immune cells (Mackaness 1962, Nathan *et al.* 1983). The definition of M1 macrophages has since been refined to include new evidence, and can be summarized as a macrophage activated by TLRs and IFN- γ /TNF that exhibits enhanced intracellular microorganism killing and increased expression of inflammatory cytokines and co-stimulatory molecules (Mosser *et al.* 2008). In contrast, in response to IL-4 or IL-13, macrophages exhibit high phagocytic activity and contribute to tissue repair and wound healing. These were named alternatively-activated macrophages (M2 macrophages) and defined by their high expression of arginase-1, MR and the IL-4 receptor (IL-4R) (Stein *et al.* 1992, Doyle *et al.* 1994). It was soon noticed that M1 macrophages were highly efficient in destroying intracellular *Leishmania* amastigotes (Green *et al.* 1990, Mauel *et al.* 1991). In contrast, M2 macrophages were permissive to parasite growth (Green *et al.* 1990). At the molecular level, these disparate outcomes resulted from differential metabolism of L-arginine by the macrophage. In classically-activated macrophages, iNOS induction converts L-arginine into microbicidal NO and citrulline (Mauel *et al.* 1991). In M2 macrophages, the increase in arginase-1 levels diverts the L-arginine metabolism towards the production of polyamines that promote parasite growth (Wanasen *et al.* 2008). By modulating a single host metabolic pathway, *Leishmania* could avoid microbicidal NO while inducing the production of polyamines. Recent studies demonstrate that the outcome of *Leishmania* infection can hardly be explained by such a simple bimodal model. Nevertheless, ample evidence suggests that arginase-1 activity correlates with disease severity in both cutaneous and visceral forms of leishmaniasis (Muller *et al.* 2008, Abebe *et al.* 2012, Takele *et al.* 2013).

More recently, the spectrum of macrophage polarization has been extended. Classic M1 macrophages still correspond to the microbicidal state initially described by Mackaness (Van-Epps 2005). The alternatively-activated group has been further subdivided to accommodate its heterogeneous nature (Mosser *et al.* 2008). As such, IL-4/IL-13-conditioned macrophages were renamed as M2a, and engage in typical Th2 responses,

such as killing of helminthes or airway inflammation (Mantovani *et al.* 2004). A second type of alternative activation, termed M2b or type II, is induced by simultaneous interactions with TLR-ligands and immune complexes. These are characterized by producing a mixed cytokine pattern with high levels of IL-10, TNF and IL-6 but low IL-12 (Martinez *et al.* 2014). A third type of alternative activation, M2c, polarizes in response to IL-10 and glucocorticoids, and plays immunoregulatory functions and tissue remodeling and matrix deposition (Mantovani *et al.* 2004). With respect to *Leishmania* infections, recent studies suggest that infected macrophages bear a phenotype that fits into the M2b group, characterized by upregulation of arginase-1, FCyRs, CLRs, IL-6, TNF, IL-1 β , IL-10 and TGF- β (Rabhi *et al.* 2012, Lefevre *et al.* 2013). However, these macrophage subsets result from defined *in vitro* stimulations and probably do not reflect the complexity of the *in vivo* environment (Martinez *et al.* 2014). In a complex immune environment such as leishmaniasis, the macrophage population probably exists in a continuum. At a given time-point, some cells should be more engaged in parasite killing while others perform tissue remodeling functions, and some macrophages ensure immunoregulation, with possible transit of individual cells between these groups (Mosser *et al.* 2008).

Early microarray studies failed to observe significant transcriptional changes in macrophages when infected with *Leishmania* (Buates *et al.* 2001, Chaussabel *et al.* 2003, Rodriguez *et al.* 2004, Depledge *et al.* 2009). Recent approaches employing modern bioinformatic tools unveiled a prominent ability of the parasite to modulate the host cell metabolism (Rabhi *et al.* 2012, Beattie *et al.* 2013, Lecoeur *et al.* 2013). Such ability is not particularly surprising, as parasite growth and replication are energetically demanding, and parasites must compete with the host for energy and nutrient sources (Brunton *et al.* 2013). Furthermore, modulation of the host cell metabolism is an effective way to dampen innate immunity, which relies on energy-demanding processes (O'Neill *et al.* 2013). The phagolysosome is relatively hexose-poor, and amastigotes are thought to generate energy mainly from amino-acids and fatty acids (Naderer *et al.* 2008, Rosenzweig *et al.* 2008). As such, parasite-mediated modulation of host metabolism should reflect these requirements. Recent microarray data suggests that *Leishmania* infection induces a coordinated increase in host cell glycolysis as revealed by the induction of several glycolytic enzymes, as well as glucose transporters. Concomitantly, mitochondrial aerobic pathways appear down-regulated (Rabhi *et al.* 2012). More strikingly, parasite infection has a significant impact in macrophage lipid metabolism. At the transcriptional level, consistent upregulation of macrophage mediators involved in lipid uptake and metabolism were observed (Rabhi *et al.* 2012, Beattie *et al.* 2013, Lecoeur *et al.* 2013). Moreover, lipid bodies and lipid droplets are found in parasitized macrophages, frequently in close association with the PV (Rabhi *et al.*

2012, Lecoeur *et al.* 2013). Cholesterol turnover appears to be particularly targeted by the parasite with reported increases in the levels of cholesterol-metabolizing enzymes and receptors for cholesterol uptake (Rabhi *et al.* 2012, Lecoeur *et al.* 2013). Intriguingly, hypocholesterolemia is commonly found in VL patients, and hyperlipidemia conferred protection against the disease (Lal *et al.* 2007, Ghosh *et al.* 2011, Ghosh *et al.* 2012). Dissecting the mechanisms through which *Leishmania* interferes with lipid metabolism should provide valuable insight into the pathogenesis of the disease.

Several *in vitro* studies demonstrated the ability of *Leishmania* to inhibit or delay macrophage cell death in response to a number of pro-apoptotic stimuli or growth factor deprivation (Moore *et al.* 1994, Akarid *et al.* 2004, Gutierrez-Kobeh *et al.* 2013, Srivastav *et al.* 2014). At the molecular level, *Leishmania* infection results in the activation of a number of pro-survival signaling pathways in the macrophage (Rodrigues *et al.* 2012). For instance, activation of the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3-K) / Protein Kinase B (PKB, Akt) pathway by *Leishmania* leads to cytoplasmic arrest of pro-apoptotic Bcl-2 family members, such as Bad (Ruhland *et al.* 2007). As a result, cytochrome c release from the mitochondria is prevented, and the infected cell protected from apoptosis (Akarid *et al.* 2004). Inhibition of host cell apoptosis allows the parasite to preserve its intracellular niche and presumably contributes to evasion of the immune system. However, *in vivo* studies are required to confirm the relevance of these observations.

4.4. *Leishmania* interactions with antigen-presenting cells

Whereas the macrophage serves as a long-term host cell for *Leishmania*, initiation of the adaptive immunity depends on parasite uptake by dendritic cells (Kaye *et al.* 2011a). In non-infected skin, dermal DCs continuously migrate through the interstitial space. Within minutes after promastigote injection in the dermis, DC migration is arrested, and the cells extend their dendritic processes towards the parasites efficiently capturing them (Ng *et al.* 2008). Compared with macrophages, DCs tend to harbor fewer parasites, which may result from limited phagocytic capacity or reflect an ability to restrict intracellular growth (von Stebut *et al.* 1998). Dermal DCs and monocyte-derived DCs activate and mature locally into TNF and iNOS-producing DCs (Tip DCs), which become the main infected population at the lesion site in mice resistant to *L. major* infection (Leon *et al.* 2007, De Trez *et al.* 2009, Olekhnovitch *et al.* 2014). Alternatively, infected DCs upregulate co-stimulatory markers (CD40, CD83, CD86) and MHC-II molecules and migrate towards the draining lymph nodes to present parasite antigens to naïve T cells (Leon *et al.* 2007, Kautz-Neu *et al.* 2012, Liu

et al. 2012). Furthermore, unlike macrophages, DCs are good producers of IL-12 after internalizing *Leishmania*, which acts along with antigen-MHC signals and co-stimulation to efficiently prime T cells (Gorak *et al.* 1998, Baldwin *et al.* 2004, Ritter *et al.* 2004, Schleicher *et al.* 2007).

Interestingly, the ability of DC subsets to produce IL-12 appears to be inversely correlated to their permissibility to intracellular *Leishmania* (Henri *et al.* 2002), and several reports unveiled the ability of the parasite to counteract DC function and interfere with T cell priming (Liu *et al.* 2012). Indeed, many of the signaling pathways that the parasite modulates in macrophages appear similarly affected in DCs (Neves *et al.* 2010). As a result, DC maturation is impaired, as revealed by poor upregulation of surface MHC, co-stimulatory molecules or inflammatory cytokine production (Prina *et al.* 2004, Vanloubbeeck *et al.* 2004, Xin *et al.* 2007).

Besides a specific signal, provided by peptide-MHC complexes, T cells require appropriate co-stimulation and cytokines for efficient priming. In the absence of one or more of these complementary stimuli, T cell activation is suboptimal, which renders T cells anergic and delays the onset of adaptive responses (Fathman *et al.* 2007, Chappert *et al.* 2010). *Leishmania* appears to be particularly effective in dampening DC functions which results either in anergic or delayed T cell responses (Qi *et al.* 2001, Vanloubbeeck *et al.* 2004, Joshi *et al.* 2009). Furthermore, parasite infection induces the expression of suppressive molecules in DCs, such as indoleamine 2,3-dioxygenase (IDO) or the ATP-catabolizing enzymes CD39 and CD73. These suppressive DCs are very poor at priming T cells, delaying the onset of adaptive immunity (Makala *et al.* 2011, Figueiredo *et al.* 2012). Impairing DC-mediated priming of T cells presumably provides a crucial window of time that allows parasite growth and dissemination to a population sufficiently large to withstand the eventual development of adaptive immunity (Khan *et al.* 2012).

Some studies employing elegant approaches are starting to reconcile previous conflicting findings concerning the outcome of *Leishmania* infection of DCs. Within a given population of DCs challenged by *Leishmania*, there is always a fraction of the cells that are exposed to parasite products or the inflammatory environment, but are not productively infected by the parasite. These bystander DCs produce high levels of IL-12, upregulate co-stimulatory molecules, and prime T cells towards the development of protective T helper-1 (Th1) cells. In contrast, their infected counterparts produce a mixed pattern of cytokines including TNF and IL-10, have their maturation arrested, and are less fit at priming T cells for type I responses (Carvalho *et al.* 2008, Resende *et al.* 2013).

5. Regulation of immunity during visceral leishmaniasis

T cell-mediated immunity is crucial for the control of all forms of leishmaniasis, as mice lacking T cells demonstrate uniform susceptibility to infection (Handman *et al.* 1979, Mitchell *et al.* 1980). Remarkably, transfer of a relatively small number of lymphoid cells (10^6) can restore protection in T cell-insufficient mice (Mitchell *et al.* 1980).

The discovery of inbred mouse strains that differ significantly in their susceptibility to *L. major* has contributed decisively to the understanding of the key immune events during infection (Kellina 1973, Sacks *et al.* 2002a). Early studies demonstrated the essential role of CD4 T cells during *L. major* infection, as CD4 T cell depletion led to the development of uncontrolled lesions in both resistant and susceptible mice (Titus *et al.* 1987). Shortly after the description of Th1 and Th2 clones *in vitro* (Mosmann *et al.* 1986), extensive empirical evidence showed that CD4 T cells were not only the main determinants of resistance to *L. major* infection, but were also the mediators of susceptibility in vulnerable strains (Reiner *et al.* 1995). In most inbred mouse strains, *L. major* causes a small lesion at the site of inoculation that heals within a few weeks. Such resistance relies on the development of type I immune responses characterized by initial production of IL-12 by antigen-presenting cells (APCs) that induce IFN- γ -secreting Th1 CD4 T cells. In contrast, some strains infected by *L. major* develop uncontrolled lesions that progress to systemic disease. In these, an early burst of APC-derived IL-4 or IL-13 polarizes the subsequent CD4 T cell response towards IL4-secreting Th2 cells that fail to activate the macrophage to kill intracellular parasites (Reiner *et al.* 1995, Sacks *et al.* 2002a). This simple model resulted from extensive experimental work in the late 1980's and 1990's. Nevertheless, it has since been challenged, modified and refined to accommodate the increasing number of cytokines and immune populations that proved to have an impact on the outcome of the infection (Sacks *et al.* 2002a, Alexander *et al.* 2005, Alexander *et al.* 2012).

Importantly, early observations in mice infected with visceral *Leishmania* species as well as direct analysis of infected organs in human patients noticed that the defect in cell-mediated immunity during VL could not be attributed to abnormal expansion of CD4 T cells with a Th2 profile (Kaye *et al.* 1991, Karp *et al.* 1993, Kenney *et al.* 1998). Instead, VL progresses even in the presence of detectable levels of Th1 cytokines, whose action is neutralized by increased levels of immunosuppressive factors (Sacks *et al.* 1987, Cillari *et al.* 1991, Carvalho *et al.* 1992). Due to the intrusive procedures required to analyze infected organs in human patients, the bulk of the knowledge concerning the regulation of immunity during VL has been obtained from mouse models, in which clinical disease differs in some important aspects from human infection (Nieto *et al.* 2011). Nevertheless, studies over the

past decades employing several experimental strategies such as transgenic mice, antibody-mediated neutralization of immune populations, and adoptive cell transfer greatly improved our knowledge into the role of the several immune factors that come into play during VL (Stanley *et al.* 2007, Nieto *et al.* 2011). Most studies in mice are based on intravenous or intraperitoneal injection of a high dose of parasites (usually more than 1000 times the average inoculum deposited by the sand-fly), thus bypassing the events in the skin and parasite navigation to the viscera (Stanley *et al.* 2007). Some comparative studies suggest that the parasite dose and inoculation route influence the kinetics of parasite colonization of the viscera and the ensuing immune response (Rolao *et al.* 2004, Kaur *et al.* 2008). In mice, VL provides a clear example of organ-restricted immunity. In the liver, infection is self-resolving, in a manner that is dependent on the development of T cell-mediated immunity and formation of granulomas (Engwerda *et al.* 2004a). In contrast, in the spleen the immune system fails to clear parasites and instead progressive immunopathology is observed (Kaye *et al.* 2004). Infection of other lymphoid organs is less well studied. Nevertheless, it is known that *Leishmania* colonizes the bone marrow and influences hematopoiesis (Cotterell *et al.* 2000b).

5.1. Compartment-specific immunity: parasite control in the liver

Resident Kupffer macrophages harbor the *majority* of parasites in the liver after intravenous injection of mice with *L. donovani* or *L. infantum* (McElrath *et al.* 1988, Honore *et al.* 1998). Kupffer cells appear to have reduced innate capacity to kill intracellular *Leishmania* and, as a result, hepatic parasite burden increases rapidly during the first weeks after infection (Leclercq *et al.* 1996, Wilson *et al.* 1996). Restriction of liver parasite numbers is coincident with the assembly of inflammatory structures, known as granulomas, constituted by a central core of fused and parasitized Kupffer cells and an outer cuff of motile lymphocytes and variable amounts of other immune cells (**Figure 10**) (Murray *et al.* 1987, McElrath *et al.* 1988, Murray 2001). Granulomas allow the local concentration of inflammatory cytokines that in turn efficiently activate the leishmanicidal mechanisms of Kupffer cells (Moore *et al.* 2013). The kinetics of granuloma maturation during experimental infection of mice with *L. donovani* has been dissected in detail (**Figure 10**) (Engwerda *et al.* 2000, Murray 2001, Moore *et al.* 2013). Within 24h of parasite injection, infected Kupffer cells appear activated and secrete several chemokines and cytokines that recruit immune populations, including monocytes, neutrophils and CD1d-restricted invariant natural killer (iNKT) cells (Cervia *et al.* 1993, Cotterell *et al.* 1999, Svensson *et al.* 2005). iNKT cells, in particular, play a major role in coordinating initial granuloma formation (**Figure 10**) (Robert-Gangneux *et al.* 2012).

Parasite infection induces the surface expression of the signal regulatory protein- α (SIRP- α) in Kupffer cells, which binds CD47 on the surface of iNKT cells, triggering early production of IFN- γ (Beattie *et al.* 2010b). Recognition of CD1d-bound LPG by the iNKT T-cell receptor (TCR) also contributes to the initial burst of IFN- γ (Amprey *et al.* 2004). Additionally, iNKT cells rapidly secrete several cytokines upon activation, including C-X-C motive chemokine-10 (CXCL10), which attracts T cells and promotes maturation of granulomas (Svensson *et al.* 2005, Beattie *et al.* 2010b, Robert-Gangneux *et al.* 2012). However, the role of iNKT cells during VL is unclear, as their activation with selective ligands resulted in disease amelioration in one study (Karmakar *et al.* 2012) and aggravation in another (Stanley *et al.* 2008). It is possible that these cells are important in the orchestration of the initial response after infection, but their chronic activation is detrimental to the host (Stanley *et al.* 2008).

By one week after infection, T cells are recruited to the granuloma and eventually become the predominant immune cell type (**Figure 10**) (Stern *et al.* 1988). Given the low level of innate parasite killing in the early stages of infection in the liver, it has long been assumed that the majority of parasite-specific T cells are primed in the spleen and subsequently migrated to the liver, guided by chemotactic gradients (Engwerda *et al.* 2000). Nevertheless, a recent study demonstrated that specific CD4 T cells can be primed in the liver and suffice to confer hepatic immunity (Bunn *et al.* 2014). Both CD4 and CD8 T cells appear indispensable for the development of mature granulomas (Stern *et al.* 1988). Two-photon imaging revealed that antigen presentation to CD8 T cells is restricted to Kupffer macrophages (Beattie *et al.* 2010a), whereas CD4 T cells may be activated by both Kupffer cells and some granuloma-associated DCs (Stanley *et al.* 2007, Moore *et al.* 2013). Some bystander-activated non-specific T cells also seem to associate to the granuloma and presumably contribute to the protective response (Moore *et al.* 2013).

A number of cytokines play critical roles in granuloma development and parasite killing. IL-12 is produced by activated Kupffer macrophages and induces IFN- γ by granuloma-associated lymphoid cells (Murray *et al.* 1992). In turn, IFN- γ maximizes the leishmanicidal capacity of Kupffer cells (Stanley *et al.* 2007). Arguably, the most important soluble factor for granuloma development and hepatic control of *Leishmania* infection is TNF, which plays a crucial role in coordinating the assembly and maturation of granulomas (Kaye *et al.* 2004). In the absence of *TNF*, parasite growth in the liver proceeds unimpaired during the first weeks due to completely absent granuloma formation. However, later in infection (6-8 weeks) there is an abrupt assembly of granulomas causing rapid death due to fulminant hepatic necrosis (Tumang *et al.* 1994, Murray *et al.* 2000). Interestingly, lack of IFN- γ may be compensated to some extent by TNF (Taylor *et al.* 1997). Additionally, lymphotoxin- α , a TNF-related cytokine, promotes the recruitment of leukocytes from the perivascular space to the sinusoidal areas, where infected Kupffer cells reside (Engwerda *et al.* 2004b).

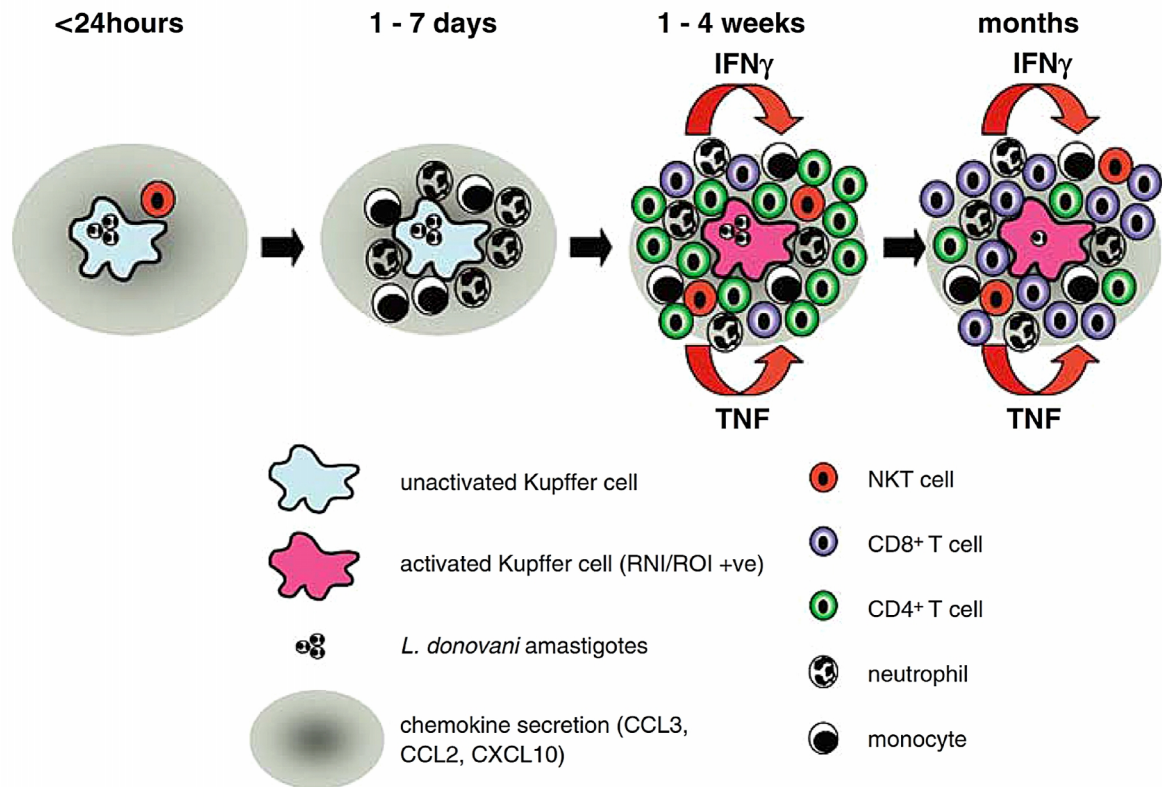


Figure 10. Kinetics of hepatic granuloma development during *L. donovani*/infantum infection in mice. Shortly after parasite inoculation, infected Kupfer cells produce a range of chemokines that recruit rapid-responding innate lymphoid cells such as iNKT cells. These play an important role in early granuloma development by providing an early source of anti-parasitic IFN- γ and by secreting further chemokines that allow the recruitment of additional immune populations. In mature granulomas, T cells predominate and, by producing TNF and IFN- γ , efficiently induce the leishmanicidal function of infected Kupffer cells. The parasite population decreases rapidly as soon as mature granulomas are formed. However, sterile cure is not achieved. Long-term immunity to relapse or re-infection is dependent on CD8 T cells. ROI- Reactive Oxygen Intermediates; RNI- Reactive Nitrogen Intermediates. Adapted from (Stanley *et al.* 2007).

Granulomas attain full maturation by 2-4 weeks after infection and hepatic parasite burden rapidly declines up to 8 weeks post-infection (Murray 2001). Importantly, sterile immunity in the liver is not achieved. However, the presence of a residual parasite population is thought to incite a small but enduring immune response that provides long-term immunity to reinfection (Stanley *et al.* 2007). In particular, CD8 T cells play a major role in the rapid resolution of hepatic re-infection (Stager *et al.* 2003a).

5.2. Compartment-specific immunity: parasite persistence in the spleen

The spleen is the major organ for initiation of the anti-*Leishmania* immune response and is also the site of parasite persistence during chronic infection (Engwerda *et al.* 2000). It has

been postulated long ago that once the splenic phase of VL starts, death is the most likely outcome (Heyneman 1971). Furthermore, splenectomy has been applied with success to cure VL (Lyngdoh *et al.* 1971, Rees *et al.* 1984, Alon *et al.* 2012). As such, a detailed understanding of the immune mechanisms that dictate parasite persistence and tissue destruction in the spleen during VL should provide valuable hints to the design of therapeutics aimed at modulating the outcome of the infection.

5.2.1. Early events in the spleen

The spleen is the body's largest blood filter. Splenic macrophages are strategically placed to remove any exogenous particle or pathogen that enters the spleen through the blood stream (Mebius *et al.* 2005). Following intravenous injection of *L. donovani*, about 95% of the parasites are phagocytized by three distinct splenic populations; red pulp macrophages, marginal zone macrophages (MZM) and marginal metallophilic macrophages (MMM) (**Figure 11**) (Gorak *et al.* 1998). Despite the wide range of strategies attributed to *Leishmania* to establish a safe niche in the host cell, macrophage populations of the spleen demonstrate a remarkable innate capacity to kill the parasite. Indeed, it is estimated that 50% of the initial parasite inoculum is killed by macrophage populations of the marginal zone within the first 24 hours after infection (Gorak *et al.* 1998). For both MZMs and MMMs this was shown to depend on IRF7 recruitment to parasite-containing phagosomes and may involve leishmanicidal mechanisms independent of NO (Phillips *et al.* 2010).

A few hours after mice infection, mature DCs appear in T cell areas at the periarteriolar lymphoid sheaths (PALS) and produce IL-12, which is crucial to initiate protective T cell responses (**Figure 11**) (Squires *et al.* 1989, Engwerda *et al.* 1998, Gorak *et al.* 1998). Priming DCs appear to not contain viable parasites supporting the notion that bystander-activation of DCs is crucial for the generation of T cell responses (De Trez *et al.* 2004, Resende *et al.* 2013). It is not clear how bystander DCs acquire parasite antigens for T cell priming. DCs may phagocytize parasite debris present in the splenic marginal zone or ingest macrophages containing digested parasites (Stanley *et al.* 2007). In mice infected with *L. donovani*, CD4 T cell activation can be detected in the first day after infection and the pool of parasite-specific splenic CD4 T cells increases several fold during the first weeks contributing to splenomegaly (Kaye *et al.* 2004, Polley *et al.* 2005b).

In CD4 T cells, IL-12 signaling leads to nuclear translocation of STAT-4 resulting in induction of the transcription factor T-bet and upregulation of IL-12 receptor (IL-12R), to prime for Th1 differentiation. T-bet, in turn, induces the cardinal Th1 cytokine IFN- γ that through autocrine signaling, activates STAT-1 and further stabilizes the Th1 lineage (Zhu *et al.* 2010). There

is substantial evidence indicating that all the components involved in Th1 differentiation are necessary for an effective response against visceral *Leishmania* (Murray 1997, Lehmann *et al.* 2000, Rosas *et al.* 2006b, Oghumu *et al.* 2014).

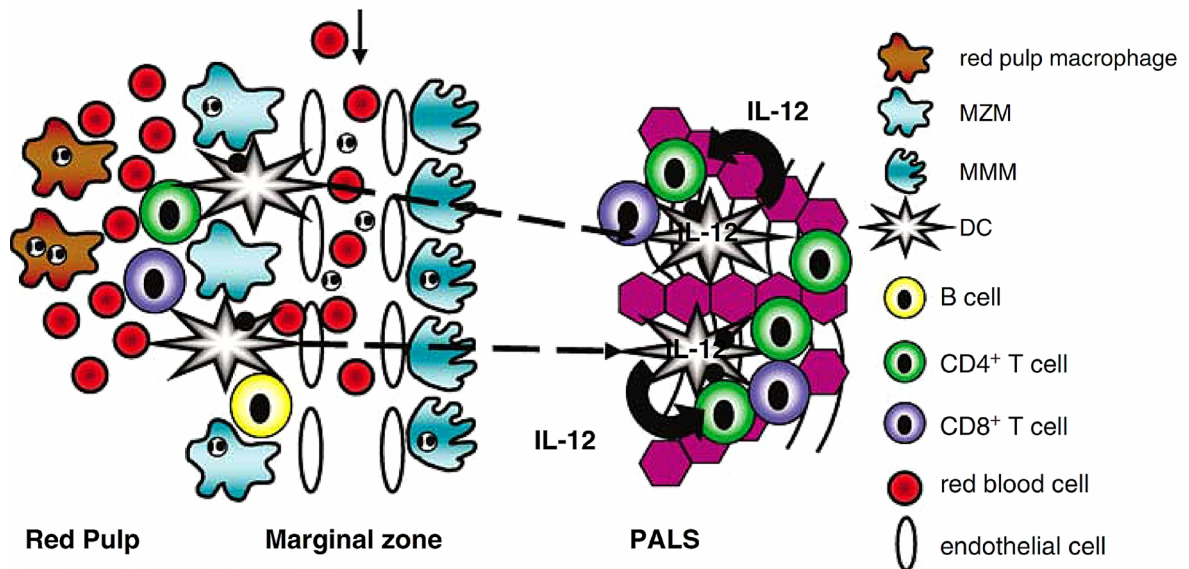


Figure 11. The splenic acute phase of experimental visceral leishmaniasis in mice. Intravenously injected parasites are efficiently removed from the circulation by distinct macrophage populations of the spleen. A substantial fraction of the parasite population is killed through innate mechanisms by splenic macrophages, originating large amounts of parasite antigen for dendritic cells to process. Mature DCs migrate to the T cell areas of the PALS and secrete IL-12 to initiate a type-I adaptive response. MZM- Marginal Zone Macrophage; MMM- Marginal Metallophilic Macrophage; DC- Dendritic Cell; PALS- Periarteriolar Lymphoid Sheath. Adapted from (Stanley *et al.* 2007).

Shortly after infection, splenic DCs are also capable of producing other members of the IL-12 family of cytokines, including IL-23p19 (Maroof *et al.* 2008b), which may pair with IL12p40 to form biologically active IL-23 (Vignali *et al.* 2012). Along with additional cytokines such as TGF- β , IL-6 or IL-1 β ; IL-23 promotes the differentiation of Th17 cells from naïve CD4 T cells (Zhu *et al.* 2010). Some evidence suggests an important protective role for Th17 cells during VL. Analysis of cytokine responses in peripheral blood mononuclear cells (PBMCs) from symptomatic and asymptomatic VL patients revealed that the expression of Th17 cytokines was strongly associated with the asymptomatic state (Pitta *et al.* 2009, Ghosh *et al.* 2013). Supporting these findings, another study noticed negligible transcript levels of Th17-associated cytokines or transcription factors in splenic tissue from patients with active disease (Ansari *et al.* 2011).

CD8 T cell-mediated immunity is equally important during experimental VL (Tsagozis *et al.* 2003, Murray 2005, Polley *et al.* 2006). The expansion of splenic CD8 T cells after infection is impressive and may reach 10-fold within a two month period (Kaye *et al.* 2004, Polley *et*

al. 2005a). Nevertheless, splenic CD8 T cell expansion and acquisition of effector/memory markers during *L. donovani* infection in mice is delayed when compared with prototypical acute viral infections (Stern *et al.* 1988, Joshi *et al.* 2009). The mechanisms underlying this delay are unknown, as the factors governing CD8 T cell differentiation remain poorly studied in VL. CD8 T cells respond to IL-12 and type I IFNs by upregulating the T-box transcription factors T-bet and Eomesodermin (Eomes). These promote the expression of type I cytokines such as TNF or IFN- γ and cytotoxic molecules such as granzymes and perforin which allow CD8 T cells to perform effector function (Kaech *et al.* 2012). There is sustained evidence pointing to an important role of CD8 T cell-derived IFN- γ in the activation of infected macrophages to control parasite growth (Tsagozis *et al.* 2003, Tsagozis *et al.* 2005, Polley *et al.* 2006). Less clear is whether CD8 T cells are capable of killing infected macrophages through cytotoxic effectors.

Splenic DCs recognize parasite DNA via TLR9 leading to rapid activation of NK cells, which provide an early source of IFN- γ (Schleicher *et al.* 2007). The activity of NK cells decreases steadily a few days after infection, highlighting their transient role during VL (Bogdan 2012). After the initial period of parasite elimination by splenic macrophages, the parasite population is maintained at a constant size throughout the next two weeks, suggesting that parasite replication is balanced by parasite killing (Engwerda *et al.* 2004a). While able to control infection, the immune response appears to be far from optimal, as it cannot eradicate the parasite. By the third week after infection, parasite load in the spleen starts to increase slowly, signaling the onset of the chronic phase. By this time, infection is already being resolved in the liver (Engwerda *et al.* 2004a).

5.2.2. Chronic VL: Regulatory cytokines and parasite persistence

Mice deficient in the immunosuppressive cytokine IL-10, or in which IL-10 signaling is blocked, are highly resistant to *L. donovani* infection (Murphy *et al.* 2001, Murray *et al.* 2003). IL-10 is found in elevated levels in the serum, splenic aspirates, lymph nodes and bone marrow of VL patients (Kenney *et al.* 1998, Nylen *et al.* 2007a, Ansari *et al.* 2011). IL-10 is produced after *Leishmania* antigen stimulation of whole blood cultures from patients with active disease, but significantly decreases after drug cure (Singh *et al.* 2012c). Neutralization of IL-10 augments IFN- γ production in whole blood assays and promotes amastigote clearance in cultured splenic cells from VL patients (Gautam *et al.* 2011, Singh *et al.* 2012c). Together, all these pieces of evidence led to the conclusion that IL-10 is the major mediator of the immunological defects observed in the spleen during chronic VL (Nylen *et al.* 2007b, Kumar *et al.* 2012).

IL-10 is a general suppressive cytokine with a range of anti-inflammatory effects in several immune lineages (Ouyang *et al.* 2011). During VL, IL-10 deactivates the leishmanicidal mechanisms of the macrophage and downregulates the expression of co-stimulatory molecules and MHC expression (Vouldoukis *et al.* 1997, Bhattacharyya *et al.* 2001, Nylen *et al.* 2007b). It also decreases the production of IFN- γ in T cells (Ito *et al.* 1999, Singh *et al.* 2012c) and inhibits DC migration to T cell areas (Ato *et al.* 2002).

IL-10 can be produced by multiple immune lineages (Saraiva *et al.* 2010, Ouyang *et al.* 2011). During VL, several cell types have been identified as sources of IL-10, including CD4 T cells, CD8 T cells, B cells, NK cells, macrophages and DCs (Peruhype-Magalhaes *et al.* 2006, Nylen *et al.* 2007a, Maroof *et al.* 2008a, Bankoti *et al.* 2012, Nandan *et al.* 2012, Resende *et al.* 2013). A major topic of interest in the past few years has been the identification of the relevant sources of IL-10. The best available evidence points to conventional IFN- γ -secreting Th1 cells as the most relevant source of pathological IL-10 during chronic experimental VL (Stager *et al.* 2006, Nylen *et al.* 2007a, Owens *et al.* 2012, Resende *et al.* 2013). IFN- γ^+ IL-10 $^+$ splenic CD4 T cells can be detected already at two weeks after parasite infection and attain a plateau by one month, representing 2 to 5% of the total splenic CD4 T cell pool (Owens *et al.* 2012, Resende *et al.* 2013).

A matter of upmost interest is to decipher the mechanisms underlying the regulatory switch that results in the induction of the double positive IFN- γ^+ IL-10 $^+$ CD4 T cells. Recent studies are unveiling a cytokinic network that works to maintain the suppressive environment during chronic VL (**Figure 12**). One study reported a role for T cell-derived IL-21 and myeloid cell-derived IL-27 in the induction of IL-10 in CD4 T cells (Ansari *et al.* 2011). Recent data points to a crucial role for DCs in promoting the regulatory switch in CD4 T cells (**Figure 12**) (Owens *et al.* 2012, Resende *et al.* 2013). Indeed, DC-derived IL-27 and IL-12 appear to be involved in the induction of IL-10 in CD4 T cells (Owens *et al.* 2012, Resende *et al.* 2013). Interestingly, the suppressive-promoting capacity is restricted to infected DCs (**Figure 12**) (Resende *et al.* 2013) and DC depletion between the third and fourth week after infection reduces pathology and enhances resistance to infection (Owens *et al.* 2012). Finally, IL-10 signaling may contribute to additional IL-10 and IL-27 secretion by the infected macrophage, to continuously fuel this suppressive loop (**Figure 12**) (Kane *et al.* 2001, Ansari *et al.* 2011). Whilst less studied than IL-10, TGF- β is another suppressive cytokine that is consistently linked with parasite persistence in VL (Barral-Netto *et al.* 1992, Wilson *et al.* 1998, Kumar *et al.* 2012). Additionally, mice resistant to *L. infantum* infection become significantly more susceptible when injected with a viral vector expressing TGF- β (Wilson *et al.* 1998).

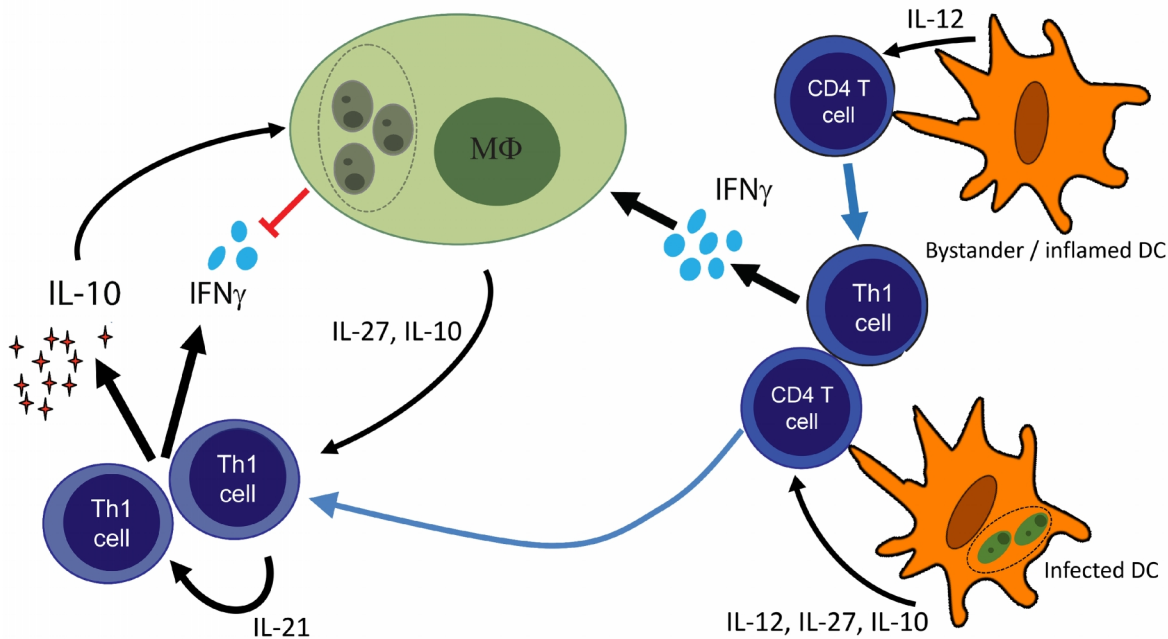


Figure 12. A regulatory circuit centered on IL-10 promotes parasite persistence in the spleen during chronic visceral leishmaniasis. Non-infected bystander and inflamed DCs secrete IL-12 and prime CD4 T cells towards a protective IFN- γ -secreting phenotype. In contrast, a mixed cytokinic profile in infected DCs characterized by IL-10, IL-12 and IL-27 promotes the differentiation of CD4 T cells capable of producing both IFN- γ and IL-10. Additionally, IL-21 produced by CD4 T cells induces, in an autocrine/paracrine manner, additional IL-10 in CD4 T cells. IL-10 reprograms the macrophage for enhanced parasite survival and renders it unresponsive to IFN- γ stimulus. The infected macrophages further contribute to the maintenance of the suppressive loop by producing more IL-10 and IL-27. Image obtained from (Kumar *et al.* 2012) and modified to include recent evidence.

5.2.3. Chronic VL: T cell exhaustion and T cell apoptosis

Chronic infections are characterized by a prominent impairment of T cell function, known as T cell exhaustion, which precludes an effective response in the long term (Wherry 2011). Exhaustion does not occur abruptly. Instead, it proceeds progressively, paralleling the increase in pathogen burden (Wherry 2011). Some functions, such as cytotoxicity, IL-2 production or proliferation are lost initially. Severe exhaustion is characterized by an inability to produce TNF, IFN- γ or to degranulate (Schieteringer *et al.* 2014). Apoptotic deletion is usually the final fate of an exhausted T cell. Nevertheless, exhausted T cells are capable of long-term survival, if their specific antigen remains present (Wherry 2011). Evidence has convincingly linked the occurrence of T cell exhaustion with progressive and sustained expression of inhibitory receptors on effector T cells (Yi *et al.* 2010). These include programmed death-1 (PD-1), cytotoxic T lymphocyte antigen-4 (CTLA-4), CD160, lymphocyte-activation gene-3 (LAG-3) and T cell immunoglobulin and mucin domain (TIM-3) (Jin *et al.* 2010, Jin *et al.* 2011, Wherry 2011). These receptors act by inhibiting T cell

effector function. PD-1 ligation, for instance, leads to recruitment and activation of tyrosine phosphatases, which efficiently dampen TCR-proximal and costimulatory signaling (Keir *et al.* 2008). T cell exhaustion was initially noticed over a decade ago in models of chronic viral infections (Gallimore *et al.* 1998, Zajac *et al.* 1998). Recent work unveiled a similar paradigm during chronic protozoan infections (Gigley *et al.* 2012), including malaria (Butler *et al.* 2012, Horne-Debets *et al.* 2013), toxoplasmosis (Bhadra *et al.* 2011), Chagas' disease (Albareda *et al.* 2009) and leishmaniasis (Esch *et al.* 2013, Gautam *et al.* 2014).

In mice infected with *L. donovani*, splenic CD8 T cells exhibit signs of functional exhaustion by the third week after infection, and severe functional impairment is evident after the fourth week, with abrogated production of IFN- γ , TNF and IL-2 and granzyme B (Joshi *et al.* 2009). Exhaustion is paralleled by increased expression of PD-1 in CD8 T cells and its ligand PD-L1 in populations of splenic DCs. Treatment with an antibody blocking the PD-1/PD-L1 interaction rescued the functionality of parasite-specific effector/memory CD8 T cells, resulting in lower splenic parasite burden (Joshi *et al.* 2009). Interestingly, the recovery of CD8 T cell effector function after α -PD-1 treatment was only partial (Joshi *et al.* 2009), suggesting that additional inhibitory receptors may contribute to the functional attrition of CD8 T cells during VL. In agreement, mice treated with a CTLA-4 blocking mAb one day after infection demonstrate significantly lower parasite burden by one month post-infection, consistent with the timing of CD8 T cell exhaustion (Gomes *et al.* 1998, Murphy *et al.* 1998). The relevance of these findings in mice has been confirmed in human VL patients, whose splenic CD8 T cells similarly exhibited functional impairment and augmented expression of PD-1 and CTLA-4. Surprisingly, blockage of either inhibitory receptor did not improve cytokine production by cultured splenic CD8 T cells from VL patients (Gautam *et al.* 2014), again suggesting the involvement of additional inhibitory receptors.

Exhaustion in CD4 T cells is less studied and less understood than in CD8 T cells (Wherry 2011). In chronic canine VL, splenic CD4 T cell exhaustion is less severe than CD8 exhaustion and appears only in aggravated clinical stages of the disease (Esch *et al.* 2013).

Apoptosis of T lymphocytes is a homeostatic mechanism that ensures that the immune system returns to the steady state once an infection has been resolved (Krammer *et al.* 2007). Activated T cells express CD95 ligand (CD95L, Fas ligand), which ligates CD95 (Fas) on the surface of the same or a neighboring activated T cell, inducing apoptosis via the death receptors, hence limiting the expansion of activated T cells. As the infectious agent is cleared, survival factors become scarce, which induces the expression of pro-apoptotic Bim in the responding T cells. Bim leads to mitochondrial outer-membrane permeabilization, an event that precedes apoptosis by the intrinsic pathway (Krammer *et al.* 2007). In acute infections, the concerted action of Fas and Bim-dependent mechanisms

ensures the deletion of most activated T cells, yet allowing the survival of small numbers of cells that will later constitute the memory pool (Krammer *et al.* 2007, Green 2008). In contrast, in chronic infections the persisting pathogen subjects T cells to continuous stimulation. In this context, a delicate control over the mechanisms of T cell death has to be ensured in order to pose sufficient immune pressure on the pathogen, but still avoiding the pathological sequelae resulting from excessive accumulation of activated T cell clones.

During VL, some evidence from human patients and experimental models suggests that T cells from the spleen or peripheral blood become more prone to apoptotic death (Potestio *et al.* 2004, Tsagozis *et al.* 2005, Banerjee *et al.* 2011, de Lima *et al.* 2012, Silva *et al.* 2013). Accordingly, Fas and FasL expression is elevated in splenic lymphocytes (Potestio *et al.* 2004). Surprisingly, ablation of *Fas* does not reduce the rate of T cell apoptosis (Alexander *et al.* 2001). A role for Bim in mediating the turnover of T cells during VL has not yet been investigated. However, in cutaneous disease caused by *L. major*, ablation of the gene encoding Bim (*BCL2L1*) provides a striking example of how T cell apoptosis negatively affects immunity to infection. Unlike WT animals, Bim-deficient mice achieve sterile cure after infection and are resistant to reinfection (Reckling *et al.* 2008).

5.2.4. Chronic VL: Loss of splenic lymphoid architecture

The most striking clinical feature of both human and experimental VL is the impressive splenomegaly (Engwerda *et al.* 2000). Concomitant with increased organ mass and size, a number of changes in the splenic microarchitecture occur (Kaye *et al.* 2004). These include disorganization of the white pulp, hypertrophy of the red pulp and disruption of the marginal zone. In the white pulp, germinal centers (GCs) disappear, and the perarteriolar lymphoid sheet (PALS) disintegrates (Smelt *et al.* 1997). Neovascularization is also prominent in both red and white pulp (Dalton *et al.* 2010, Yurdakul *et al.* 2011).

In mice, the structural changes in the spleen start as the infection enters the chronic phase (around 3 to 4 weeks post-inoculation) (**Figure 13**) (Engwerda *et al.* 2000). Disorganization of the PALS is mediated by TNF and results mainly from the loss of gp38⁺ stromal cells. These are crucial for establishment and maintenance of the PALS by producing the chemokines CCL19 and CCL21, which attract CCR7-expressing cells, mainly naïve and memory T cells that form the bulk of the PALS, but also activated DCs (Cyster 1999, Ato *et al.* 2002, Nolte *et al.* 2003). Destruction of the stromal cell network is concomitant with decreased expression of CCL19 and CCL21 and loss of reticular fibers that form the scaffold along which the PALS is organized (**Figure 13**) (Ato *et al.* 2002).

Concomitant with the disassembly of the PALS, an extensive remodeling of the splenic marginal zone also occurs, characterized by depletion of the MZM population (**Figure 13**) (Engwerda *et al.* 2002). Again, TNF appears to mediate the loss of MZMs, through a mechanism that is not clearly elucidated but may involve a direct apoptotic effect (Engwerda *et al.* 2002). It is intriguing to note that TNF, the crucial cytokine responsible for the maturation of protective granulomas in the liver, is similarly the major factor responsible for the histopathological sequelae of chronic infection in the spleen (Stanley *et al.* 2007). A final significant alteration in the lymphoid architecture of the spleen during chronic VL is the loss of follicular dendritic cells (FDCs), leading to loss of GCs and B cell follicles, which become occupied by parasitized macrophages and plasma cells (Stanley *et al.* 2007).

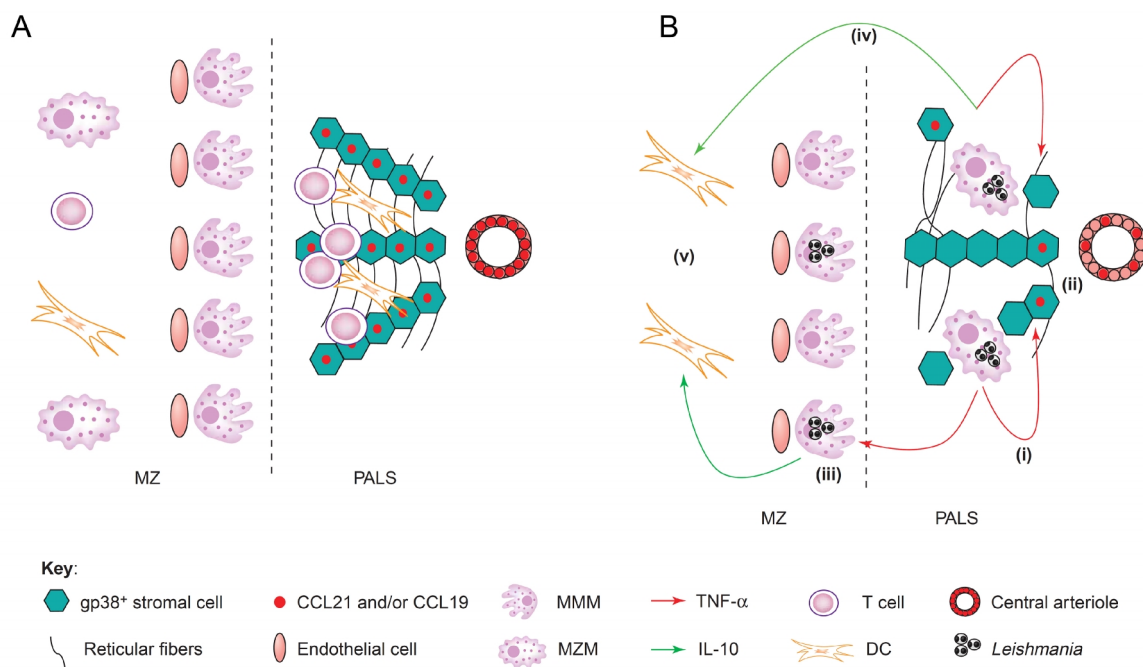


Figure 13. Extensive remodeling of the splenic micro-architecture accompanies chronic visceral *Leishmania* infection. The picture depicts a comparison between the normal splenic lymphoid architecture (**A**) and the alterations occurring during the chronic phase of visceral leishmaniasis in the spleen (**B**). Loss of gp38⁺ stromal cells in the PALS is mediated by TNF and results in disassembly of the reticular fibers that form its scaffold. Decreased production of the chemokines CCL19 and CCL21 precludes normal immune cell trafficking to the PALS. In the marginal zone, TNF also mediates the loss of MZMs. From (Engwerda *et al.* 2004a).

5.3. Humoral response

5.3.1. Germinal centers and T follicular helper cells

Most vaccines currently approved for human use rely on the induction of -specific antibodies capable of neutralizing pathogens (Tangye *et al.* 2013). The production of high affinity

antibody by B cells depends on T cells and the development of GCs (MacLennan 1994). B cells resident in the follicles of secondary lymphoid organs initially contact antigen deposited at the surface of FDCs (MacLennan 1994). Upon recognition and internalization of specific antigen, B cells transiently upregulate CCR7, gaining the ability to migrate to the border between the B cell follicle and the T cell area (**Figure 14**) (Craft 2012, Victora *et al.* 2012). Meanwhile, some CD4 T cells primed by conventional DCs in the PALS, upregulate CXCR5 and migrate from the PALS towards the follicle (Craft 2012). At the border between the follicle and the PALS, B cells present peptide antigen to activated CD4 T cells and the cognate pair engages in long-term interactions mediated by the several surface molecules (Ma *et al.* 2012). As a result of productive interactions, B cells migrate back to the follicle where they start to proliferate vigorously to form a GC (**Figure 14**) (Victora *et al.* 2012).

In germinal centers, two fundamental processes of B cell biology occur: affinity maturation and class-switch recombination, both initiated by activated-induced deaminase (AID), which cause mutations in defined regions of the Immunoglobulin (Ig) gene (Muramatsu *et al.* 2000, Zotos *et al.* 2012). Class-switch recombination allows B cells to express an antibody isotype distinct from IgM or IgD, hence tailoring antibody functions to the invading pathogen (Stavnezer *et al.* 2008). Affinity maturation results from AID-induced mutations in the variable region of the Ig genes. As a result, B cells with slightly different variable regions are produced and selected according to their relative affinities (Victora *et al.* 2012).

In essence, the GC reaction is a Darwinian selection process that occurs sequentially and over many iterative rounds (Victora *et al.* 2012). Histologically, the GC is divided into dark and light zones (MacLennan 1994). In the dark zone, B cells proliferate and undergo somatic hypermutation in the variable regions of their Ig genes. Mutations leading to a non-functional B cell receptor (BCR) result in the apoptotic deletion of the B cell. Survival of B cells requires their migration to the light zone and recognition of antigen deposited at the surface of FDCs (Tarlinton *et al.* 2000). It is theorized that high-affinity B cells internalize higher quantities of antigen and present higher quantities of peptide antigen to the CD4 T follicular helper (Tfh) cells that are present in the light zone of the GC (Victora *et al.* 2010, Victora *et al.* 2012). Due to the relatively small number of Tfh cells in the GC, only B cells with the highest affinity for the antigen are able to establish long-term interactions with Tfh cells. As such, only B cells with this competitive advantage will receive the signals necessary to either initiate another round of proliferation or, instead, exit the GC as a memory B cell or a plasma cell (Victora *et al.* 2014). Low-affinity B cells are outcompeted and unable to receive signals from CD4 T cells and die by apoptosis (Zotos *et al.* 2012).

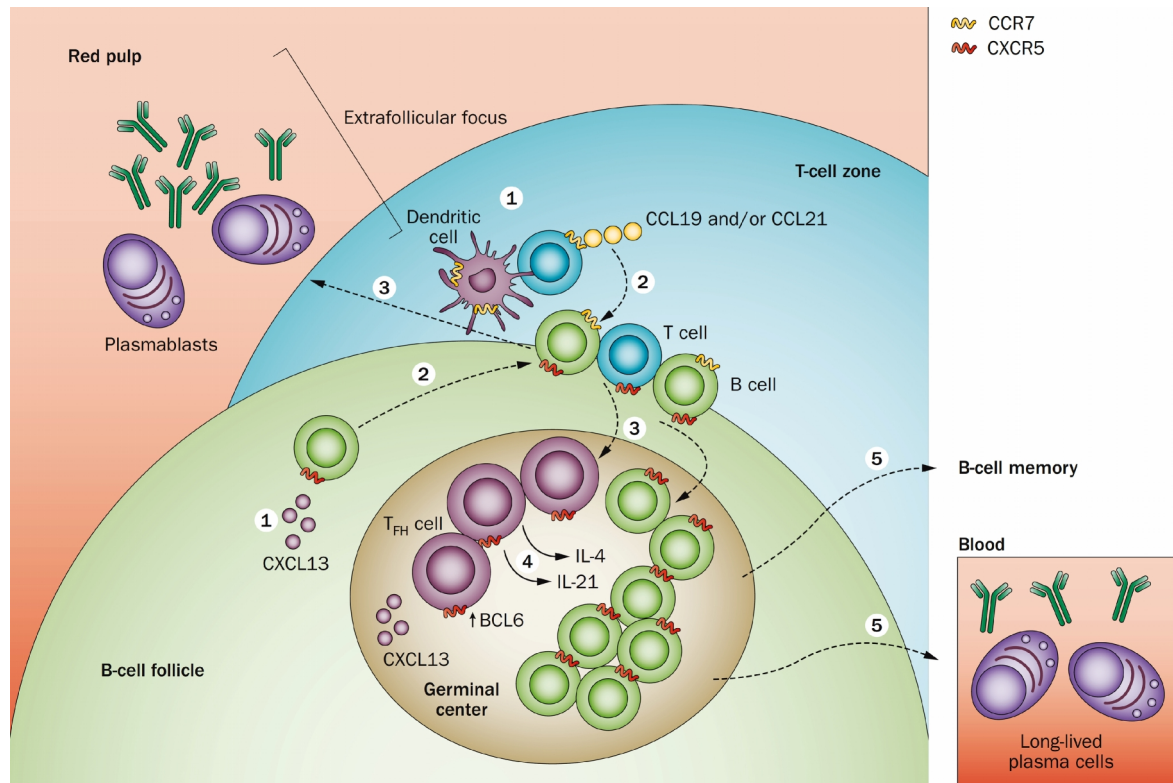


Figure 14. CD4 T cell-dependent antibody production. (1) Follicle B cells recognize and internalize antigen deposited on the surface of follicular dendritic cells. Concomitantly, CD4 T cells are primed by conventional DCs in the PALS. (2) Activated B and CD4 T cells migrate towards the border between the T and B cell areas, where they engage in cognate interactions. (3) B cells that receive productive signals from CD4 T cells can either differentiate to an extrafollicular plasmablast or migrate back to the follicle and form a germinal center. In a similar manner, CD4 T cells that engage in long-term interactions with B cells are able to infiltrate the follicle and become T follicular helper cells. (4) Inside the germinal center, B cells are selected according to their affinity for antigen, in a process mediated by CD4 T cells. (5) As a result of the germinal center reaction, B cells differentiate into long-lived plasma cells that produce high affinity antibodies and memory B cells that rapidly differentiate to plasma cells upon secondary exposure to the same pathogen. From (Craft 2012).

GC B cells possess a number of unique phenotypic and functional characteristics. For instance, they express high levels of CD95 (Fas) and bind to peanut agglutinin with high affinity. CD38 expression appears downregulated in murine GC B cells but increases in humans (Vences-Catalan *et al.* 2011). Crucial for the identity of GC B cells is their high expression of the transcriptional repressor B cell lymphoma-6 (Bcl-6) (Dent *et al.* 1997). Bcl-6 represses DNA damage sensors, such as p53, hence allowing the occurrence of somatic hypermutation. It also represses anti-apoptotic genes, to maintain a proapoptotic state in GC B cells, and antagonizes the plasma cell master regulator, BLIMP-1 (Victora *et al.* 2012). As referred, follicular helper CD4 T cells (T_{fh} cells) are crucial for the selection process occurring in GCs (Crotty 2011). As with GC B cells, Bcl-6 is the master transcription factor for T_{fh} differentiation (Nurieva *et al.* 2009). In T_{fh} cells, Bcl-6 appears to regulate genes distinct from those it controls in GC B cells, possibly due to its ability to interact with different

transcription factors in both cell types (Crotty 2011). Indeed, additional transcription factors, such as the cellular-musculoaponeurotic fibrosarcoma (c-maf), IRF4, STAT3 or basic leucine zipper transcription factor (BATF), have been identified as playing important roles in Tfh development and function (Tangye *et al.* 2013). The process of naïve CD4 T cell differentiation into a Tfh cell is complex, involving several cytokines and co-stimulatory molecules (**Figure 15**) (Linterman *et al.* 2012). It starts with the priming of a naïve CD4 T cell by DCs in the PALS. The interaction between the inducible T cell costimulator (ICOS), expressed in CD4 T cells and its ligand (ICOSL), expressed in DCs, is crucial for directing initial Tfh differentiation. ICOS engagement, acting along with cytokines that activate STAT3, such as IL-6 or IL-21, converge to induce the expression of Bcl-6 in CD4 T cells (Craft 2012). Bcl-6 upregulates the expression of CXCR5, and the primed CD4 T cell migrates towards the border between the T and B cell zones (Crotty 2011). In contrast, cytokines that use STAT5 for signaling, such as IL-2, repress Tfh differentiation by upregulating the Bcl-6 antagonist, Blimp-1 (**Figure 15**) (Nurieva *et al.* 2012).

At the T-B border, primed CD4 T cells interact with cognate B cells and commit themselves to the Tfh lineage (Crotty 2011). In fact, B cells are crucial for Tfh differentiation and Tfh cells do not develop in mice depleted of B cells (Haynes *et al.* 2007). Multiple pairs of co-stimulatory and adaptor molecules are involved in the long-lasting contacts between CD4 T cells and B cells. Of particular importance for Tfh differentiation is the ligation of ICOS, CD40L and PD-1 expressed in CD4 T cells by ICOSL, CD40, and PD-L1 expressed in B cells (**Figure 15**) (Tangye *et al.* 2013). These interactions provide signals that further stabilize the Tfh lineage. CD4 T cells at the T-B border are frequently named *pre-Tfh* cells and, upon migration to the GC, become *bona-fide* Tfh cells (Tangye *et al.* 2013).

Fully polarized Tfh cells are localized in the GC, where they mediate the selection of high-affinity B cell clones (Crotty 2011). They express the highest levels of Bcl-6 and the other Tfh-associated markers and also produce high levels of IL-21, a crucial cytokine involved in the differentiation of GC B cells into plasma cells (Ding *et al.* 2013). Strong and sustained interactions between GC B cells and Tfh cells are required for the differentiation of plasma cells, due to the strong inhibition exerted by Bcl-6 on the plasma cell master regulator Blimp-1. As such, only B cells with the highest affinity for the antigen are capable of inducing the expression of Blimp-1 to a level sufficiently high to initiate plasma cell differentiation (Victora *et al.* 2012). In contrast, the signals dictating the exit of a B cell from the GC to the memory pool are currently not understood (Crotty 2011).

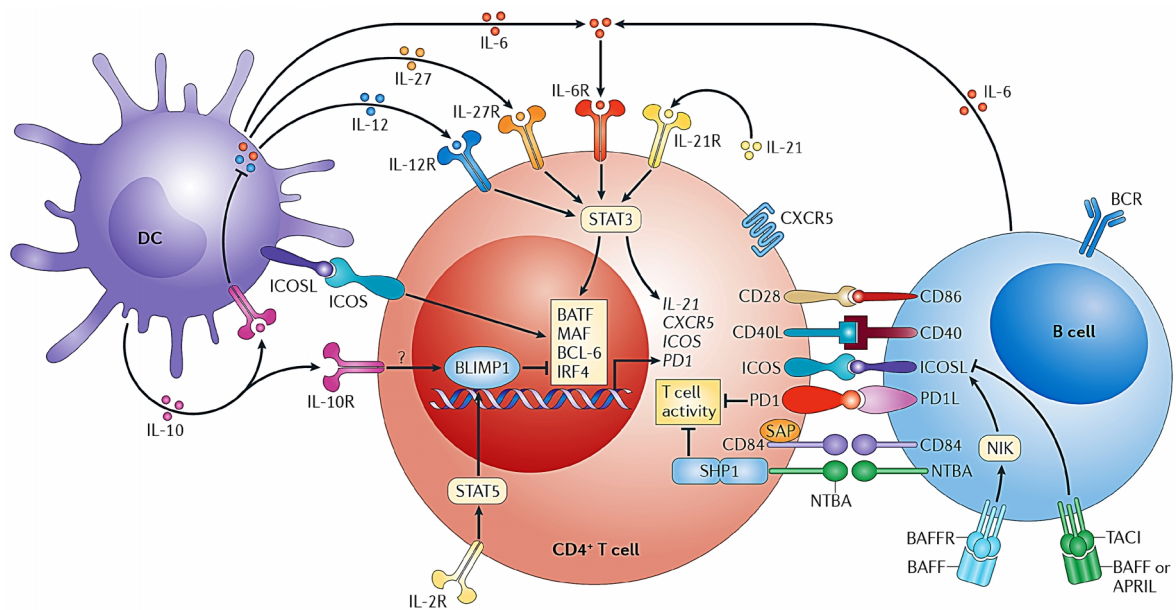


Figure 15. Differentiation of follicular helper CD4 T cells. A complex network of signals converges to induce the differentiation of Tfh cells from naive CD4 T cells. These include co-stimulatory molecules and cytokines produced by conventional DCs (priming phase) and B cells (commitment phase). From (Tangye *et al.* 2013).

5.3.2. B cells and antibodies during visceral leishmaniasis

Hypergammaglobulinemia has long been recognized as one of the cardinal signs of VL (Cooper *et al.* 1946, Campos-Neto *et al.* 1982). Early studies also demonstrated that most of the circulating IgGs are not parasite-specific, but instead result from polyclonal B cell activation (Chavas *et al.* 1966, Ghose *et al.* 1980). Indeed, autoantibodies are a recurrent finding in VL patients (Kager *et al.* 1982, Pearson *et al.* 1983, Galvao-Castro *et al.* 1984), frequently associated with proliferative glomerulonephritis (Agu *et al.* 1981). The mechanisms underlying polyclonal B cell activation are not completely understood, but may in part result from parasite-derived factors that act as B cell mitogens and induce secretion of polyreactive antibodies (Cordeiro-Da-Silva *et al.* 2001, Rico *et al.* 2002).

A number of studies demonstrated that B cell-deficient mice have enhanced resistance to visceral *Leishmania* infection as evidenced by accelerated parasite clearance in the liver and reduced chronic splenomegaly (Smelt *et al.* 2000, Deak *et al.* 2010, Bankoti *et al.* 2012). However, the augmented resistance comes at the cost of increased immunopathology, mediated by extensive neutrophil infiltration in both the liver and spleen (Smelt *et al.* 2000). Remarkably, transfer of immune serum to B cell-deficient mice alleviates pathology without affecting the rate of parasite clearance, suggesting a host-protective role for antibodies (Smelt *et al.* 2000, Bankoti *et al.* 2012).

The splenic marginal zone is populated by a particular type of innate-like B cell, termed marginal zone B (MZB) cell (Pillai *et al.* 2005). Due to the broad affinities of their BCRs, MZB cells recognize multiple microbial patterns and rapidly differentiate into antibody-producing cells (Cerutti *et al.* 2013). A recent study demonstrated that MZB cells interacted with *L. donovani* and presented parasite antigen to T cells, which led to suppression of T cell function, by a mechanism that is not mediated by antibodies and possibly involves IL-10 production by B cells. Specific depletion of MZ B cells led to enhanced CD4 and CD8 T cell responses and reduced splenic parasite burden (Bankoti *et al.* 2012).

5.4. Vaccines for visceral leishmaniasis

The development of a prophylactic human vaccine likely provides the most cost-effective approach for the control of VL (Kedzierski 2010, Hotez 2011). It is consensual that the leishmaniasis are amenable to control by vaccination, as individuals that recovered from cutaneous or visceral disease are usually protected from re-infection (Kaye *et al.* 2011b).

5.4.1. Correlates of protection

Central to the development of an effective vaccine for VL is the accurate definition of the correlates of protection. Classically, vaccine candidates for all forms of leishmaniasis have been evaluated for their capacity to induce memory CD4 T cells that produce IFN- γ upon stimulation with parasite antigen (Handman 2001). As the understanding of the immunobiology of leishmaniasis advanced, further elements have been added as surrogate markers of immunity (Costa *et al.* 2011). For instance, absence of IL-10 is now regarded as having the same importance as induction IFN- γ for the efficiency of a *Leishmania* vaccine (Kedzierski *et al.* 2006). Additional elements that are integral to a type I immune response, such as CD8-mediated immunity or the production of inflammatory cytokines as TNF, IL-2, or IL-17 should also be considered as predictive of protection (Stager *et al.* 2003b). Finally, specific antibodies capable of promoting parasite neutralization or its clearance with concomitant macrophage activation are increasingly regarded as providing an additional layer of protection (Kaye *et al.* 2011b, Das *et al.* 2012). Perhaps as important as evaluating surrogate markers of immunity in experimental models is to consider the factors that confer protection in subclinical infections (Nylen *et al.* 2009). Despite being infected, these individuals control parasite burden and do not develop disease-mediated pathology. As

such, vaccine design may benefit from lessons taken from this natural resistance (Costa *et al.* 2011).

5.4.2. Vaccines and parasite life cycle

A close inspection of the *Leishmania* life cycle provides multiple points at which vaccines may intersect and block the infection (Kaye *et al.* 2011b). Ideally, a vaccine for leishmaniasis should be capable of neutralizing the parasite at the transmission site, hence minimizing the period of host-parasite interaction and reducing the possibilities of successful colonization. These strategies aim to neutralize metacyclic promastigotes and products derived from them, such as the PSG (Kaye *et al.* 2011b). Additionally, the presence of immunomodulatory components in sandfly saliva has long been regarded as an important factor for successful *Leishmania* transmission (Oliveira *et al.* 2013) and vaccinal approaches directed against salivary proteins have been tested (Das *et al.* 2012). This issue becomes of crucial importance after the demonstration that mice previously immunized with a vaccine candidate were protected when challenge was performed by needle injection, but not when sandflies were used for parasite challenge (Peters *et al.* 2009).

On alternative, vaccines may be developed against the intracellular amastigote stage, with protection relying on T cell-mediated immunity and IFN- γ production. However, a role for antibodies should not be excluded. For instance, amastigotes are vulnerable to antibody neutralization when they egress from heavily infected macrophages (Kaye *et al.* 2011b).

5.4.3. Vaccinal approaches in leishmaniasis

5.4.3.1. Live and whole parasite vaccines

Attempts to protect individuals against leishmaniasis have been practiced since antiquity. Indeed, Bedouin and Kurdistani tribes protected their babies from facial lesions by exposing their bottoms to sandflies (Handman 2001). Throughout the 20th century, controlled leishmanization with cultured parasites was performed in the Soviet Union and Israel with high levels of protection against CL achieved (Nadim *et al.* 1983). Leishmanization, obviously, carries elevated risks, and standardization of the inoculum is hard to accomplish (Handman 2001). Leishmanization never demonstrated efficacy for VL (Kaye *et al.* 2011b), which led to a refocusing on vaccinal approaches based on inactivated whole parasites (Handman 2001). However, data compiled from field trials demonstrates that whole killed

parasites have low protective efficacy, despite their good safety profiles (Noazin *et al.* 2008). Strikingly, in mice, injection of killed promastigotes abrogates previously-established immunity to *L. major*, due to the expansion of IL-10-producing CD4 T cells at the lesion site and draining lymph node (Okwor *et al.* 2009a), which possibly explains the failure of whole-parasite killed vaccines. Nevertheless, the use of killed parasites holds promise as immunotherapeutic agents (Okwor *et al.* 2009b).

Alternatively, live-attenuated parasites may be used with the hopes of inducing a more natural and improved response that may lead to enhanced protection (Handman 2001, Silvestre *et al.* 2008, Kedzierski 2010). Importantly, the use of recombinant DNA technology to alter the parasite's genome in a targeted and controlled manner is essential to avoid the phenotype reversion that is always a risk when attenuation is performed with chemical or physical methods (Das *et al.* 2012). In the last years, several parasite lines lacking genes essential for normal physiology or virulence have been generated. Some of these mutants have been proposed as vaccine candidates for experimental VL in mice, with a few achieving promising results associated with induction of protective patterns of cytokine production (Titus *et al.* 1995, Silvestre *et al.* 2007, Selvapandiyan *et al.* 2009, Carrion *et al.* 2011). To date, none of these candidates has advanced to clinical studies.

5.4.3.2. Component vaccines

Live attenuated vaccines always carry the risk of causing disease, particularly in immunocompromised hosts, such as those infected with HIV (Das *et al.* 2012). Furthermore, vaccinal approaches based on whole parasites, either killed or alive, involve the introduction in the host of parasite factors that may have immunosuppressive activity and will possibly interfere with the development of protective immunity (Das *et al.* 2012). In this context, vaccines based on parasite fractions/components or proteins/subunits aim to circumvent these issues by reducing the complexity of the immunizing agent (Kaye *et al.* 2011b).

Selecting the parasite antigens that provide the optimal long-term protective immunity while maintaining an acceptable safety profile is the central aspect in subunit-based vaccination (Kaye *et al.* 2011b). Several techniques have been used to identify the most immunogenic parasite components. For instance, the fucose mannose ligand (FML) is the main constituent of the product Leishmune®, the first vaccine licensed for canine VL, available in Brazil (Dantas-Torres 2006). FML is a glycoprotein fraction of *L. donovani* promastigotes, discovered by chemical fractionation of the parasite in a search for ligands of macrophage receptors (Palatnik *et al.* 1989). A distinct strategy was used in the elaboration of the product

Canileish®; the first vaccine for canine VL approved in Europe, which is constituted by the excreted/secreted proteins of *L. infantum* (Holzmüller *et al.* 2005, Moreno *et al.* 2012). Despite their relative success, these two examples are still based on complex and ill-defined components of the parasite. The classical approach for antigen selection is to generate expression libraries of the parasite's genome and screen for their immunogenicity against serum or T cells from patients (Skeiky *et al.* 1995, Wilson *et al.* 1995). Following this strategy, several molecularly-defined parasite antigens have been tested as experimental vaccines against VL (Das *et al.* 2012). Despite some promising candidates, a general drawback for vaccines based on a single antigen is the relatively short duration of protective immunity (Costa *et al.* 2011). An attempt to circumvent this limitation is through the engineering of poly-component or poly-protein vaccines constituted by several immunogenic antigens. Indeed, a poly-protein resultant from the fusion of three conserved *Leishmania* antigens is the main component of the vaccine Leish-111f (Skeiky *et al.* 2002). After demonstrating promising results in mice and non-human primates (Coler *et al.* 2007), Leish-111f has recently passed phase I trials for safety and immunogenicity (Chakravarty *et al.* 2011).

DNA-based vaccines offer a number of advantages over their recombinant protein counterparts (Das *et al.* 2012). They are able to provide antigen for longer periods and are superior to protein vaccines in eliciting CD8 T cell responses. Furthermore, the host cell machinery should ensure proper folding of the protein; an aspect not always granted with recombinant expression in bacteria. Finally, the stability and long-shelf life of DNA is advantageous for large scale programs (Handman 2001). Several DNA vaccine candidates were tested for VL during the last decade. As with recombinant vaccines, multivalency appears to improve the efficacy of genetic vaccines (Das *et al.* 2012).

5.4.4. Challenges and future directions for vaccine design for VL

With the genome sequences for several *Leishmania* species publicly available, vaccinologists may now mine the parasite's genome for new antigens as vaccine candidates (Herrera-Najera *et al.* 2009). Genome sequences allow the identification of antigens conserved across multiple *Leishmania* species and that lack a homologue in mammals. Furthermore, genome sequences combined with proteomic data provide information on the levels of expression of a particular protein or whether its expression is stage dependent (Kaye *et al.* 2011b).

Vaccines for leishmaniasis should preferentially stimulate cellular immunity by generating specific memory T cells. Even considering that the humoral response is also desirable;

stimulating T cells is highly recommended, given the prominent role that CD4 T_{fh} cells play in tuning the production of high-affinity antibodies. A major challenge in the design of T cell vaccines is the enormous variability on the MHC genes among individuals (Kaye *et al.* 2011b). The candidate vaccine must provide peptides able to be presented by the most common MHC alleles, to induce maximum protection within a heterogeneous population. Several freely-available algorithms are capable of predicting T cell epitopes by calculating binding affinities to MHC molecules (Kaye *et al.* 2011b). Recent investigations are increasingly using these reverse vaccinology approaches for the identification of promising antigens across several *Leishmania* species (John *et al.* 2012, Lakshmi *et al.* 2014).

A crucial aspect in vaccine development for VL is the longevity of the memory T cells (or B cells) generated by immunization. By definition, a memory cell is one that persists long after antigen clearance and is capable of recall responses upon re-exposure to the antigen (Jameson *et al.* 2009). However, there is no minimum durability that suffices to categorize a cell as memory, and the use of surface markers to distinguish between memory and effector cells has significant limitations (Jameson *et al.* 2009). As such, progression to pre-clinical and clinical evaluation should be encouraged for vaccine candidates that provide long-term protection in experimental models. This issue is further complicated in *Leishmania* infections as it is postulated that protection from re-infection relies on the persistence of a small parasite population that residually but continuously stimulates the immune system (Uzonna *et al.* 2001, Belkaid *et al.* 2002a). Rather than being mediated by *bona fide* memory T cells, immunity to *Leishmania* results from the continuous generation of effector T cells promoted by the few persisting parasites (Sacks *et al.* 2002a). This view has been challenged, however, by studies in mice showing that long-lived parasite-specific central memory T cells are preserved in the host after antigen removal and capable of conferring protection to *L. major* re-infection (Zaph *et al.* 2004, Reckling *et al.* 2008). Whether the same antigen-independent protection is achievable in humans is unknown, but the prevailing view argues that cured patients rely on parasite persistence for life-long immunity to re-infection (Kedzierski 2010). The notion that persistent parasites are required for long-term protection is supported by the fact that component or subunit vaccines usually fail to induce long-lasting immunity (Das *et al.* 2012). Indeed, both Leishmune® and CaniLeish®, which are non-defined multicomponent vaccines, require annual boost to maintain the response. As such, some authors preconize that only live attenuated vaccines will be able to provide lifelong immunity to individuals (Costa *et al.* 2011). If it turns out to be the case, then exhaustive safety testing in pre-clinical models will be required before advancing to human trials (Kedzierski 2010). Nevertheless, component vaccines still have room for improvement, particularly with the discovery of newer and effective adjuvants capable of directing protective responses (Coffman *et al.* 2010, Das *et al.* 2012).

6. Animal models for the study of the immune response during visceral leishmaniasis

The requirements for an appropriate animal model for VL are similar to those for any other infection, or disease, in general. The model should mimic human disease in its pathophysiological components. Nevertheless, given the long duration of the disease course in humans, a model with faster disease progression might be preferable for practical reasons related with the feasibility of the experiments (Loria-Cervera *et al.* 2014). Furthermore, animals that are easier to handle and less costly are preferable, as well as those for which ample tools and reagents are available. Whenever possible, models that are natural hosts for the parasite are desirable as these should provide a more accurate representation of the host-pathogen interaction (Garg *et al.* 2006). For immunological studies, the primary requirement is a detailed knowledge of the model's immune system, and the ability to translate changes in immune populations or markers during the course of infection to their direct counterparts in humans. This aspect is intimately linked with the phylogenetic distance between the animal model and humans (Garg *et al.* 2006). In practical terms, the choice of the model is usually a compromise between the purpose of the study, the accuracy of the model in mimicking human infection and the aspects related with the costs and tools available for each model. For instance, studies aiming to dissect particular fundamental mechanisms related to the immune response benefit from models for which abundant immune tools exist. In contrast, pre-clinical studies for drug or vaccine candidates in which the main readout is reduction in parasite load or protection from re-infection, probably do not require such sophisticated tools but must closely mimic human disease (Loria-Cervera *et al.* 2014).

6.1. Mouse

Mice have been exhaustively explored as experimental models of VL throughout the past decades. Indeed, most of the knowledge presented in the previous section concerning the regulation of immunity during VL came from lessons from mice. The reasons for their wide use in VL research are certainly related to those that made mice the most successful laboratory animal. Mice are low-priced and of easy husbandry and handling. Their small size allows the housing of large numbers of animals in a single facility. The existence of inbred strains with genetically similar individuals eliminates confounding factors related with the host background, allowing a more straightforward interpretation of experimental data (Szatkiewicz *et al.* 2008). Furthermore, the mouse immune system has been exhaustively

studied; something made possible after the development of the most sophisticated tools for immunological studies. Indeed, a vast panel of antibodies exists for the detection of numerous mouse antigens, allowing the characterization of immune populations to the finest detail. Also, the ability to manipulate the mouse's genome led to the development of transgenic animals of increasing complexity, including conditional or lineage-restricted gene knock-outs (Babinet 2000, Jaisser 2000).

Outbred laboratory mice are generally resistant to infection by visceral *Leishmania* species (Garg *et al.* 2006). In contrast, the use of inbred animals allowed the delineation of susceptible and resistant strains. Importantly, in the context of VL, susceptibility does not mean fatal infection. Instead, the terms resistance and susceptibility refer to the ability of the host to rapidly control parasite load (Wilson *et al.* 2005). For instance, resistant strains as C3H or CBA are capable of limiting parasite growth very rapidly and do not develop any infection-mediated pathology. In contrast, in susceptible Balb/c or C57Bl/6 mice, parasites replicate rapidly in the liver but are ultimately controlled with the development of adaptive immunity. As described before, these animals are unable to eradicate the parasite from the spleen and bone marrow, developing a life-long latent infection that, in contrast with human disease, is not fatal (Wilson *et al.* 2005). Susceptibility to VL in mice is genetically determined and has been mapped to the *Slc11a1* gene that encodes the transporter NRAMP1 (Bradley 1974, Wilson *et al.* 2005). Resistant strains express a functional NRAMP1, whereas in susceptible mice, a point mutation renders the pump non-functional (Vidal *et al.* 1995). Polymorphisms in *Slc11a1* have also been linked with susceptibility to VL in humans as previously referred (Mohamed *et al.* 2004). However, in contrast with susceptible mouse strains, human polymorphisms are confined to the promoter region of *Slc11a*, which translates into altered protein expression rather than a non-functional protein (Mohamed *et al.* 2004).

Clearly, the main limitation of the mouse model is its inability to reproduce fatal human VL. Nevertheless, studies in mice have been instrumental in predicting or confirming many of the immune factors that regulate the disease in humans (Nylen *et al.* 2007b, Stanley *et al.* 2007). Mice naturally infected with *L. infantum* or *L. donovani* have been identified (Mohebbi *et al.* 2004, Helhazar *et al.* 2013), suggesting that the interaction mouse / *Leishmania* does not occur solely in the laboratory and this host / pathogen pair has co-evolved. Interestingly, as with animals infected in the lab, naturally-infected mice had parasites in the liver, spleen and skin but demonstrate a healthy body condition (Helhazar *et al.* 2013). This asymptomatic long-term infection combined with the presence of parasites in the skin suggests a role for mice in maintaining the transmission cycle (Helhazar *et al.* 2013).

6.2. Hamster

The Syrian or golden hamster (*Mesocricetus auratus*) demonstrates an exquisite susceptibility to a number of intracellular pathogens and has long been recognized as an excellent model for the pathophysiology of human VL (Harrison *et al.* 1946, Fulton *et al.* 1950). Intracardiac injection of a high dose of *L. donovani* or *L. infantum* results in the typical clinical signs of VL in less than 3 months, as evidenced by marked weight loss, splenomegaly, anemia, hypergammaglobulinemia, immune complex-mediated glomerulonephritis and disseminated amyloidosis (Lafuse *et al.* 2013). At advanced stages of the disease, the animals exhibit severe cachexia and asthenia and eventually succumb (Wilson *et al.* 2005). Unlike mice, hamsters are completely unable to control parasite replication in the liver, spleen, bone marrow and lymph nodes (Dea-Ayuela *et al.* 2007). However, gene expression analysis demonstrated a strong induction of type I cytokines, such as IFN- γ , TNF or IL-2 as early as the first week after infection. These are balanced by the induction of regulatory IL-10 and TGF- β by the third to fourth week after infection (Melby *et al.* 2001). As the disease advances, a strong suppressive environment is established in infected tissues as evidenced by impaired T cell proliferation to common mitogens (Evans *et al.* 1990). In lymph nodes, this T cell anergy was recently shown to be mediated by TGF- β (Banerjee *et al.* 2011). Perhaps more relevant, the lack of control over parasite growth in hamster results from an inability to induce iNOS expression in macrophages upon IFN- γ engagement (Perez *et al.* 2006). Further work described that differences at the *NOS2* promoter of mice and hamsters account for the much reduced expression of iNOS in hamster macrophages (Perez *et al.* 2006, Saldarriaga *et al.* 2012). Such observation is remarkable given that human macrophages also produce less NO than mouse macrophages upon IFN- γ + LPS stimulation and the defect has been similarly attributed to differences at the *NOS2* promoter (Zhang *et al.* 1996).

From all the above considerations, it seems clear that the hamster model should be the first choice for studies on the immunopathology of VL. However, the striking lack of immune reagents for hamsters, such as antibodies specific for hamster antigens or recombinant cytokines precludes its wide use as a tool for VL studies (Loria-Cervera *et al.* 2014). Furthermore, few developments have occurred towards the generation of transgenic hamsters. Nevertheless, recent efforts, acknowledging the remarkable physiological and metabolic similarities between the golden hamster and humans, are starting to optimize protocols for the generation of transgenic hamsters (Gao *et al.* 2014). For the reasons exposed, nowadays, hamsters are used in VL research mostly as an abundant source of amastigotes for infection in other species.

6.3. Dog

Canine VL is a serious veterinary problem, and the dog is the main reservoir of zoonotic VL in the Mediterranean basin and Latin America (Alvar *et al.* 2004, Otranto *et al.* 2013). Similar to human disease, canine VL has a long asymptomatic period (Garg *et al.* 2006). Furthermore, disease manifestation in naturally-infected dogs is highly diverse, ranging from a total asymptomatic condition to a systemic disease that often leads to death (Reis *et al.* 2009, Otranto *et al.* 2013). Initial signs are revealed by hypertrophy of the lymph nodes and dermatitis. Full blown disease is characterized by some of the cardinal signs of human VL such as fever, splenomegaly, weight loss, hypergammaglobulinemia or loss of lymphoid architecture in the spleen (Alvar *et al.* 2004). Nevertheless, canine VL produces a number of unique symptomatic manifestations, including several skin disorders, keratoconjunctivitis that may progress to total blindness, nasal discharge or muscular atrophy (Alvar *et al.* 2004, Nicolato Rde *et al.* 2013). Another singular characteristic of canine VL is the existence of a large proportion of animals that exhibit partial expression of the disease, the so-called oligosymptomatic (Reis *et al.* 2009). These animals, which may represent about one-third of a naturally-infected population, usually exhibit ulceration of the skin at the tip of the ears and other cutaneous disorders, but appear normal otherwise (Mancianti *et al.* 1988, Reis *et al.* 2009, Travi *et al.* 2009).

Parasite colonization of several organs underlies some of the peculiar manifestations of canine VL. Indeed, parasites are usually observed in the skin, kidneys, heart, lung or cerebrospinal fluid, besides more common locations, such as the spleen, liver or bone marrow (Alvar *et al.* 2004, Alves *et al.* 2010). Furthermore, there is a strong correlation between the level of parasite load in the organs and the clinical status of the animals (Reis *et al.* 2006).

Varying degrees of susceptibility to experimental infection with visceral *Leishmania* have been described among distinct dog breeds (Abranches *et al.* 1991). Inbred animals are generally more susceptible (Alvar *et al.* 2004), but even within a particular breed, the result of the infection is highly variable, which is an important limitation for an experimental model (Alvar *et al.* 2004, Reis *et al.* 2009).

Immunologically, canine VL is characterized by high levels of type I cytokines such as IFN- γ and TNF are present in infected tissue during infection. Suppressive factors such as IL-10 and TGF- β appear to promote parasite persistence and chronicity (Reis *et al.* 2009).

In-depth studies of VL in dogs have been hampered by the lack of appropriate immune tools. Fortunately, during the last years, many monoclonal antibodies against canine antigens have been generated, and many cytokines and chemokines have been cloned and expressed (Cobbold *et al.* 1994, Moreno *et al.* 2002). With these tools in hand, descriptive

studies of increasing complexity have been performed with the aim of characterizing the immune populations and factors that mediate resistance and susceptibility during VL in dogs (Reis *et al.* 2009). However, widespread use of the model has been hampered by the inherent difficulties of doing experimental work with dogs, related to the difficulties in husbandry, housing and handling. These aspects limit the number of animals per experimental group, which also decreases the statistical power of the experiments (Moreno *et al.* 2002).

6.4. Non-human primate

Arguably, non-human primates provide the best possible animal models of human disease. The close phylogenetic relationship and the similar physiological and anatomical systems often result in analogous pathology in a disease state (Kennedy *et al.* 1997, Gardner *et al.* 2008). In the context of infectious diseases, the use of great apes has been discontinued, mostly due to ethical reasons (Evans *et al.* 2013). In contrast, old world macaques have been extensively explored over the past century as models of infections caused by bacteria, virus, parasites or prions (Gardner *et al.* 2008).

Experimental infections in macaques provide models of remarkable similarity with human disease and in which invasive studies can be performed. Also, macaque models allow for the control of variables that are impossible or extremely difficult to assess in human patients, such as the inoculum size or the time after infection, as human patients only seek medical attention once the symptomatic phase starts. In this sense, macaques provide a window to the early events after an infection, which are usually not available in patients (Ambrose *et al.* 2007). Furthermore, their relative large size, compared to mice, allows a single animal to be sampled for blood or tissue several times over the course of an experiment, which is of crucial importance in longitudinal experiments (Gardner *et al.* 2008).

A major boost in the use of macaques for studies on the immunology to infection was driven by the emergence of the AIDS pandemic. In the mid-80's and in the face of a new and deadly disease caused by HIV infection, a quest was made to find an animal model capable of recapitulating the condition in humans (Lifson *et al.* 2012). Soon after, it was discovered that Asian macaques infected with the HIV closely-related simian immunodeficiency virus (SIV) developed a progressive immunodeficiency similar to AIDS (Benveniste *et al.* 1986, Murphey-Corb *et al.* 1986). Since then, countless studies with SIV-infected macaques have been instrumental in characterizing the immune events that lead to AIDS (Sui *et al.* 2013). In parallel, much work was made to characterize the macaque immune system and many

reagents and tools specific for macaque have been developed (Pitcher *et al.* 2002, Gansuvd *et al.* 2008, Kuhrt *et al.* 2011, Onabajo *et al.* 2013).

Concerning *Leishmania* infections, the use of non-human primates has been mostly limited to the pre-clinical evaluation of some drug or vaccine candidates (Porrozzi *et al.* 2004, Grimaldi *et al.* 2010). With respect to vaccine testing, macaques have served as models to test the safety, immunogenicity or protective capacity against challenge of vaccine candidates (Grimaldi Jr 2008).

There are few published studies describing natural infections by *Leishmania* in macaques, but the available ones nevertheless shown that these animals can be natural hosts of the parasite (Herrer *et al.* 1973, Lainson *et al.* 1988, Souza *et al.* 2014). More commonly, disease is induced by experimental infection. Macaques demonstrate a wide range of susceptibilities to visceral *Leishmania* infection. Furthermore, the magnitude of disease expression varies with the parasite strain, dose or route of inoculation (Grimaldi Jr 2008). On general manner, New World simians appear to be more susceptible. Indeed, the owl monkey (*Aotus trivirgatus*), the squirrel monkey (*Saimiri sciureus*) or the common marmoset (*Callithrix jacchus*) demonstrate an exquisite susceptibility to infection by parasites of the *L. donovani* complex and succumb rapidly to fulminating infections (Chapman *et al.* 1981a, Chapman *et al.* 1981b, Marsden *et al.* 1981). These species have nevertheless been useful for drug testing (Madindou *et al.* 1985, Berman *et al.* 1986).

Attempts to produce experimental VL by injecting visceral *Leishmania* into Old World monkeys resulted in highly variable disease manifestation. For instance, experimental infection with *L. donovani* in baboons (*Papio cynocephalus*) or the Sykes monkey (*Cercopithecus mitis*) resulted in a large proportion of “failed” infections and these have been proposed as models of asymptomatic human infection (Githure *et al.* 1986). However, a few Old World macaques developed, upon experimental infection with *L. donovani* or *L. infantum*, a chronic condition sharing many features with VL (Grimaldi Jr 2008). These included the Indian langur monkey (*Presbytis entellus*) (Dube *et al.* 1999), the African green monkey (*Chlorocebus sabaeus*) (Binhazim *et al.* 1993) and the rhesus macaque (*Macaca mulatta*) (Porrozzi *et al.* 2006).

The rhesus macaque is widely used in biomedical research as it is not an endangered species and is relatively easy to keep in captivity (Clarence *et al.* 2006). It is the most frequently used primate species in both AIDS and leishmaniasis research (Gardner *et al.* 2008, Grimaldi Jr 2008).

When infected intravenously with *L. infantum* amastigotes, rhesus macaques manifested fever, weight loss, hypergammaglobulinemia and anemia, but the occurrence of hepatomegaly and splenomegaly was variable. Parasites disseminated to the spleen, liver and bone marrow and hepatic granulomas were apparent as early as 2 weeks after

infection. In the spleen, disorganization of the white pulp was also visible as well as elevated levels of serum IFN- γ (Porrozzzi *et al.* 2006). Apart from this work, no other study has so far investigated immunological markers during visceral *Leishmania* infection in macaques. The main limitations of rhesus macaques as experimental models are related with the costs and the difficulties in the husbandry of these animals (Bennett *et al.* 1995). In 1959, Russel and Burch proposed a series of guidelines for the ethical use of animals in research, based on the principle of the 3Rs (Replacement, Reduction and Refinement) (Russell *et al.* 1959). In this context, macaques, and non-human primates in general, are the laboratory animals for which the concept of the 3Rs is more strictly applied (Bennett *et al.* 1995). Current legislation only supports research with non-human primates when no other model is available (European Comission 2010). While the need for non-human primates in AIDS research is clear and widely accepted, it may not be so obvious for VL, where alternative models are available. However, as outlined above, all currently-used models in *Leishmania* research have limitations and failed to provide effective new drugs and vaccines. Given the neglected status of the leishmaniasis, the use of non-human primates to study the host-pathogen interaction during VL may offer new insights into the regulation of immunity during infection. These models should also provide improved correlates of protection for the development of newer vaccines and a very relevant platform for the test of promising drug candidates

Chapter II | Objectives

The mouse model of VL has been instrumental in characterizing numerous aspects of the immune response during VL. Unfortunately, it is unable to faithfully mimic the progressive and fatal disease that affects humans. As such, a more frequent employment of alternative experimental models is imperative as a mean to complement the valuable research performed in mice. While the Syrian hamster develops a condition that closely mimics human disease, it lacks immunological reagents and tools, which precludes its use in studies aiming to characterize immune mechanisms beyond the simple description of the main immune populations. In dogs, VL has unique features and, as such, caution has to be taken when translating observations from canine VL to human disease.

Enormous progress has been made over the past decades in the characterization of the rhesus macaque immune system and numerous infectious models employing macaques have been developed (Gardner *et al.* 2008). Unfortunately, such has not been the case for VL with only a few studies taking advantage of the close phylogenetic relationship of these animals with humans. Nevertheless, the available studies suggest that, at the clinical level, the rhesus macaque may provide a very attractive model for fundamental studies on the regulation of immunity during VL (Grimaldi Jr 2008). These observations incited us to develop a non-human primate model for VL taking advantage of the vast experience on macaque models of infectious diseases accumulated in our group over the years (Estaquier *et al.* 2000, Hurtrel *et al.* 2005, Campillo-Gimenez *et al.* 2010).

Immune events at the early phase of infection, which are usually not accessible in human studies, may have a defining impact on the immunological defects during chronicity. As such, we allocated a significant proportion of the experimental animals to obtain information on these initial stages and to integrate these observations while interpreting the immune dysfunctions occurring at the chronic phase.

Most studies in VL in mice focus in a single infected organ. Taking advantage of the abundant availability of biological material in rhesus macaques, we recovered and analyzed the main organs known to be involved in human VL pathogenesis with the aim of obtaining a complete picture of VL in macaques.

Our initial objective, while elaborating this work, was to confirm the suitability of the rhesus macaque as a host model that closely mimics the symptomatology of human VL. Such purpose was pursued through close clinical surveillance of the animals, hemogram elaboration, quantification of the parasite burden in parasitized organs over the course of infection and histological observation of infected organs.

Our second major aim was to obtain a detailed picture of CD4 and CD8 T cell populations over the course of infection, with respect to their differentiation phenotype, cytokine production and localization in infected organs.

Barely anything is known about the kinetics of germinal center development during VL. Furthermore, longitudinal studies examining B cell differentiation during the course of infection are also missing, and the dynamics of Tfh cells remains elusive. Such studies would be very informative and could provide mechanistic insight into the observed defects in the humoral response occurring during the disease, such as hypergammaglobulinemia. As such, our third major goal in this work was to elucidate the cellular mechanisms underlying the dysfunctional humoral response during VL, by exploring the dynamics of B and Tfh cells over the course of infection.

Chapter III | Results

Article 1:

Abortive T Follicular Helper Development Is Associated with a Defective Humoral Response in *Leishmania infantum*-Infected Macaques

PLOS Pathogens 2014, 10(4):e1004096

In this first article, we introduce a macaque model for visceral leishmaniasis, based on the intravenous injection of a high dose of *L. infantum* promastigotes. Our aim was to confirm the suitability of the rhesus macaque as a host model for visceral *Leishmania* infections, at the clinical and immunological level. Additionally, by following longitudinally the B cell and Tfh cell responses we aimed at providing a cellular basis for the defects observed in the humoral response during VL.

Main findings

- In the early stages of *L. infantum* infection of rhesus macaques, parasites appear contained in visceral compartments (spleen and liver). At the chronic phase, however, the parasite population disseminated to the bone marrow and peripheral lymph nodes;
- *L. infantum* infection elicits an acute phase response characterized by transient neutrophilia and elevated serum levels of acute phase proteins and chronic hepatocellular damage in rhesus macaques, as denoted by elevated levels of serum alanine transaminase at the chronic phase of infection;
- *L. infantum* infection of rhesus macaques drives the expansion and differentiation of splenic CD4 T cells, which exhibit a Th1-polarized cytokinic profile during the early stages of infection. However, at the chronic phase their molecular signature is characterized by elevated levels of *IL10*;
- Infection leads to prominent production of non-specific IgG and poor specific humoral responses. Concomitantly, B cell maturation appears defective and the development of germinal centers is curtailed;
- The transient expansion of a CXCR5⁺BCL6⁺ splenic CD4 T cell population that localizes to B cell areas is associated with memory B cell differentiation and parasite-specific antibody production. The loss of these populations at the chronic phase underlies the short-lived specific-IgG response;



Abortive T Follicular Helper Development Is Associated with a Defective Humoral Response in *Leishmania infantum*-Infected Macaques

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Abstract

Leishmania infantum causes a chronic infectious disease named visceral leishmaniasis (VL). We employed a non-human primate model to monitor immune parameters over time and gain new insights into the disease. Rhesus macaques were infected with *L. infantum* and the T helper and B cell immunological profiles characterized during acute and chronic phases of infection. Parasite detection in visceral compartments during the acute phase was associated with differentiation of effector memory CD4 T cells and increased levels of Th1 transcripts. At the chronic phase, parasites colonized novel lymphoid niches concomitant with increased expression of *IL10*. Despite the occurrence of hypergammaglobulinemia, the production of parasite-specific IgG was poor, being confined to the acute phase and positively correlated with the frequency of an activated memory splenic B cell population. We noticed the expansion of a splenic CD4 T cell population expressing CXCR5 and Bcl-6 during acute infection that was associated with the differentiation of the activated memory B cell population. Moreover, the number of splenic germinal centers peaked at one month after infection, hence paralleling the production of specific IgG. However, at chronic infection these populations contracted impacting the production of parasite-specific IgG. Our study provides new insights into the immune events taking place in a physiologically relevant host and a mechanistic basis for the inefficient humoral response during VL.

Citation: Rodrigues V, Laforge M, Campillo-Gimenez L, Soundaramourty C, Correia-de-Oliveira A, et al. (2014) Abortive T Follicular Helper Development Is Associated with a Defective Humoral Response in *Leishmania infantum*-Infected Macaques. PLoS Pathog 10(4): e1004096. doi:10.1371/journal.ppat.1004096

Editor: Guido Silvestri, Emory University, United States of America

Received: May 11, 2013; **Accepted:** March 12, 2014; **Published:** April 24, 2014

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Funding: This work was supported by an ANR (Agence Nationale de la Recherche) grant (LeishApo) and the Seven Framework Programme (KINDReD) to JE. JE thanks the Canada Research Chair program for financial assistance. VR is supported by a doctoral fellowship from FCT (Fundação para a Ciência e Tecnologia); code SFRH/BD/64064/2009. RS is supported by Programa Ciência – financed by Programa Operacional Potencial Humano POPH – QREN– Tipologia 4.2 – Promoção do Emprego Científico, co-funded by Fundo Social Europeu and National funding from Ministry of Science, Technology and Higher Education (MCTES). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Visceral leishmaniasis (VL) is a chronic and potentially fatal infectious disease caused by the protozoan parasite *Leishmania infantum*/*chagasi* or *L. donovani*. The generalized spread of the parasite to the reticuloendothelial system (spleen, liver and bone marrow) results in a clinical picture including weight loss, cyclic fever, hepatosplenomegaly, anemia and hypergammaglobulinemia [1]. Studies in murine models revealed that recovery from VL is crucially dependent on the development of a robust cellular-mediated immunity, with the production of cytokines such as IFN- γ and TNF as central components of the protective response [2]. In contrast, parasite persistence and chronicity are associated with the induction of immune suppressive mediators, such as IL-10 and TGF- β [3,4].

Inbred mice strains invariably control infections with viscerotropic *Leishmania* species and develop a life-long latent infection [5],

contrasting with the potentially fatal human VL in which progressive illness develops, even in the presence of detectable levels of IFN- γ and TNF in lesional tissue [3,6–8]. Therefore, despite the notable usefulness of murine models, new insights into the immunopathogenesis of VL would potentially benefit from a more frequent employment of alternative animal models [9]. Non-human primates (NHP) constitute powerful experimental models for understanding host-pathogen interactions that are not directly observable in human patients, particularly the early events after infection which are usually poorly characterized in humans [10–12]. Concerning leishmaniasis, the Asian rhesus macaque has already been shown to mimic human VL [13], and NHP models are routinely used for pre-clinical evaluation of novel drug and vaccine candidates [14].

The role played by antibodies and B cells during leishmaniasis has always been contentious. High titers of both *Leishmania*-specific

Author Summary

We introduced a non-human primate model for visceral leishmaniasis by intravenous injection of *L. infantum* promastigotes in rhesus macaques and followed the animals for a period of eight months. In this model, parasites dock to the liver and spleen shortly after inoculation and remain in these visceral compartments during all the acute phase of infection. However, at the chronic phase, additional body locations appeared colonized (lymph nodes, bone marrow). During the acute phase, a Th1-polarized CD4 T cell response develops in the spleen, but, and concomitant with parasite growth, it waned at the chronic phase. Furthermore, we observed the acute expansion of a splenic T follicular helper (Tfh) cell population, a CD4⁺ T cell subset specialized to assist B cells in the production of antigen-specific antibody. These cells were localized in close association with B cell follicles but, interestingly, the Tfh population is lost at the chronic phase. Nevertheless, there was a close association between the development of Tfh cells and the differentiation of B cells that produce *L. infantum*-specific IgG. Thus, our results suggest that Tfh cells are important in instructing B cells to produce parasite-specific antibodies during VL, but their abortive differentiation precludes the continuous production of specific-IgG.

and non-specific antibodies are a recurrent finding in patients [15], implying the development of strong humoral response during infection. While several reports in mice models have revealed an increased resistance to infection upon B cell depletion [16–18], others have proposed protective roles for B cells and/or antibodies [19,20].

Upon binding and internalization of specific antigen, B cells generally depend on cognate interactions with CD4 T cells to differentiate into antibody-producing plasma cells (PCs) [21]. The activated B cell can either follow the follicular pathway and form a germinal center (GC), or differentiate into an extra-follicular focus of immunoglobulin (Ig)-secreting PCs. While the GC pathway generates long-lived memory B cells as well as PCs that produce antibodies with high affinity for the antigen, the extra-follicular pathway is generally associated with short-lived plasmablasts and PCs that secrete antibodies of modest affinity, but nevertheless provides an early source of antibody that might be critical during infection [21,22]. Recent studies have greatly increased our knowledge on the biology of GC-associated CD4 T cells, also known as T follicular helper cells (Tfh). Tfh cells are phenotypically characterized by expression of the follicular-homing chemokine receptor CXCR5, the transcriptional repressor Bcl-6 and an array of surface molecules that include ICOS, CD40L and PD-1 [23,24]. These cells produce high levels of IL-21, a crucial mediator in the development of affinity-matured and class-switched B cells, as well as in the differentiation of long-lived plasma cells [21,25]. In contrast, much less is known about the functional and phenotypic characteristics of the CD4 T cell helpers associated with extra-follicular antibody responses. Furthermore, infections with several types of pathogens, including *Leishmania spp.*, induce strong polyclonal B cell activation, in a process independent of T cell help, that results in copious secretion of non-specific and potentially auto-reactive antibody [26]. Despite the strong humoral response that is usually associated with VL, the mechanisms underlying antibody production remain poorly explored.

To address these questions we performed a detailed immunological analysis in rhesus macaques infected with *L. infantum*.

Tracking the CD4 T cell responses overtime revealed that parasite containment in visceral compartments during the acute phase was associated with the differentiation of splenic CD4 T cells and their increased expression of Th1-related transcripts. The acute expansion of a splenic CD4 T cell population expressing CXCR5 and Bcl-6, but not PD-1, was associated with the differentiation of activated memory B cells and production of parasite-specific IgG. These cells were localized in B cell areas and closely paralleled the development of germinal centers. In the chronic phase, parasite dissemination and growth were concomitant with *IL10* mRNA accumulation in lymphoid tissues. Furthermore, the splenic CXCR5⁺Bcl-6⁺ CD4 T cell population contracted, which was paralleled by loss of the activated memory B cells, impacting the production of parasite-specific antibodies, despite the chronic persistence of hypergammaglobulinemia.

Results

Parasite load dynamics and pathology in *L. infantum*-infected rhesus macaques

First, we monitored over time the progression of a variety of parameters in our model of rhesus macaques intravenously infected with a high dose of *L. infantum* promastigotes. Parasite load was assessed during the course of infection employing a quantitative PCR (qPCR) assay [27]. Parasite clearance was evident in the blood during the first weeks of infection, with a steady decrease in parasitemia from about 400 parasites per million of host cells at day 7 post-infection (pi), to less than 20 at day 28 (Fig. 1A). Yet, blood parasite numbers rebounded as the infection progressed towards late stages, being, by day 250 pi, at a level comparable to that of day 7 and significantly higher than at day 28 pi (Fig. 1A, $P < 0.05$).

Parasites were hardly detectable in lymph nodes (LNs) during the first weeks of infection (Fig. 1B). However, a significant increase in the parasite burden occurred during the chronic phase ($P < 0.001$), revealing a pattern of parasite growth and/or infected cell migration to the LNs during chronic infection (Fig. 1B). In the bone marrow (BM), the parasite load kinetics was reminiscent of that found during the early weeks in peripheral blood, with an apparent early clearance phase resulting in a scarcity of parasites by day 28 pi. Similarly to the situation in LNs, a significant increase in parasite burden was found as the infection advanced towards the chronic phase ($P < 0.001$; Fig. 1C).

In the spleen and liver the parasite burden remained relatively constant during the acute phase (Fig. 1D–E). Yet, during chronic infection we found an increase in parasite numbers in these organs, albeit not statistically significant (Fig. 1D–E).

Weight loss and intermittent fever were not observed in infected animals during the time-course of our experiments (not shown). Yet, the animals developed a transient state of anemia during the first weeks after inoculation, with a reduction in erythrocyte number (Fig. 2A), hematocrit values and blood hemoglobin (Fig. S1A–B). Additionally, an early and transient neutrophilia was detected (Fig. 2B), accompanied by increased levels of serum markers of acute phase response such as C-reactive protein (CRP; Fig. 2C) and the complement factors C3 and C4 (Fig. 2D–E). Hepatocellular damage was detected at late stages of infection, as revealed by elevated serum levels of alanine transaminase (ALT; Fig. 2F), albeit without any signs of biliary tract disease, as indicated by normal levels of γ -glutamyl-transaminase (Gamma-GT) and total bilirubin (TBil, Fig. S1C–D) or the absence of hepatic synthetic function abnormalities (normal levels of serum albumin and total serum protein; Fig. S1E–F).

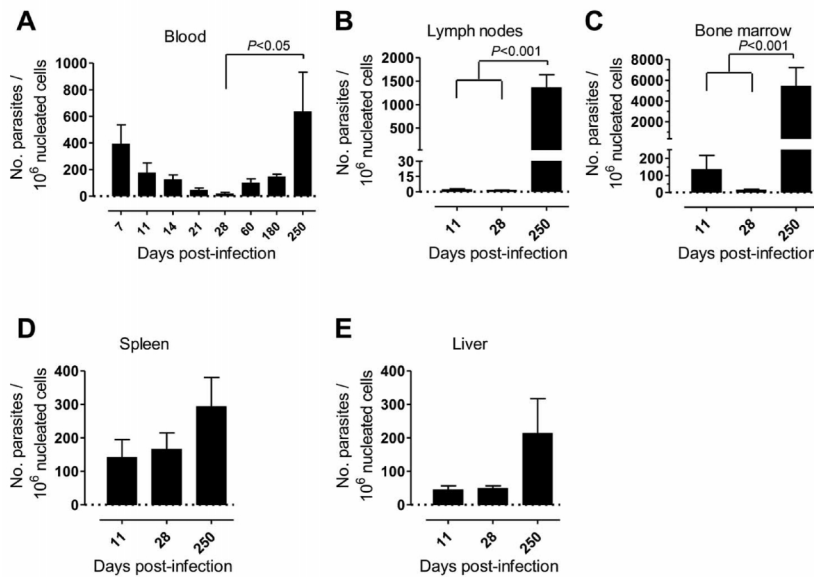


Figure 1. Kinetics of parasite load in *L. infantum*-infected rhesus macaques. Parasite kinetoplast DNA was quantified during the course of infection by qPCR in the (A) blood, (B) lymph nodes, (C) bone marrow, (D) spleen and (E) liver of infected macaques. Data presented as mean $\pm$ SEM of 4–8 (blood) or 2–4 (tissues) macaques per time-point. Statistics performed using a one-way analysis of variation (ANOVA) followed by a Bonferroni's post-hoc test.
doi:10.1371/journal.ppat.1004096.g001

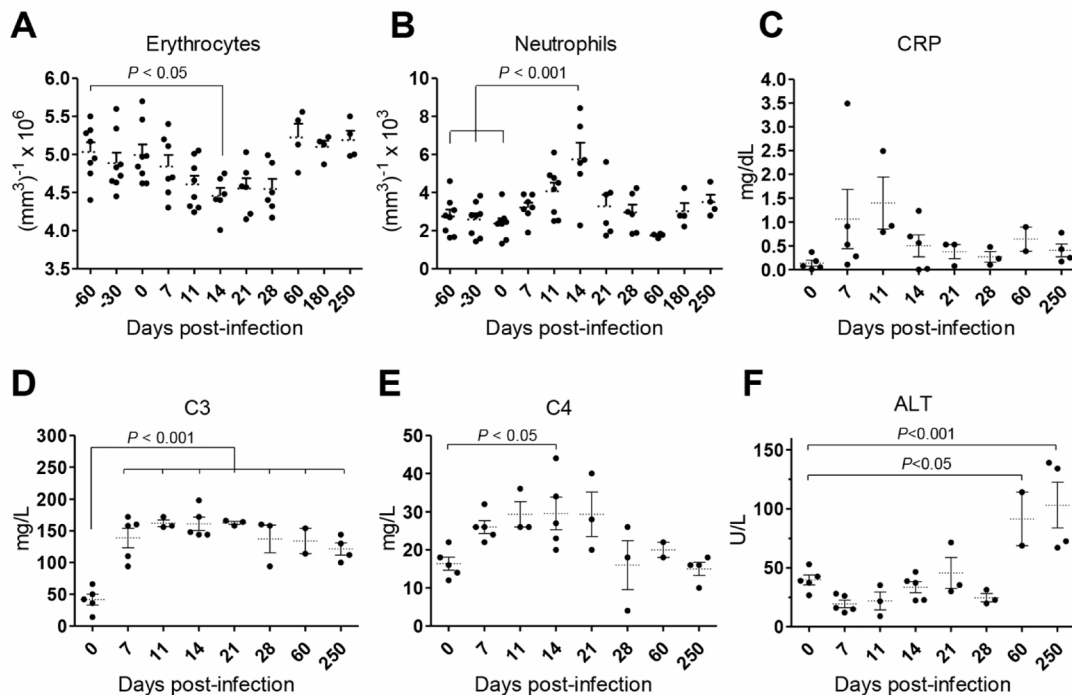


Figure 2. *L. infantum* infection elicits an acute phase response and chronic hepatocellular damage in rhesus macaques. (A–B) Blood samples were collected for cellular enumeration at the indicated time points using a Coulter LH 500 hematology analyzer; (A) erythrocytes, (B) neutrophils. (C–F) Serum samples from rhesus macaques were collected at the indicated time points and the following analytes quantified; (C) c-reactive protein (CRP), (D) complement component 3 (C3), (E) complement component 4 (C4) and (F) alanine transaminase (ALT). Each dot represents an individual macaque. Statistics performed using one-way ANOVA followed by a Bonferroni's post-hoc test.
doi:10.1371/journal.ppat.1004096.g002

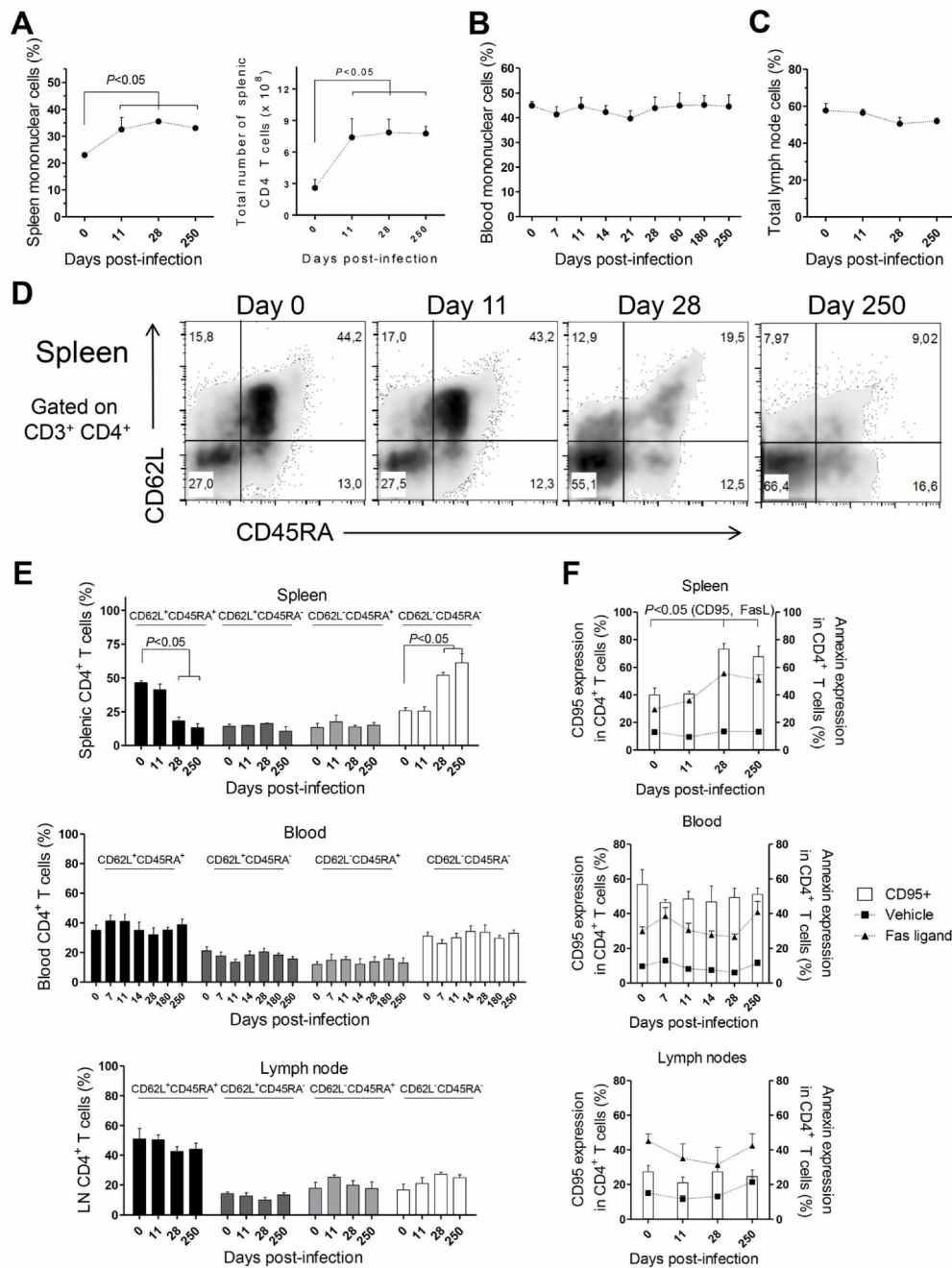


Figure 3. CD4 T cell dynamics in *L. infantum*-infected rhesus macaques. Flow cytometry was employed to determine the percentage of CD3⁺CD4⁺ T cells in the (A, left panel) spleen, (B) blood and (C) lymph nodes. (A, right panel) Total number of splenic CD3⁺CD4⁺ T cells. (D) Representative plots depicting the expression of CD62L and CD45RA in splenic CD3⁺CD4⁺ T cells during infection. (E) Histograms present the mean $\pm$ SEM percentage during infection of naïve (CD45RA⁺CD62L⁺), central memory (CD45RA⁻CD62L⁺), effector memory (CD45RA⁺CD62L⁻) and terminally differentiated (CD45RA⁺CD62L⁻) CD3⁺CD4⁺ T cells in the spleen (upper panel), blood (middle panel) and lymph node (lower panel) CD4⁺ T cells. (F) Graphics show the mean $\pm$ SEM of CD95 surface expression (white bars) and the percentage of annexin-positive CD3⁺CD4⁺ T cells after treatment with Fas ligand, 100 ng/ml (closed triangles), or vehicle (closed squares) in the spleen (upper panel), blood (middle panel) and LNs (lower panel). Plots present data from 4–8 (blood) or 2–4 (spleen, lymph node) macaques per time-point. Statistical analysis performed using one-way ANOVA followed by a Bonferroni's post-hoc test.

doi:10.1371/journal.ppat.1004096.g003

Collectively, our data point to a time-dependent and organ-specific establishment of *L. infantum* in rhesus macaques, with early parasite colonization of visceral compartments and posterior migration and/or growth in LNs.

L. infantum infection of rhesus macaques drives the expansion and differentiation of splenic CD4 T cells

CD4 T cells are crucial mediators of both protective and pathological immune responses during VL [2]. In rhesus macaques infected with *L. infantum*, the percentage of CD4 T cells in the spleen was significantly increased 11 days after infection (Fig. 3A, $P < 0.05$), which resulted in a 3-fold increase in their total numbers that were thereafter maintained at constant levels (Fig. 3A). Despite the drastic increase in splenic CD4 T cells, the occurrence of splenomegaly was not evident in infected animals during the course of the experiments (not shown). In contrast, no significant variations occurred in the total numbers (not shown) or frequencies of CD4 T cells in the blood (Fig. 3B) and LNs (Fig. 3C).

By assessing the differentiation phenotype of CD4 T cells we observed that, in the spleen, *L. infantum* infection induced a significant decrease in the percentage of naïve (CD62L⁺ CD45RA⁺) CD4 T cells, at days 28 and 250 pi (Fig. 3D–E upper panel, $P < 0.05$). This was paralleled by an increase in the percentage of CD4 T cells with an effector memory phenotype (CD62L[−] CD45RA[−], $P < 0.05$), while the frequencies of terminal effector (CD62L[−] CD45RA⁺) and central memory (CD62L⁺ CD45RA[−]) CD4 T cells remained roughly constant throughout infection (Fig. 3D–E, upper panel). In contrast, no significant alterations were detected in the differentiation phenotype of CD4 T cells in the blood or LNs during both acute and chronic stages of infection (Fig. 3E middle and lower panels).

T cell differentiation is usually associated with increased susceptibility to Fas-mediated apoptosis [28]. By quantifying annexin V binding, we observed that the susceptibility of splenic CD4 T cells to undergo apoptosis, upon exposure to exogenous FasL, increased significantly at days 28 and 250 pi (Fig. 3F, upper panel, $P < 0.05$). Interestingly, the surface expression of CD95 (Fas receptor) was found to parallel the susceptibility to FasL-mediated death (Fig. 3F, upper panel, $P < 0.05$). As expected, no significant alterations were found in the sensitivity of blood and LNs CD4 T cells to FasL-mediated apoptosis or in their CD95 expression (Fig. 3F, middle and lower panels). Despite the differentiation and increased sensitivity of splenic CD4 T cells to FasL-mediated apoptosis, splenic, blood or LN CD4 T cells of infected macaques were not more prone to apoptotic death in the absence of the apoptotic stimulus (Fig. 3F). This suggested the inexistence of death-receptor signaling *in vivo*, prompting us to analyse the levels of FasL in infected macaques. In agreement, neither the serum FasL levels, nor its splenic or LN transcript were found increased after infection (Fig. S2A–C). Globally, the results presented point to an early expansion and differentiation of the splenic CD4 T cell pool towards an effector memory phenotype after *L. infantum* infection.

A Th1-polarized cytokinic profile is induced in the spleen early after infection, but converts to an *IL10*-dominated environment during the chronic phase

We evaluated the gene expression levels of a panel of cytokines and transcription factors in total splenic mononuclear cells (SMCs). The qPCR analysis demonstrated a biphasic response with induction of Th1-related transcripts during the acute phase that converted to an *IL10*-enriched environment during the

chronic phase (Fig. S3A–I). In agreement with previous studies [3,8], we did not observe any modification in the transcript levels of the genes encoding the Th2-related cytokines IL-4 and IL-13 or the Th2 master regulator GATA-3 (Fig. S3D–F).

Given the prominent expansion and differentiation of CD4 T cells in the spleen of *L. infantum*-infected macaques (Fig. 3D–E), we evaluated the expression of the same genes in sorted splenic CD4 T cells. We detected significant 3- and 2.5-fold elevations for *IFNG* and *TBX21* (T-bet) transcripts, respectively and a non-significant 2-fold increase in *TNF* expression at day 28 pi (Fig. 4A–B, $P < 0.05$), indicating CD4 T cells as a source of these Th1-associated factors during the acute phase. This molecular signature was found to be transient and the expression of *IFNG* and *TBX21* declined during the chronic phase (Fig. 4A–B). As before, no significant modifications were observed in the transcript levels of Th2-associated transcripts, in sorted splenic CD4 T cells, even though non-significant 2-fold increases in *IL4* and *GATA3* occurred at day 28 pi (Fig. 4D–F).

Emerging evidence has associated increased lymphoid expression of the immunosuppressive IL-10 and TGF- β cytokines as underlying factors responsible for parasite persistence and chronicity in leishmaniasis [29–31]. We observed a significant 2.5-fold increase in the *IL10* transcript in sorted splenic CD4 T cells at day 250 after infection ($P < 0.05$), with no changes in the expression of *TGFB1* (Fig. 4H). Moreover, we failed to detect any significant modification in the expression levels of *FOXP3*, the master transcription factor for Treg differentiation, suggesting a FoxP3 negative phenotype for the splenic IL-10-producing CD4 T cells during VL in NHP, as previously suggested in both experimental mice models and natural human infections [31–33].

In LNs, we failed to detect any significant changes in the mRNA levels of Th1 or Th2-related cytokines or transcription factors in both total and sorted CD4 T cells during infection (Fig. 4A–F and Fig. S3A–F). Interestingly, a significant 2-fold increase on the expression of *IL10* occurred at day 250 pi in total LN cells (Fig. S3G, $P < 0.05$), concomitant with the parasite multiplication in these organs (Fig. 1B). We further observed a marginally non-significant increase in the transcript of *TGFB1* during the chronic phase (Fig. S3H, $P = 0.058$). However, in sorted LN CD4 T cells none of these transcripts underwent significant induction at the chronic phase (Fig. 4G–H). Finally, we observed an increase, albeit not-statistical, in the *FOXP3* transcript both in total LN cells and sorted CD4 T cells (Fig. 4H–I and Fig. S3H–I). An environment enriched in IL-10 and TGF- β may provide a safe niche for the parasite, hence explaining the ramping increase in parasite load in LNs during chronic infection.

L. infantum infection of rhesus macaques induces the production of non-specific IgG concomitant with defective B cell maturation and curtailed development of germinal centers

To further characterize the immune events in *L. infantum*-infected rhesus macaques we quantified the serum levels of total and *L. infantum*-specific IgM and IgG (Fig. 5A–B). A non-significant increase in total serum IgM occurred during acute infection, with the levels falling back to steady state by day 60 pi (Fig. 5A). This was paralleled by a significant increase of *L. infantum*-specific IgM up to the 11th day pi (Fig. 5A), which subsequently decreased to pre-infection levels. The total levels of serum IgG were significantly elevated at every time point evaluated after infection (Fig. 5B), demonstrating that the classical hypergammaglobulinemia observed during VL [1] rapidly develops upon infection. Nevertheless, this does not reflect the

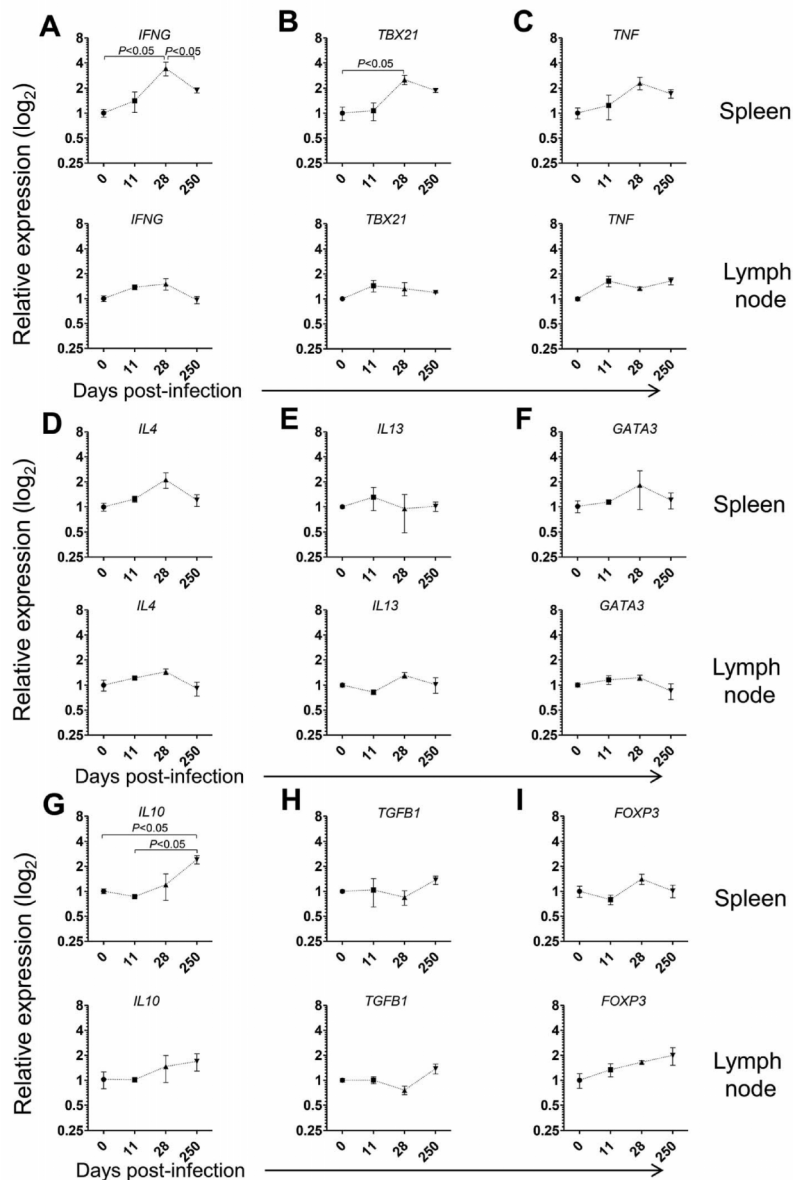


Figure 4. Gene expression profile of CD4 T cells in the spleen and LNs during *L. infantum* infection of rhesus macaques. The relative transcript levels in sorted CD4 T cells from the spleen (upper panels) and LNs (lower panels) were determined by qPCR in non-infected animals and after 11, 28 and 250 days of infection. Results are shown as mean $\pm$ SEM of the fold change over the non-infected samples. (A) *IFN*, (B) *TBX21*, (C) *TNF*, (D) *IL4*, (E) *IL13*, (F) *GATA3*, (G) *IL10*, (H) *TGFB1* and (I) *FOXP3*. Plots present data from 2–4 animals per time-point. Statistics performed by one-way ANOVA followed by a Bonferroni's post-hoc test.

doi:10.1371/journal.ppat.1004096.g004

development of a sustained *L. infantum*-specific IgG response, as only modest increases were observed in the serum levels of *L. infantum*-specific IgG, and these were confined to the early stages of infection (Fig. 5B). Importantly, during the chronic phase, while total IgG remained high, the levels of *L. infantum*-specific IgG returned to pre-infection levels, indicating a weak production of parasite-specific IgG.

Given that the spleen was colonized by parasites during both early and late stages of infection (Fig. 1C) and that it is the major organ of B cell differentiation [34], we sought to explore in detail the splenic B cell dynamics during the course of infection. No significant variations occurred in the total number of splenic B cells during infection (Fig. 5C). Next, we discriminated four distinct B cell populations, based on their differentiation status,

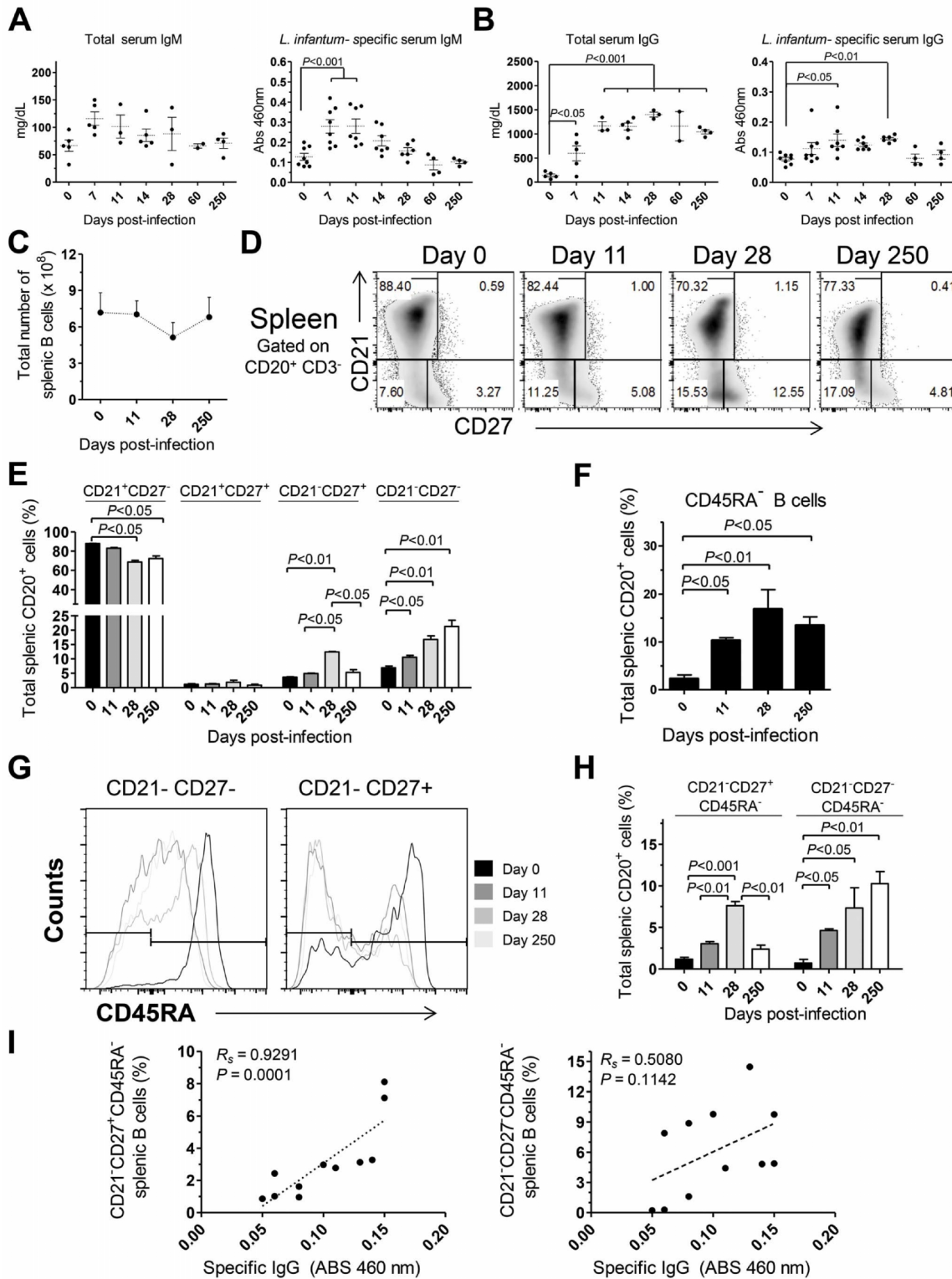


Figure 5. Humoral response and splenic B cell dynamics in *L. infantum*-infected rhesus macaques. (A–B) Serum levels of total (left panel) and *L. infantum*-specific (right panel) IgM (A) and IgG (B) during infection. **(C)** Total splenic B cell number was calculated from the frequency of CD3⁺CD20⁺ cells and the total number of SMCs. **(D)** Representative flow cytometry dot plots illustrating the expression of CD21 and CD27 in splenic B cells during infection. **(E)** Graphics depict the mean $\pm$ SEM for each B cell subset: naïve (CD21⁺CD27[−]), resting memory (CD21⁺CD27⁺), effector memory (CD21[−]CD27⁺) and immature (CD21[−]CD27[−]), as defined in panel (D). **(F)** Percentage (mean $\pm$ SEM) of CD3⁺CD20⁺CD45RA[−] splenic cells during infection. **(G)** Representative histograms depicting the expression of CD45RA in effector memory (CD21[−]CD27⁺) and immature B cells (CD21[−]CD27[−]) during the course of infection. **(H)** Percentage (mean $\pm$ SEM) of the activated memory (CD21[−]CD27⁺CD45RA[−]) and activated immature (CD21[−]CD27[−]CD45RA[−]) among the total splenic B cell pool. **(I)** Correlation between the frequency of CD21[−]CD27⁺CD45RA[−] splenic B cells (left panel) or the frequency of CD21[−]CD27[−]CD45RA[−] splenic B cells (right panel) with the serum levels of *L. infantum*-specific IgG. (A–B, I) Each dot represents an individual macaque. (C–H) Data obtained from 2–4 animals per time point. Significant differences were assessed by a one-way ANOVA followed by a Bonferroni's post-hoc test and the Spearman's rank test was used for correlations. doi:10.1371/journal.ppat.1004096.g005

[35,36] and followed their dynamics throughout infection. A significant decrease in the percentage of naïve (CD21⁺CD27[−]) splenic B cells was observed at days 28 and 250 after infection ($P < 0.05$; Fig. 5D–E). This decrease was accompanied by increases in the frequencies of immature (CD21[−]CD27[−]) and effector memory (CD21[−]CD27⁺) B cells (Fig. 5D–E). Interestingly, during the chronic phase only the immature population remained significantly elevated when compared with the level at $t = 0$ ($P < 0.05$), while the effector memory population contracted (Fig. 5D–E). Finally, no significant variations occurred in the resting memory (CD21⁺CD27⁺) subset of B cells throughout infection (Fig. 5D–E).

Loss of CD45RA expression in B cells is an early marker of B cell activation and differentiation into an Ig-secreting cell [37,38]. Indeed, we observed a significant increase in the percentage of splenic B cells having lost CD45RA expression after infection (Fig. 5F). We thus addressed the expression of CD45RA in the previously defined B cell subsets. In the naïve and resting memory B cell subsets, no significant loss of CD45RA was observed throughout infection (not shown). On the other hand, a marked downregulation of CD45RA occurred after infection in the immature and effector memory subsets (Fig. 5G–H). Overall, among the total splenic B cell pool, *L. infantum* infection induced a significant increase in the frequency of immature/activated B cells (here defined as CD21[−]CD27[−]CD45RA[−]) that persisted during chronic infection. In contrast, the activated/memory (CD21[−]CD27⁺CD45RA[−]) B cell population peaked at day 28 pi but declined at the chronic phase. We found a significant positive correlation between the levels of *L. infantum*-specific serum IgG and the frequency of CD21[−]CD27⁺CD45RA[−] splenic B cells ($R_s = 0.9291$, $P = 0.0001$) (Fig. 5I), but not with the percentages of CD21[−]CD27[−]CD45RA[−] B cells (Fig. 5I). Thus, our results suggest that a failure to maintain the activated memory B cell population may underlie the poor production of parasite-specific IgG.

As increased expression of CD27 in splenic B cells is considered an indication of GC experience [39], we performed tissue immunofluorescence in splenic sections retrieved from naïve, acutely and chronically-infected monkeys to explore the dynamics of GC development during infection (Fig. 6). The visualization of B and T cell areas as well as GC morphology and numbers during the course of infection was achieved by multiparametric analysis of splenic sections stained for CD3, CD20, IgD and Ki-67 [40]. The number and relative size of the GCs observed in naïve animals increased shortly after infection (Fig. 6A–B and 6E–F), peaking by one month after parasite inoculation (Fig. 6C, 6E–F and S4). At this time point, the increase in GC size was clear as noted by the enlarged central area harboring Ki-67⁺ proliferating cells and the peripheral localization of IgD⁺ naïve B cells that are excluded from the ongoing GC reaction (Fig. 6C). At the chronic phase, we observed a moderate remodeling of splenic architecture with less defined T and B cell areas (Fig. 6D). Additionally, we observed an

increased number of Ki-67⁺ cells scattered throughout both B and T cell areas, suggestive of widespread immune activation. Overall, the decrease in the frequency of splenic activated memory B cells (CD21[−]CD27⁺CD45RA[−]) at the chronic phase (Fig. 5H) was paralleled by a decrease in the number and average size of germinal centers (Fig. 6D and 6E).

Contrasting with the spleen, in lymph nodes, B cell differentiation was negligible with essentially all cells maintaining a naïve CD21⁺CD27[−]CD45RA⁺ phenotype throughout infection (Fig. S5). Accordingly, tissue immunofluorescence and GC quantification revealed no relevant changes in the number, size or morphology of lymph node-associated GCs (Fig. S6).

L. infantum infection induces transient expansion of a CXCR5⁺BCL6⁺ splenic CD4 T cell population associated with parasite-specific antibody production

The shortened duration of the *Leishmania*-specific antibody response associated with defective differentiation of the splenic B cell pool and curtailed development of germinal centers (Fig. 5) prompted us to explore the dynamics of Tfh-associated factors in the spleen of *L. infantum*-infected rhesus macaques. The transcript levels of *CXCR5* and *BCL6* were significantly augmented in sorted splenic CD4 T cells at day 28 pi, with *CXCR5* levels remaining elevated during the chronic phase (Fig. 7A, $P < 0.05$), while no variation was observed in expression of the *PDCD1* gene (PD-1; Fig. 7A). We further observed a 5-fold increase in the transcript levels of *IL21* in sorted splenic CD4 T cells at day 28 pi (Fig. 7A, $P < 0.05$) followed by a decrease in the chronic phase. Interestingly, the serum levels of IL-21 were found persistently elevated, from the 11th day after infection until the chronic phase (Fig. S7A) and qPCR analysis of SMCs revealed increased abundance of the *IL21* transcript as early as day 11 pi (Fig. S7B), thus suggesting that additional populations may produce IL-21 in the early acute phase as well as in the chronic phase.

Flow cytometric analysis indicated a significant increase in the frequency of splenic CD4 T cells expressing CXCR5 at days 28 ($P < 0.01$) and 250 pi ($P < 0.05$) and of Bcl-6 at day 28 pi ($P < 0.05$; Fig. 7B–C). However, no significant variations were observed in surface PD-1 expression throughout infection (Fig. 7B–C), consistent with qPCR analysis. Additionally, the percentage of splenic CD4 T cells expressing both CXCR5 and Bcl-6 peaked at day 28 pi and decreased at chronic infection ($P < 0.05$; Fig. 7B and D). Interestingly, we failed to detect any significant variation in the expression of PD-1 among the CXCR5⁺ or CXCR5⁺Bcl-6⁺ CD4 T cell populations (Fig. 7B–D). Significant positive correlations were found when plotting the frequency of the DP CXCR5⁺Bcl-6⁺ splenic CD4 T cell population against the activated memory splenic B cell population (Fig. 7E, $R_s = 0.8091$, $P = 0.0039$) and the serum levels of *L. infantum*-specific IgG (Fig. 7F, $R_s = 0.8055$, $P = 0.0039$).

To gain additional insight into the spatial dynamics of Tfh differentiation, splenic tissue sections were stained for CD4, PD-1

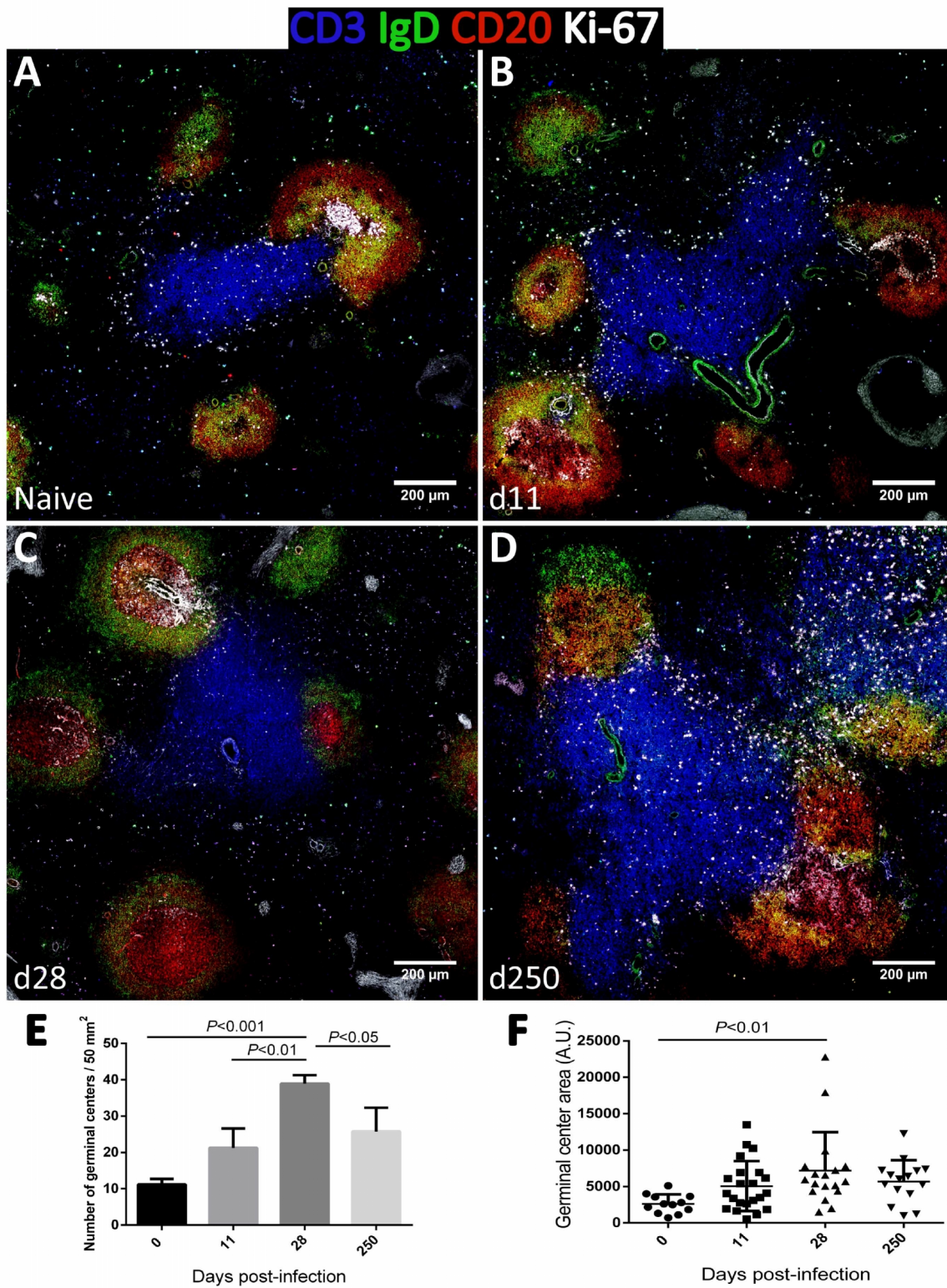


Figure 6. Dynamics of splenic germinal center development in macaques infected with *Leishmania infantum*. (A–D) Splenic tissue sections were stained with antibodies against Ki-67 (white), IgD (green), CD3 (blue) and CD20 (red) and imaged by confocal microscopy. Shown are representative pictures of a naïve animal (A) and at 11 (B), 28 (C) and 250 (D) days after infection. (E) Splenic tissue sections were stained with an antibody against Ki-67 by immunohistochemistry and germinal centers were counted in naïve macaques ($n=3$), and at days 11 ($n=2$), 28 ($n=2$) and 250 ($n=4$) after infection. Three distinct sections per animal were examined. Bars depict mean $\pm$ SEM. (F) Quantification of GC area in Ki-67-stained splenic tissue sections from naïve and infected macaques at the indicated time points. Statistical analysis was performed by one-way ANOVA, followed by a Bonferroni's post-hoc test. doi:10.1371/journal.ppat.1004096.g006

and CxCR5 (Fig. 8). In our hands, no commercially available Bcl-6 antibody was able to detect the protein by immunofluorescence in rhesus macaques (not shown). As expected, in naïve macaques very few CD4⁺ T cells were seen infiltrating CxCR5⁺ areas that define the B cell follicle (Fig. 8A). By day 11 pi, and paralleling the development of GCs (Fig. 6B), an increased number of CD4⁺ T cells were present inside the follicle, with a few expressing PD-1 in addition to CxCR5 (Fig. 8B). CD4⁺ T cells progressively accumulated inside follicles as acute infection progressed. By one month after parasite inoculation, hence at the peak of the GC response, increased numbers of CD4⁺ T cells could be visualized in B cell areas (Fig. 8C). Interestingly, some of the follicle-infiltrating CD4⁺ T cells were expressing PD-1, but not CxCR5 and, conversely, follicle-associated CD4⁺ T cells expressing CxCR5 did not express PD-1 (Fig. 8C and Fig. 8E). Spatially, CD4⁺ PD-1⁺ CxCR5⁺ cells occupied a more central position in the follicle, relative to the peripheral localization of CD4⁺ CxCR5⁺ PD-1⁺ cells (Fig. 8C and Fig. 8E). Finally, and consistent with the dynamics of GC development, CxCR5⁺ areas were largely devoid of CD4⁺ T cells by day 250 pi, despite continuous parasite presence (Fig. 8D).

The expression of the Tfh classical markers CxCR5 and PD-1 in lymph node CD4⁺ T cells increased progressively after infection at the transcript (Fig. S8A) and protein levels (Fig. S8B–D), though without statistical value. Expression of Bcl-6 in lymph node CD4⁺ T cells was negligible (S8B–D) and no changes were observed at the transcript level (Fig. S8A). Tissue immunofluorescence revealed the presence of some CD4⁺ T cells in follicular areas, particularly at one month after infection and during the chronic phase. The majority of these cells appeared to express PD-1 but not CxCR5, with only a minute number expressing both surface markers (Fig. S9C–E).

Overall, our results point to a model in which *L. infantum* infection induces an acute expansion of a CxCR5⁺Bcl-6⁺ CD4⁺ T cell population in the spleen associated with the production of parasite-specific IgG. The kinetics of expansion and contraction of the double positive CxCR5⁺Bcl-6⁺ CD4⁺ T cell population closely paralleled the development and resolution of germinal centers in the spleen. Hence, we provide a detailed description of the immune events underlying the suboptimal parasite-specific humoral response in rhesus macaques infected with *L. infantum*.

Discussion

The lack of efficient vaccines or immune therapies for human VL highlights the need for alternative animal models able to complement the extensive research performed in rodents over the past 30 years. In this study, we show that *L. infantum* infection of rhesus macaques drives an early expansion and differentiation of splenic CD4⁺ T cells with a Th1 molecular signature that is concomitant with parasite containment in visceral compartments. Furthermore, we provide evidence of the lack of a robust Tfh response throughout infection, which possibly underlies the poor and short-lived production of *Leishmania*-specific antibodies. Finally, the emergence of an immunosuppressive environment may facilitate parasite dissemination and/or growth in additional

niches during chronic infection. In a general manner, the non-human primate model introduced here confirmed some previous observations made in murine models and, more importantly, in human patients. Particularly, the expansion of the CD4⁺ T cell pool associated with a Th1 polarization during the acute phase and the chronic persistence of the parasite associated with augmented expression of IL-10. More importantly, we provided evidence that defects in B cell and T follicular helper differentiation comprise the mechanistic basis for the occurrence of hypergammaglobulinemia and inefficient specific humoral response during VL.

The acute stage of infection is characterized by rapid parasite clearance from the blood and BM, with parasite containment in the spleen and liver. Studies in mice evidenced that half of the intravenously inoculated parasites are eliminated in splenic phagocytes within 24 hours after infection [41]. We propose that a rapid decrease in parasite numbers may occur in a similar manner in rhesus macaques, providing large quantities of parasite antigens to initiate an adaptive response. Indeed, we observed that the acute phase of infection is characterized by expansion and differentiation of splenic CD4⁺ T cells towards effector memory phenotypes associated with increased levels of Th1-related factors (IFN- γ and T-bet). This differentiation of the splenic T cell pool is consistent with increased susceptibility to FasL-mediated apoptosis. However, in the absence of FasL, these cells are not prone to die. Thus, the higher levels of T cell apoptosis previously reported in VL [42] may be the consequence of T cell differentiation/activation processes rather than being the direct cause of pathogenesis. In LNs, which were minimally colonized during the acute phase, no changes were observed in the extent of T cell differentiation and of level of apoptosis. Altogether, our results denote a mobilization of the immune system early after infection associated with elimination or at least containment of the parasite in visceral organs.

In the chronic phase, we observed increased parasite loads in the spleen and liver, associated with signs of hepatocellular damage (elevated serum levels of ALT), as well as evidence of parasite growth in previously low-colonized organs such as the LNs. These changes were associated with an immune context distinct from the one observed during the acute phase. Splenic CD4⁺ T cells maintained an effector-memory phenotype, but shifted from an IFN- γ -producing phenotype towards enrichment in IL-10. The expression of the latter has long been associated with chronicity and disease progression in VL [43,44]. Importantly, conventional Th1 cells have recently been identified as the main source of IL-10 during VL, in a regulatory mechanism that presumably becomes operative to avoid excessive damage associated with pro-inflammatory cytokine secretion [31–33,45].

In striking contrast with the spleen, the increased parasite load detected in the LNs during the chronic phase does not result in the differentiation of the CD4⁺ T cell pool, a phenotype that can be ascribed to the presence of immunosuppressive cytokines - IL-10 and TGF- β . Accordingly, the anergic behavior of lymph node CD4⁺ T cells during chronic infection was shown, in a hamster model of VL, to result from the activity of macrophage-derived TGF- β [4]. Thus, our results are strongly suggestive of compart-

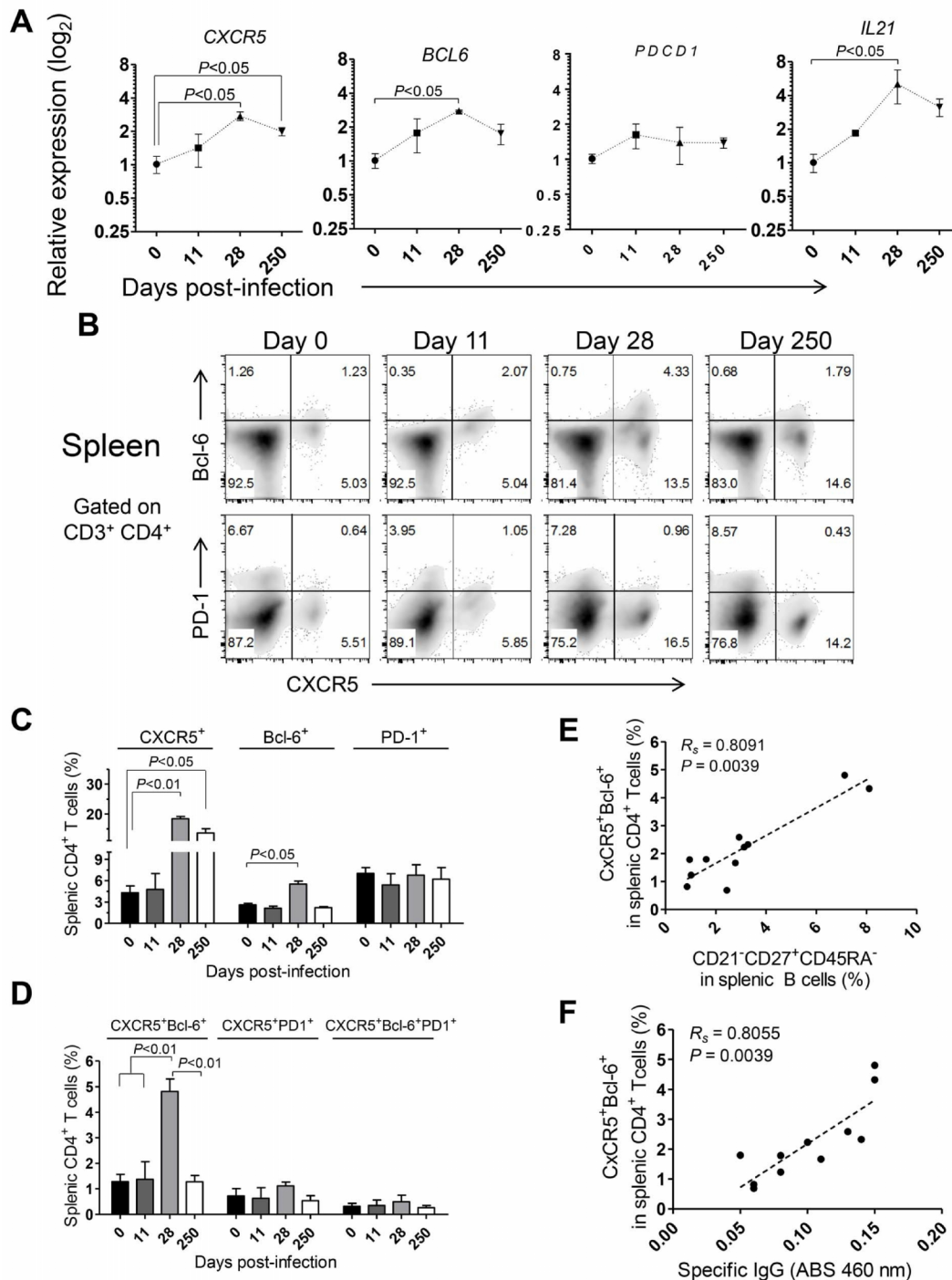


Figure 7. Abortive differentiation of splenic Tfh cells in *L. infantum*-infected rhesus macaques. (A) The relative transcript levels of *CXCR5*, *BCL6*, *PDCD1* and *IL21* in sorted splenic CD4 T cells were quantified by qPCR. Results are shown as fold change $\pm$ SEM over non-infected samples. (B) Representative density plots depicting the expression of CXCR5 and Bcl-6 (upper panels) or CXCR5 and PD-1 (lower panels) in splenic CD4 T cells during the course of infection. (C) Expression (mean $\pm$ SEM) of CXCR5, Bcl-6 and PD-1 among splenic CD4 T cells during the course of infection. (D) Percentage (mean $\pm$ SEM) of expression of the double positive CXCR5⁺Bcl-6⁺ or CXCR5⁺PD-1⁺ populations and the triple positive CXCR5⁺Bcl-6⁺PD-1⁺ population among splenic CD4 T cells. (E–F) Correlation between the frequency of CXCR5⁺Bcl-6⁺ splenic CD4 T cells and the frequency of

CD21⁺CD27⁺CD45RA⁺ splenic B cells (E) or the serum levels of *L. infantum*-specific IgG (F). (A–D) Data obtained from 2–4 animals per time point. (E–F) Each dot represents an individual animal. Statistical analysis was performed by one-way ANOVA, followed by a Bonferroni's post-hoc test. The Spearman's rank test was used for correlations. doi:10.1371/journal.ppat.1004096.g007

mentalized immune responses, underlying the complexity of the immune response in NHPs, which is reminiscent of VL in humans.

A dysfunctional humoral immune response has long been recognized in VL [46,47]; however the mechanisms behind such dysregulation remain poorly studied. In *L. infantum*-infected rhesus macaques we observed a dramatic increase in the circulating levels of total IgG. Such increase is not paralleled by the serum levels parasite-specific IgG, which peak at one month after infection and decrease thereafter. While the decline in the levels of specific IgG may reflect the loss of the GC and Tfh responses, as discussed below, it may also be a consequence of accelerated decay in the serum of the infected animals. Although we have not examined the occurrence of proteinuria, there is ample evidence of renal involvement in VL associated with tubular and glomerular damage that may lead to accelerated clearance [48]. Splenic B cells are activated and exhibit differentiation towards two main subsets: an immature subset that persists throughout infection and an activated memory subset that peaks at day 28 and declines in the chronic phase. Tissue imaging revealed that splenic germinal centers initially develop, peak in number and size by one month after infection, but ultimately fail to be maintained at the chronic phase, hence closely paralleling the kinetics of memory B cell frequency. Interestingly, B cell differentiation in the lymph nodes was minimal. Additionally, we detected a non-significant increase in the number of lymph node-associated germinal centers, suggesting a minor contribution of these organs to the production of parasite-specific IgG.

The transient expansion of a splenic CD4⁺ T cell population expressing CXCR5 and Bcl-6 correlates with the emergence of activated memory B cells and the levels of parasite-specific IgG. Concomitant with the expansion of the CXCR5⁺Bcl-6⁺ CD4⁺ T cell population is an increase in the transcript levels of *IL21* in splenic CD4⁺ T cells. IL21 mediates production of high affinity antibodies and also plays an essential role in the differentiation of plasma and memory B cells [49]. Interestingly, we observed that the serum levels of IL-21 remained elevated during the chronic phase, despite the decline in the *IL21* transcript in splenic CD4⁺ T cells, suggesting that additional immune populations may produce the transcript. Moreover, elevated serum levels of IL-21 are a biomarker for the risk of developing autoimmunity [50] and the presence of autoantibodies is a recurrent finding in VL patients [51,52].

GC-associated Tfh cells have classically been defined by their follicular localization and simultaneous expression of CXCR5, Bcl-6 and PD-1 [23]. We observed the transient expansion of CD4⁺ T cells expressing CXCR5 and Bcl-6, but lacking PD-1. Interestingly, a recent report has identified IL-21-producing, Bcl-6⁺PD-1^{low} CD4⁺ T cells, located at the interface between the T cell zone and the follicle, as providers of B cell help in T-dependent extra-follicular B cell responses [53]. These cells were named pre-GC Tfh cells as they appear early after immunization and are progressively replaced by Bcl-6⁺PD-1^{hi} CD4⁺ T cells that locate within GCs [53]. Furthermore, recent studies have proposed that full expression of the Tfh differentiation program depends on cognate interactions between primed CD4⁺ T cells and antigen-activated B cells [25,54,55]. In this sense, B cells play a crucial role for the survival of Tfh cells and commitment to the Tfh lineage [25]. In the absence of B cells, Tfh cells are still developed, albeit in significantly lower numbers that fail to express PD-1 [54]. We

observed an augmentation in the serum level of total, but not specific, IgG as early as day 7 pi, as well as the expansion of an immature/activated splenic B cell population detected at day 11. Thus, it is conceivable that *L. infantum* infection induces an early skewing of the B cell pool favoring the inappropriate differentiation of plasma cells from low-affinity B cells, and preventing the entry of a sufficient number of activated B cells in the follicle to sustain the Tfh response. Indeed, several *Leishmania*-derived factors have been identified as polyclonal activators of B cells [56,57] and, in a murine intradermal model of VL, an early polyclonal B cell response was associated with disease progression [17]. Similarly, infection of mice with the related parasite *Trypanosoma cruzi* induces a massive extra-follicular splenic B cell response associated with the production of non-specific antibodies [58]. In this sense, the CXCR5⁺Bcl-6⁺PD-1⁺ CD4⁺ T cell population that we detect at the end of the acute phase may represent a pre-GC-Tfh state that does not mature to a *bona fide* Tfh population due to the lack of cognate interactions with B cells. It is however worth referring that confocal imaging revealed the presence of some CXCR5⁺PD-1⁺ CD4⁺ T cells inside B cell areas, at early time points after infection, suggesting that some *bona fide* Tfh cells might engage into the GC pathway, even though their numbers appear compromised. We also observed that some follicle-associated CD4⁺ T cells expressed PD-1 but not CXCR5, particularly by one month after infection in the spleen, and at the chronic phase in lymph nodes. Given their clearly atypical phenotype, considering the current definition of Tfh cells, further studies would be required to completely elucidate their nature.

Interestingly, the results we present here concerning *L. infantum* infection are clearly distinct from recent findings regarding SIV/HIV infections, in which a pathological accumulation of Tfh cells occurs that accounts for the abnormalities in the B cell compartment observed during infection [59–61].

In the chronic phase, the decline of CXCR5⁺Bcl-6⁺ CD4⁺ T cells may also be related to increased IL-10, as it was shown to regulate the expression of Bcl-6 and IL-21 in CD4⁺ T cells [62]. Additionally, IL-10 enhances proliferation of activated human B lymphocytes and induces secretion of high amounts of immunoglobulin [63]. Thus, an IL-10-enriched environment combined with the early skewing of the B cell response may represent a biased environment that precludes maintenance of a Tfh response and production of specific-IgG, while sustaining the production of non-specific antibodies. We could not unfortunately provide definitive evidence for the presence of extrafollicular plasmablasts in the spleen due the absence of a clear phenotypic definition in non-human primates. Nevertheless, the global picture points to a humoral response dominated by low-affinity or irrelevant antibodies produced by polyclonally or extrafollicularly-activated B cells. Although the contribution of specific antibodies to a protective response against an intracellular pathogen such as *Leishmania* remains under debate, some studies have suggested that specific antibodies are required for an efficient uptake of the parasite [20], and protection, in an experimental vaccine against *L. infantum* [64]. Furthermore, the formation of a functional germinal center and IL-21 production were associated with lesion resolution in a model of cutaneous leishmaniasis [65]. Thus, one may envision that the loss of parasite-specific antibodies observed in the chronic phase may facilitate parasite dissemination and promote chronicity.

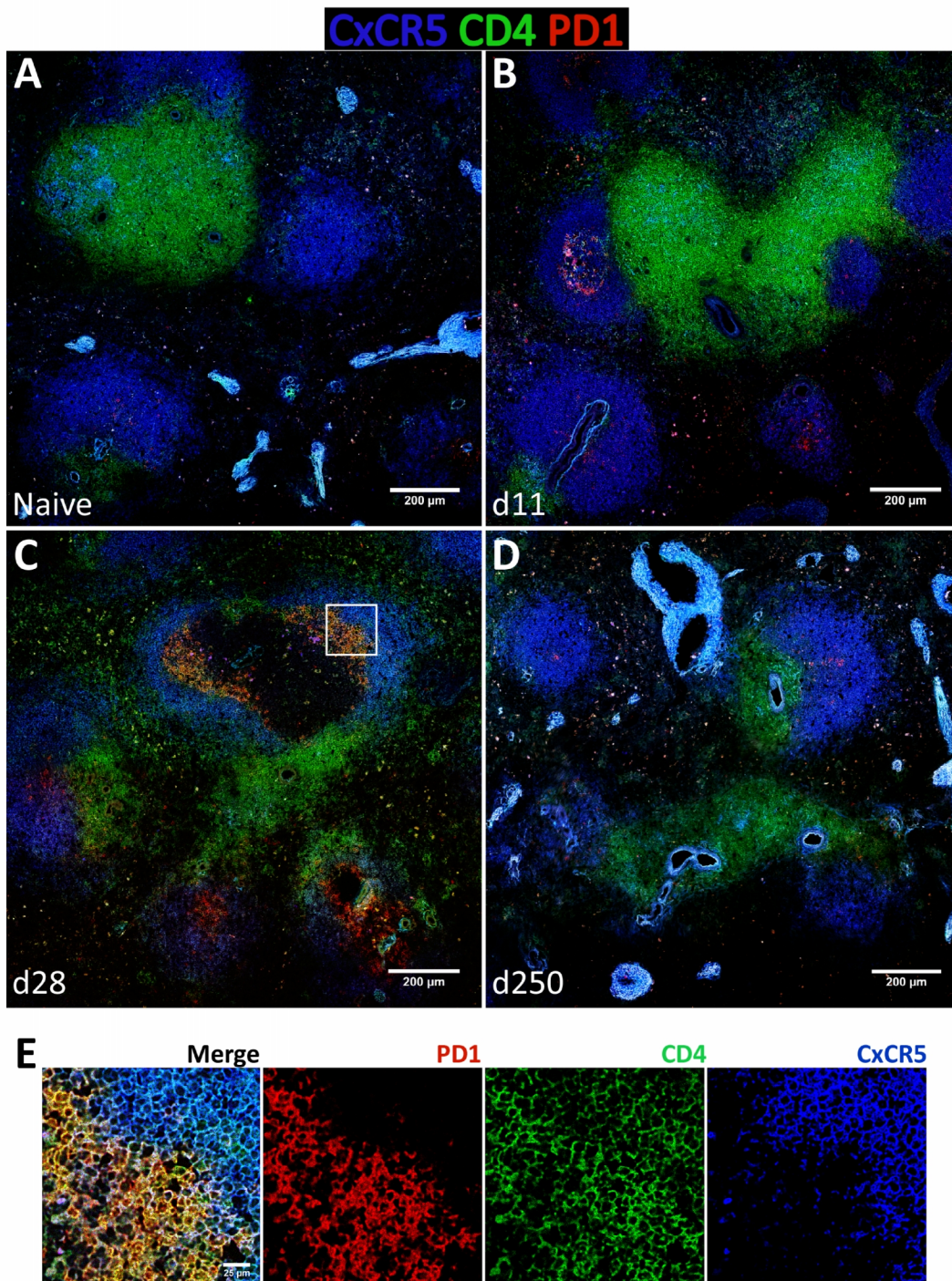


Figure 8. Follicular helper T cells infiltrate B cells follicles during the acute phase of *L. infantum* infection of rhesus macaques but the Tfh response is not sustained until the chronic phase. (A–D) Splenic tissue sections were stained with antibodies against CxCR5 (blue), CD4 (green) and PD-1 (red) and imaged by confocal microscopy. Shown are representative pictures of a naïve animal (A) and at 11 (B), 28 (C) and 250 (D) days after infection. (E) Inset from figure 8C as defined by the white square.
doi:10.1371/journal.ppat.1004096.g008

In conclusion, we used here a NHP model to decipher the immune events associated with parasite establishment and chronicity in VL. Our results indicate that despite the differentiation of effector memory CD4 T cells in the main parasitized organs early after infection, the establishment of an IL-10 enriched environment in the chronic phase and the absence of a fully matured and sustained Tfh response may participate in the immunodeficiency associated with VL chronicity.

Materials and Methods

Animal, parasites and infections

Eleven colony-outbred young adult (3–5 kg) rhesus macaques (*Macaca mulatta*) of Chinese origin, seronegative for STLV-1 (Simian T Leukemia Virus type-1), SRV-1 (type D retrovirus), herpes-B viruses and SIVmac were used in this study. A group was left as non-infected control (n = 3) and the remaining animals were inoculated intravenously via the saphenous vein with 2×10^7 stationary-phase *L. infantum* promastigotes (clone MHOM/MA/67/ITMAP-263) per kg of body weight. Subgroups of infected animals were euthanized at three time points after infection covering both acute and chronic phases (n = 2 for days 11 and 28 pi and n = 4 for day 250 pi). Peripheral blood and internal organs (axillary and inguinal lymph nodes, spleen, liver and bone marrow) were recovered for cellular analysis. Blood sampling was performed at additional time points before and after infection. For each blood-sampling point, a hemogram was elaborated using a LH750 hematology analyzer (Beckman Coulter).

Parasite quantification

DNA was extracted from cell pellets of blood or organs using the QIAamp DNA Mini Kit (QIAGEN). A TaqMan-based qPCR assay for detection and quantification of *L. infantum* kinetoplastid DNA was adapted from a described protocol [27]. Reaction mixtures were composed of ABI TaqMan PCR 2× (Applied Biosystems), 375 nM of direct primer (CTTTTCTGGTCCCTCCGGGTAGG), 375 nM of reverse primer (CCACCCGGCCCC-TATTTTACACCAA), 250 nM of hydrolysis probe (5'FAM-T-TTTCGACAGACGCCCTACCCGC-3'TAMRA) and 100 ng of sample DNA. Thermocycling settings consisted of one hold of 10 min at 95°C followed by a two-step temperature (95°C for 15 s and 60°C for 60 s) over 40 cycles in an ABI Prism 7900 HT (Applied Biosystems). A standard curve was established corresponding to a range of 50,000 to 0.01 parasites.

Sample normalization was performed by quantifying a host gene (macaque albumin), in 10 µL parallel reactions consisting of SYBR Green ROX Mix 2× (Thermo Scientific), 100 nM of forward primer (CCATTGGTGAGACCAGAGGT), 100 nM of reverse primer (GAGGCAGGCAGCTTTATCAG), 100 ng of DNA and the same thermal profile used for parasite quantification. A calibration curve ranging from 10,000 to 0.1 cells was established and parasite load expressed as the number of parasites per million of host cells.

Quantification of serum analites

Quantification of ALT, CRP, C3, C4 and total IgG and IgM were all performed on an AutoAnalyzer (PRESTIGE 24i, PZ Cormay S.A.). The detailed protocols employed are described in the Supporting Material and Methods section.

ELISA for *L. infantum*-specific immunoglobulins

The relative titers of *L. infantum*-specific antibodies in the serum of infected macaques were quantified adapting a protocol described elsewhere [66]. Briefly, 96-well plates were coated

overnight with 10 µg/mL of soluble axenic amastigote *Leishmania* antigen (prepared as described before [66]) and blocked with 200 µL of PBS/low-fat-milk 5%/FCS 5%. Sera from individual macaques were analyzed at a 1:200 dilution. Horseradish peroxidase-conjugated anti-macaque IgG (1:5,000) and anti-macaque IgM (1:10,000) was then added to each well and the tetramethyl benzidine substrate solution was used to detect antigen-specific antibody by absorbance at 492 nm.

Immunophenotyping

Fresh cell suspensions were prepared from macaque spleen and LNs (a pool of axillary and inguinal LNs). Peripheral blood was collected to EDTA-coated tubes. Cells were stained with a panel of monoclonal antibodies. The fluorochrome-conjugated antibodies used are provided in Supporting Table 1 (Table S1). After lysing erythrocytes in a hypotonic solution, fifty thousand events corresponding to mononuclear cells were acquired in a Cytomics FC500 (Beckman Coulter) and further analyzed using FlowJo software (Tree Star, Inc.). Intracellular Bcl-6 staining was performed after fixing and permeabilizing the cells with the FoxP3 staining buffer set (eBiosciences).

Ex-vivo apoptosis

Peripheral blood was recovered to heparin-coated tubes. PBMCs and SMCs were isolated by density gradient centrifugation using LymphoPrep (PAA Laboratories). PBMCs, SMCs and LN cells were cultured overnight in complete media in the presence of FasL, 100 ng/ml, or vehicle (control). The percentage of apoptotic CD4 T cells was determined by flow cytometry after surface staining with FITC-labeled annexin-V combined with surface staining for CD4 and CD3, as described previously [67].

Quantitative-PCR

Approximately 500,000 CD4 T cells from SMCs or LN cell suspensions were sorted using a FACS Aria II cell sorter (BD Biosciences), lysed in RLT buffer (RNeasy Micro Kit, QIAGEN) and stored at –80°C until further use. A similar number of total SMCs or total LN cells were lysed and stored. RNA was purified and reverse transcribed using the AffinityScript QPCR cDNA synthesis kit (Stratagene). Gene expression was analyzed by qPCR in 10 µL reactions, using 100 ng of cDNA. The thermal profile consisted of a hold of 15 min at 95°C, followed by 40 cycles of denaturation (95°C, 15 sec), annealing (60°C, 30 sec) and extension (72°C, 30 sec). Ct values were normalized by quantifying the levels of two macaque reference genes, *GAPDH* and *RPS14* and results expressed as fold change in gene expression relative to non-infected samples. Macaque-specific primers were designed using the AutoPrime software. A list of sequences, gene accession numbers and predicted amplicon size of the oligonucleotides used is provided in Table S2. The obtained sizes for the PCR products are depicted in Fig. S10.

Immunofluorescence confocal microscopy of tissue sections

Optimal cutting temperature compound (OCT)-embedded tissues (spleen and peripheral lymph nodes) were sectioned (7.5 µm thickness) in a frozen cryostat and stored unfixed at –80°C until use. A double fixation procedure was employed and consisted of 4% PFA (15 minutes at room temperature) followed by acetone (20 minutes at –20°C). Slides were saturated in blocking solution (5% normal goat serum, 0.3% triton X-100) for 1 hour at RT. Fluorochrome-conjugated antibodies were diluted in antibody dilution buffer (1% BSA, 0.3% triton X-100) and

incubated overnight with tissue sections at 4°C. Table S1 provides detailed information on the antibodies used for tissue immunofluorescence. After washing, slides were mounted with antifade mounting medium. Sections were imaged in a Zeiss LSM 710 confocal microscope. Tiled Z-stacks were acquired with a 20× objective and stitched using the Image J stitching plugin [68]. Average intensity projections were obtained from the stitched tiles using built-in Image J tools. Images were further analyzed and processed using Image J and Adobe Photoshop.

Quantification of germinal centers by tissue immunohistochemistry

Splenic and peripheral lymph node sections were fixed in 4% PFA (15 minutes at RT) and saturated in blocking solution. Sections were incubated for one hour at room temperature with Ki-67 antibody (clone MIB-1, 1/50 dilution in antibody incubation buffer). After washing, sections were incubated with HRP-coupled secondary antibody for 1 hour at RT and the 3,3'-diaminobenzidine (DAB) substrate added for revelation. Germinal centers were identified as Ki-67⁺ cell aggregates and manually counted under a magnifying glass coupled to a digital camera and normalized to the number of GCs per 50 mm² of area. For determination of germinal center area, micrographs at 40× magnification were acquired and the average area of each GC, defined by Ki-67 staining, was quantified manually using Image J. Two micrographs from two distinct animals per time point were analyzed and the results pooled. Representative micrographs are shown in Figure S4.

Statistical analysis

Statistics were performed with the GraphPad Prism 5 software. Data is presented as means ± SEM. A one-way analysis of variance (ANOVA) followed by a Bonferroni's post hoc test was employed for comparison between naïve and infected animals at different time points after infection. A Spearman's rank test was employed for correlations.

Ethics statement

All the animal experiments described in the present study were conducted at the MIRcen platform according to the European Union guidelines for the handling of laboratory animals (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm). The animal care and use protocol issued by the IACUC/ethics committee (MIRcen, CAJ-10-30) that approved the study.

Supporting Information

Figure S1 (A–B) Blood samples from rhesus macaques were collected for cellular enumeration at the indicated time points using a Coulter LH 500 analyzer; (A) hematocrit, (B) concentration of blood hemoglobin. **(C–F)** Serum samples from rhesus macaques were collected at the indicated time points and the following analytes quantified using an automated analyzer; (C) γ -glutamyl transpeptidase (Gamma-GT), (D) total bilirubin (TBil), (E) albumin and (F) total serum protein. Statistics assessed by one-way ANOVA followed by a Bonferroni's post-hoc test. (TIF)

Figure S2 (A) Serum levels of Fas ligand were quantified using a commercial ELISA assay. **(B–C)** The relative transcript levels of Fas ligand in the spleen and lymph nodes were determined by qPCR. Results are shown as fold change ± SEM over non-infected samples. (TIF)

Figure S3 Gene expression profile in total spleen mononuclear cells (SMCs) and total lymph node cells during *L. infantum* infection of rhesus macaques. The relative transcript levels from total SMCs (upper panels) and total lymph node cells (lower panels) were determined by qPCR in non-infected animals and after 11, 28 and 250 days of infection. Results are shown as mean ± SEM of the fold change over the non-infected samples, which were attributed a normalized value of 1. **(A)** *IFNG*, **(B)** *TBX21*, **(C)** *TNF*, **(D)** *IL4*, **(E)** *IL13*, **(F)** *GATA3*, **(G)** *IL10*, **(H)** *TGFB1* and **(I)** *FOXP3*. Statistics assessed by one-way ANOVA followed by a Bonferroni's post-hoc test. (TIF)

Figure S4 Representative micrographs of Ki-67-stained splenic tissue sections from naïve **(A)** and days 11 **(B)**, day 28 **(C)**, day 250 **(D)**, used for quantification of germinal center number and area. (TIF)

Figure S5 Lymph node B cell dynamics in *L. infantum*-infected rhesus macaques. **(A)** The percentage (mean ± SEM) of lymph node B cells was determined by flow cytometry. **(B)** Representative flow cytometry dot plots illustrating the expression of CD21 and CD27 in lymph node B cells during infection. **(C)** Histograms depict the mean ± SEM for each B cell subset: naïve (CD21⁺CD27[−]), resting memory (CD21⁺CD27⁺), effector memory (CD21[−]CD27⁺) and immature (CD21[−]CD27[−]), as defined in panel (B). **(D)** Percentage (mean ± SEM) of CD3[−]CD20⁺CD45RA[−] cells throughout infection. Data obtained from 2–4 animals per time point. Significant differences were assessed by a one-way ANOVA followed by a Bonferroni's post-hoc test and the Spearman's rank test was used for correlations. (TIF)

Figure S6 Dynamics of lymph node germinal center development in macaques infected with *Leishmania infantum*. **(A–D)** Lymph node sections were stained with antibodies against Ki-67 (white), IgD (green), CD3 (blue) and CD20 (red) and imaged by confocal microscopy. Shown are representative pictures of a naïve animal **(A)** and at 11 **(B)**, 28 **(C)** and 250 **(D)** days after infection. **(E)** Lymph node tissue sections were stained with an antibody against Ki-67 by immunohistochemistry and germinal centers were quantified in naïve macaques (n = 3), and at days 11 (n = 2), 28 (n = 2) and 250 (n = 4) days after infection. Three distinct sections per animal were examined. Bars depict mean ± SEM. Statistical analysis was performed by one-way ANOVA, followed by a Bonferroni's post-hoc test. (TIF)

Figure S7 (A) Serum levels of IL-21 were quantified using a commercial ELISA assay. Each dot represents sera from an individual animal **(B)** The relative transcript levels of IL-21 in total spleen mononuclear cells, shown as fold change ± SEM (n = 2–4) over non-infected samples, were determined by qPCR. (TIF)

Figure S8 Dynamics of Tfh cell differentiation in the lymph nodes of *L. infantum*-infected rhesus macaques. **(A)** The relative transcript levels of *CXCR5*, *BCL6*, *PDCD1* and *IL21* in sorted lymph node CD4 T cells were determined by qPCR. Results are shown as fold change ± SEM over non-infected samples. **(B)** Representative density plots depicting the expression of CXCR5 and Bcl-6 (upper panels) or CXCR5 and PD-1 (lower panels) in lymph node CD4 T cells during the course of infection. **(C)** Expression (mean ± SEM) of CXCR5, Bcl-6 and PD-1 among splenic CD4 T cells during the course of infection. **(D)** Percentage (mean ± SEM) of expression of the double positive CXCR5⁺Bcl-6⁺ or CXCR5⁺PD-1⁺ populations and the triple positive

CXCR5⁺Bcl-6⁺PD-1⁺ population among lymph node CD4 T cells. Statistical analysis was performed by one-way ANOVA, followed by a Bonferroni's post-hoc test.

(TIF)

Figure S9 Follicular helper T cell imaging in lymph nodes during *L. infantum* infection of rhesus macaques. (A–D) Lymph node tissue sections were stained with antibodies against CXCR5 (blue), CD4 (green) and PD-1 (red) and imaged by confocal microscopy. Shown are representative pictures of a naïve animal (A) and at 11 (B), 28 (C) and 250 (D) days after infection. (E) Inset from figure S8D as defined by the white square.

(TIF)

Figure S10 QPCR products were separated in a 2% agarose gel. The 100 bp DNA markers are shown alongside the bands.

(TIF)

Material and Methods S1 Detailed description of the protocols employed for quantification of serum analytes.

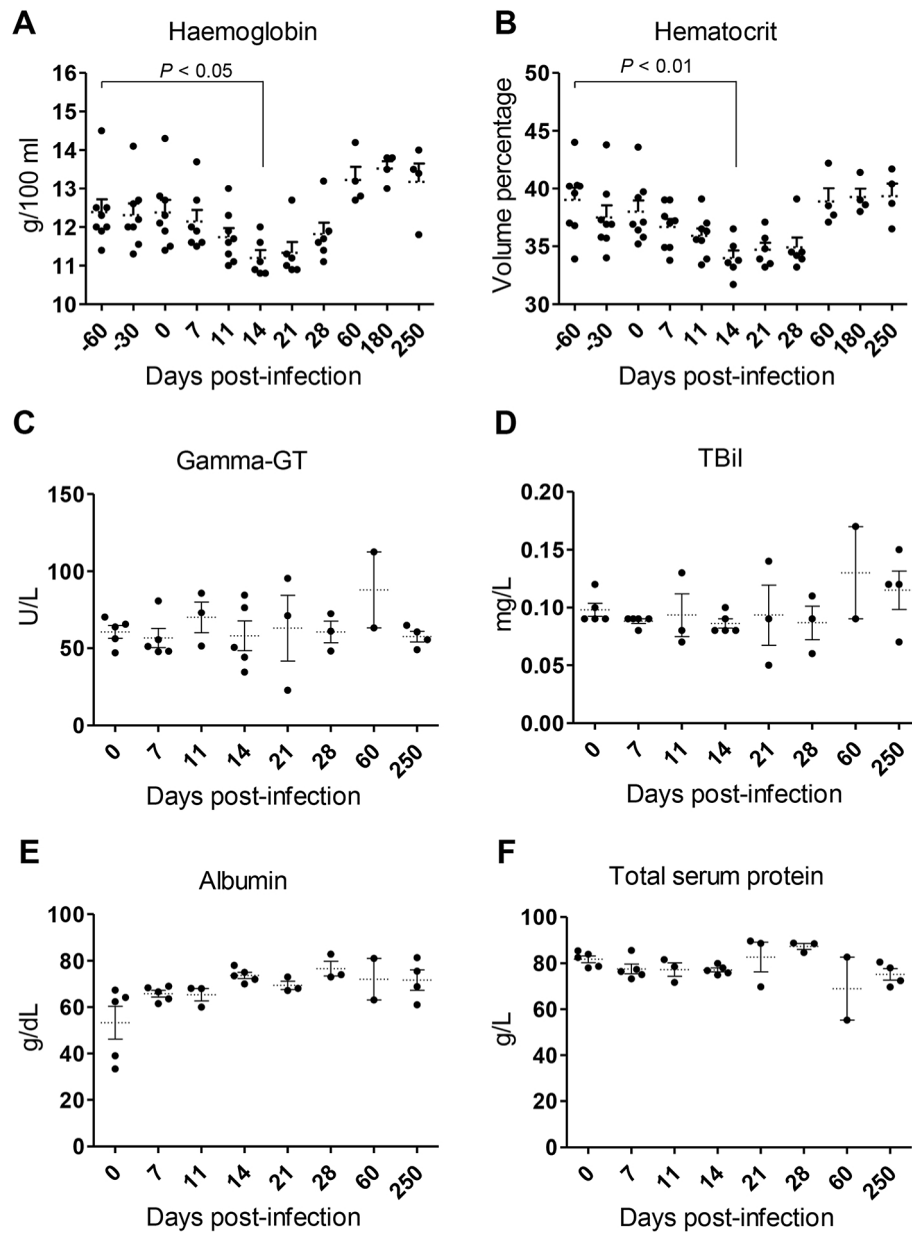
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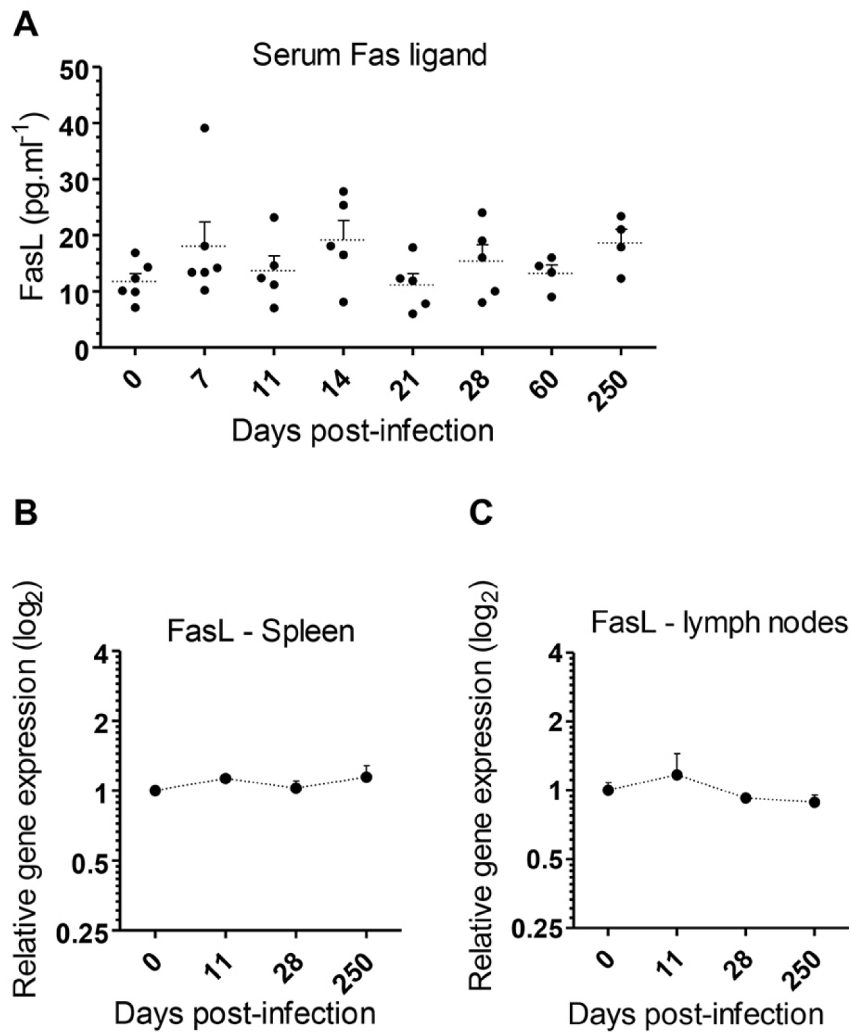
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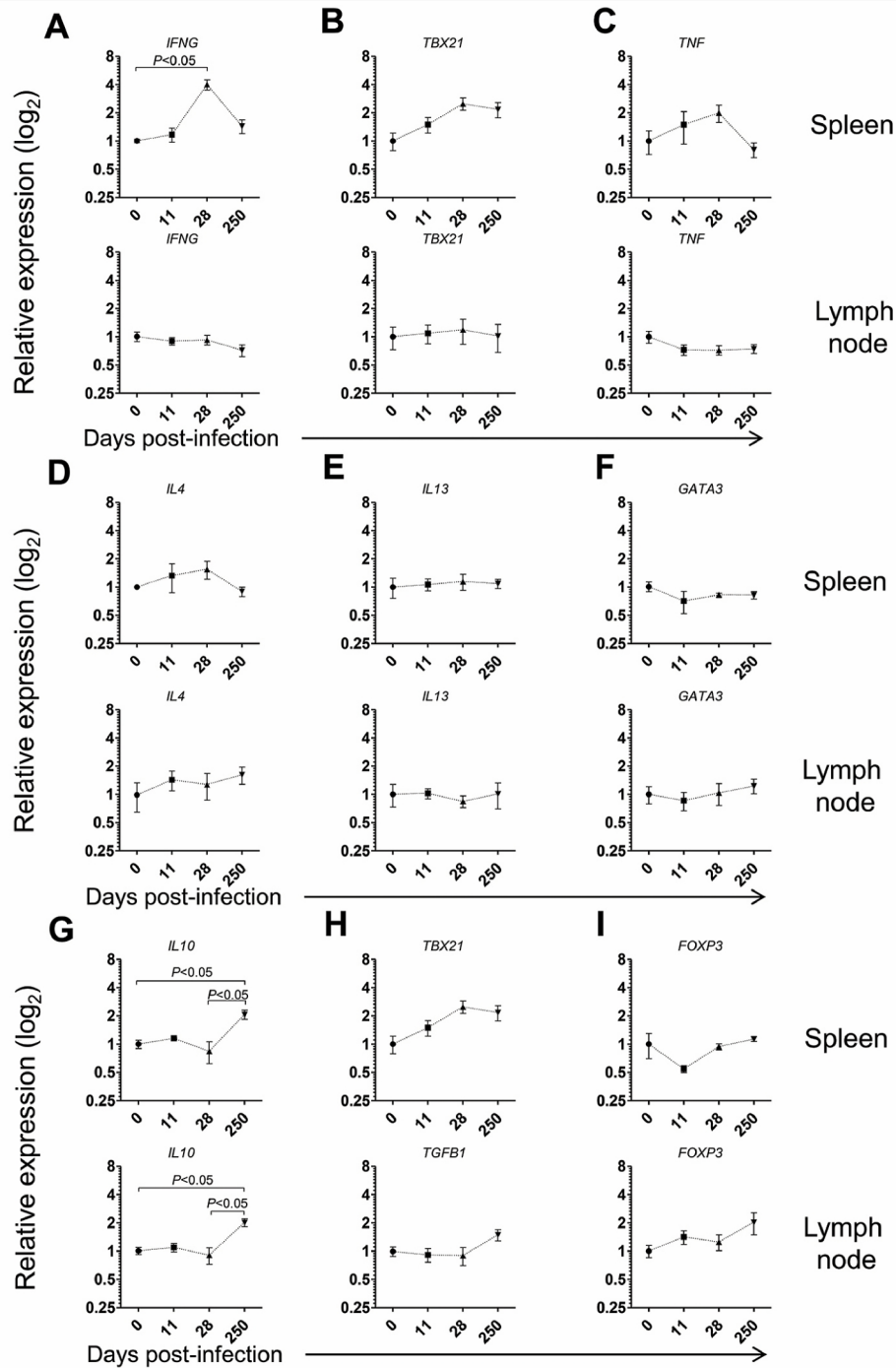
Supporting Information



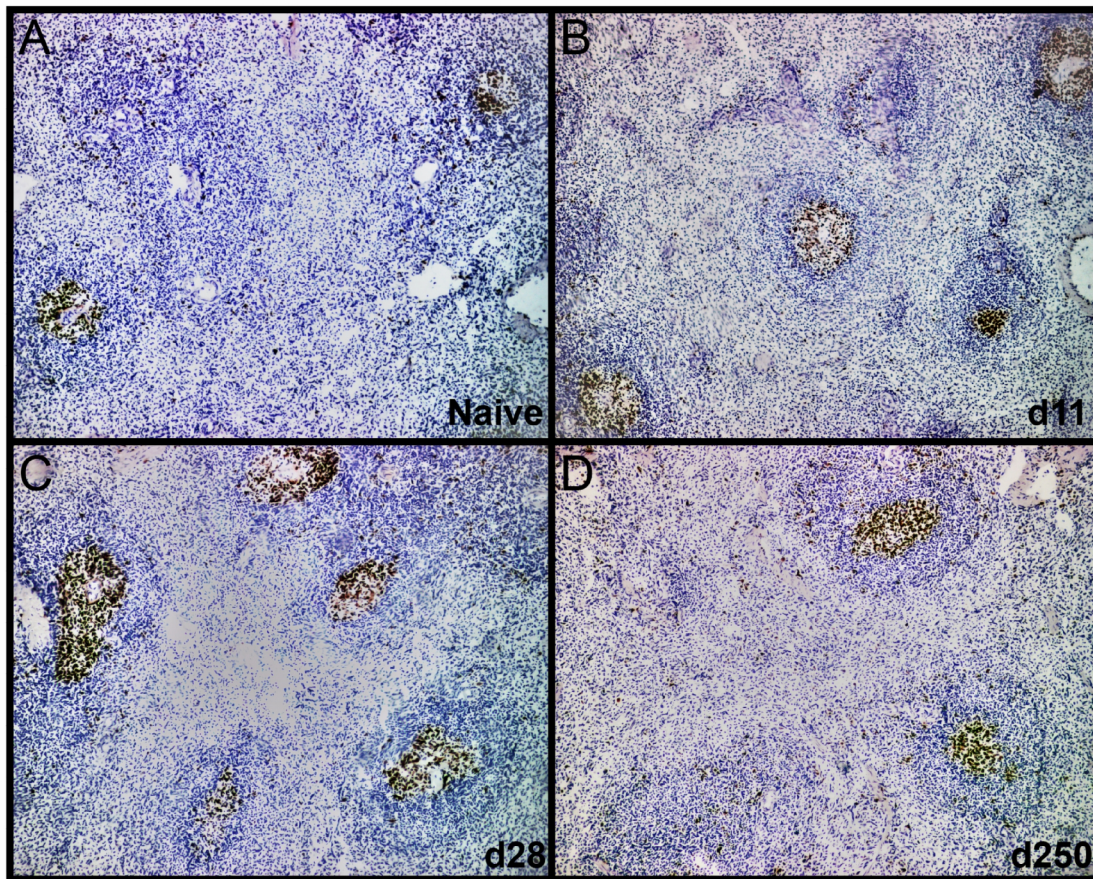
Supporting Figure 1. (A-B) Blood samples from rhesus macaques were collected for cellular enumeration at the indicated time points using a Coulter LH 500 analyzer; (A) hematocrit, (B) concentration of blood hemoglobin. (C-F) Serum samples from rhesus macaques were collected at the indicated time points and the following analytes quantified using an automated analyzer; (C) γ -glutamyl transpeptidase (Gamma-GT), (D) total bilirubin (TBil), (E) albumin and (F) total serum protein. Statistics assessed by one-way ANOVA followed by a Bonferroni's post-hoc test.



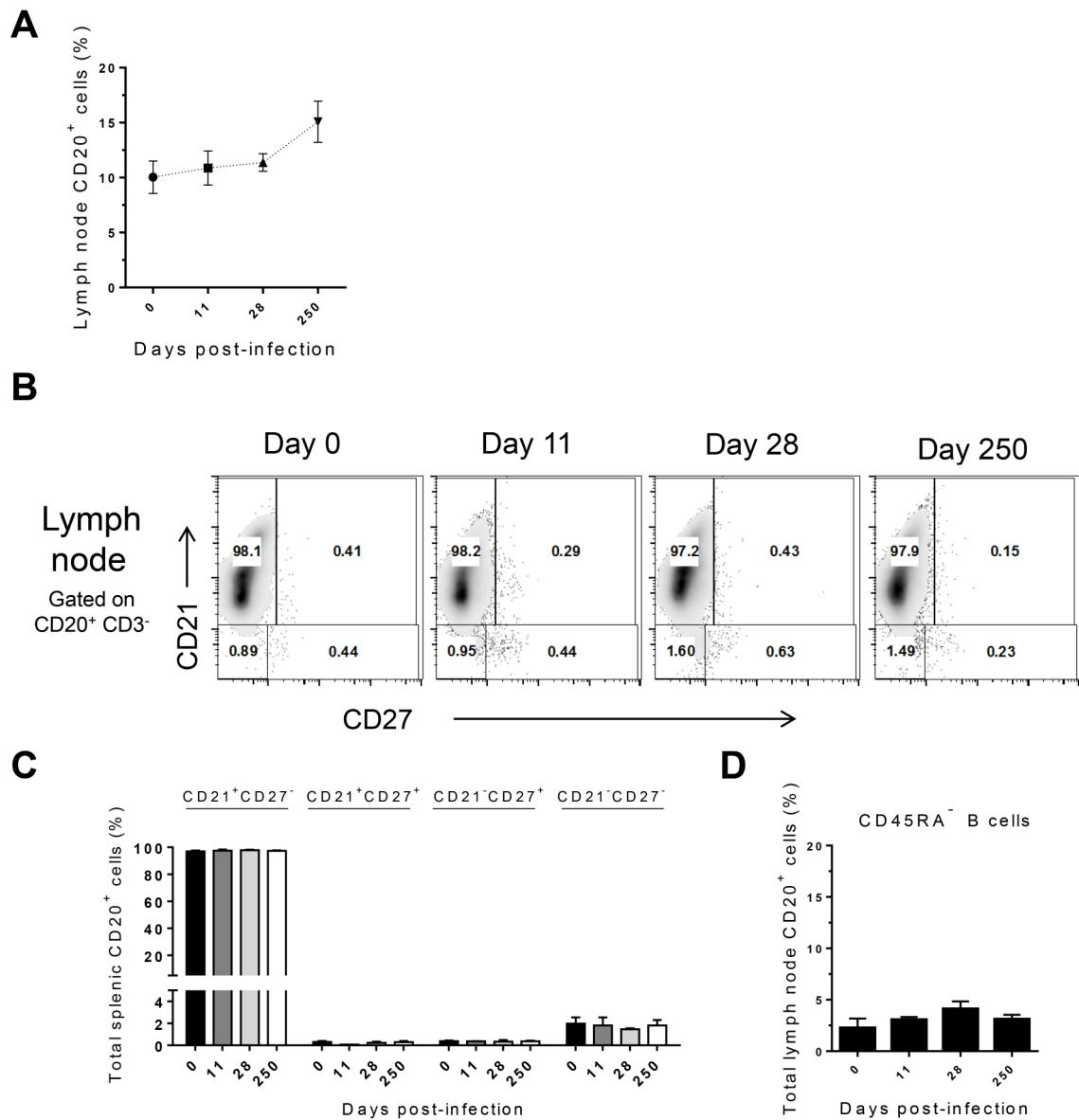
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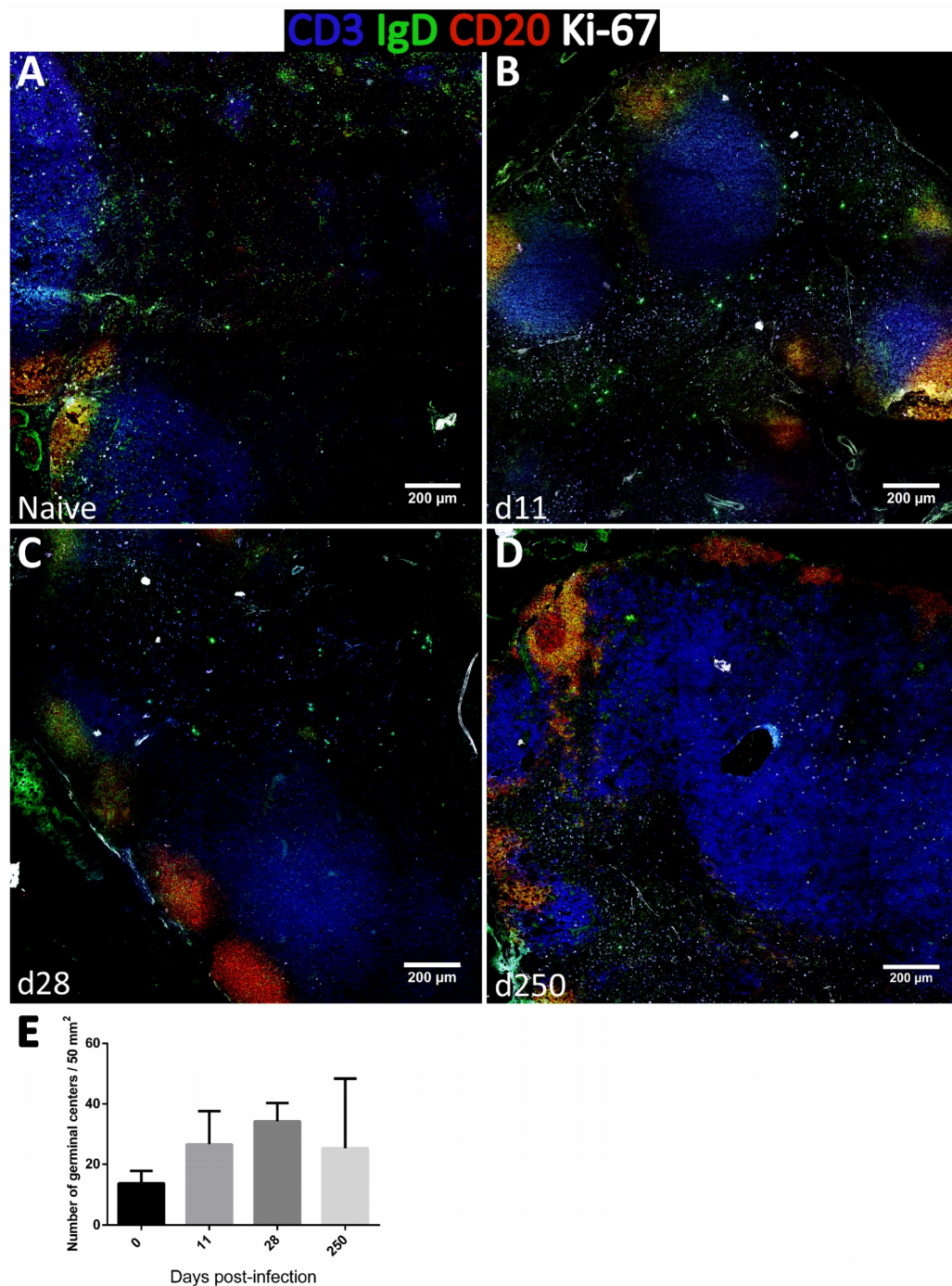
Supporting Figure 3. Gene expression profile in total spleen mononuclear cells (SMCs) and total lymph node cells during *L. infantum* infection of rhesus macaques. The relative transcript levels from total SMCs (upper panels) and total lymph node cells (lower panels) were determined by qPCR in non-infected animals and after 11, 28 and 250 days of infection. Results are shown as mean $\pm$ SEM of the fold change over the non-infected samples, which were attributed a normalized value of 1. (A) *IFNG*, (B) *TBX21*, (C) *TNF*, (D) *IL4*, (E) *IL13*, (F) *GATA3*, (G) *IL10*, (H) *TGFB1* and (I) *FOXP3*. Statistics assessed by one-way ANOVA followed by a Bonferroni's post-hoc test.



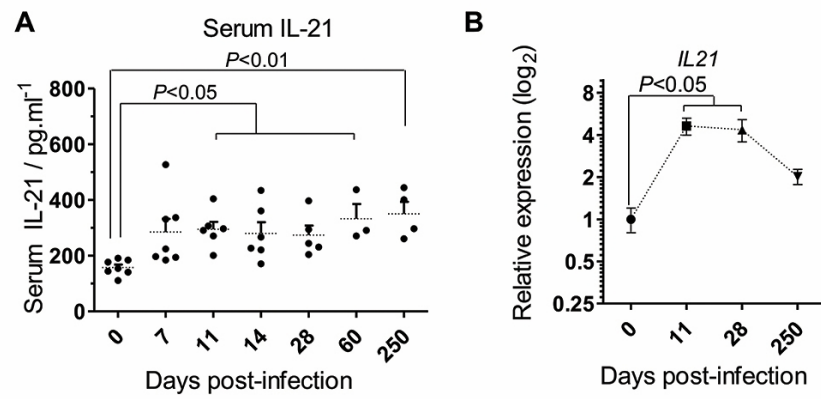
Supporting Figure 4. Representative micrographs of Ki-67-stained splenic tissue sections from naïve (A) and days 11 (B), day 28 (C), day 250 (D), used for quantification of germinal center number and area.



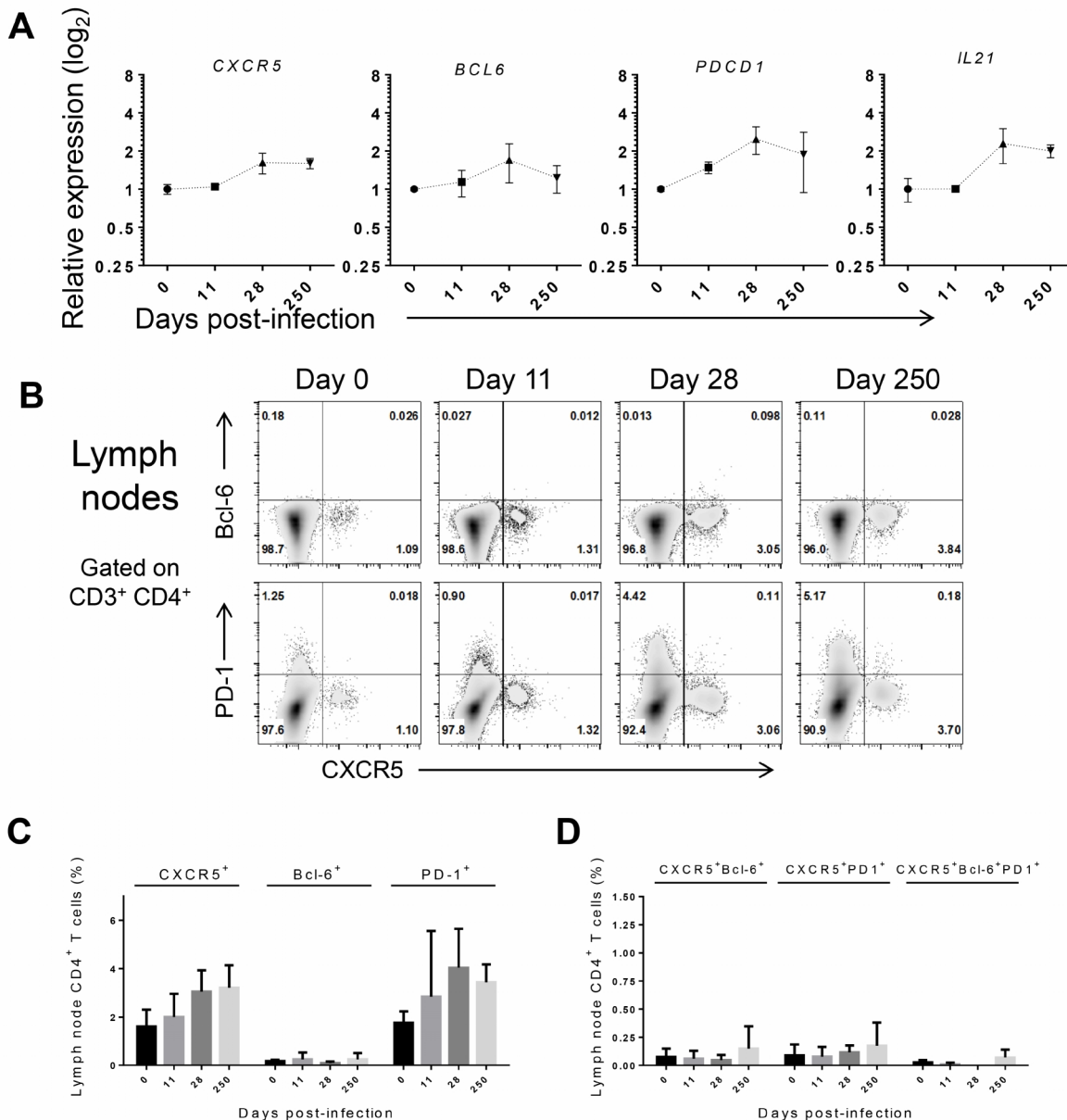
Supporting Figure 5. Lymph node B cell dynamics in *L. infantum*-infected rhesus macaques. **(A)** The percentage (mean $\pm$ SEM) of lymph node B cells was determined by flow cytometry. **(B)** Representative flow cytometry dot plots illustrating the expression of CD21 and CD27 in lymph node B cells during infection. **(C)** Histograms depict the mean $\pm$ SEM for each B cell subset: naive (CD21⁺CD27⁻), resting memory (CD21⁺CD27⁺), effector memory (CD21⁻CD27⁺) and immature (CD21⁻CD27⁻), as defined in panel (B). **(D)** Percentage (mean $\pm$ SEM) of CD3⁻CD20⁺CD45RA⁻ cells throughout infection. Data obtained from 2-4 animals per time point. Significant differences were assessed by a one-way ANOVA followed by a Bonferroni's post-hoc test and the Spearman's rank test was used for correlations.



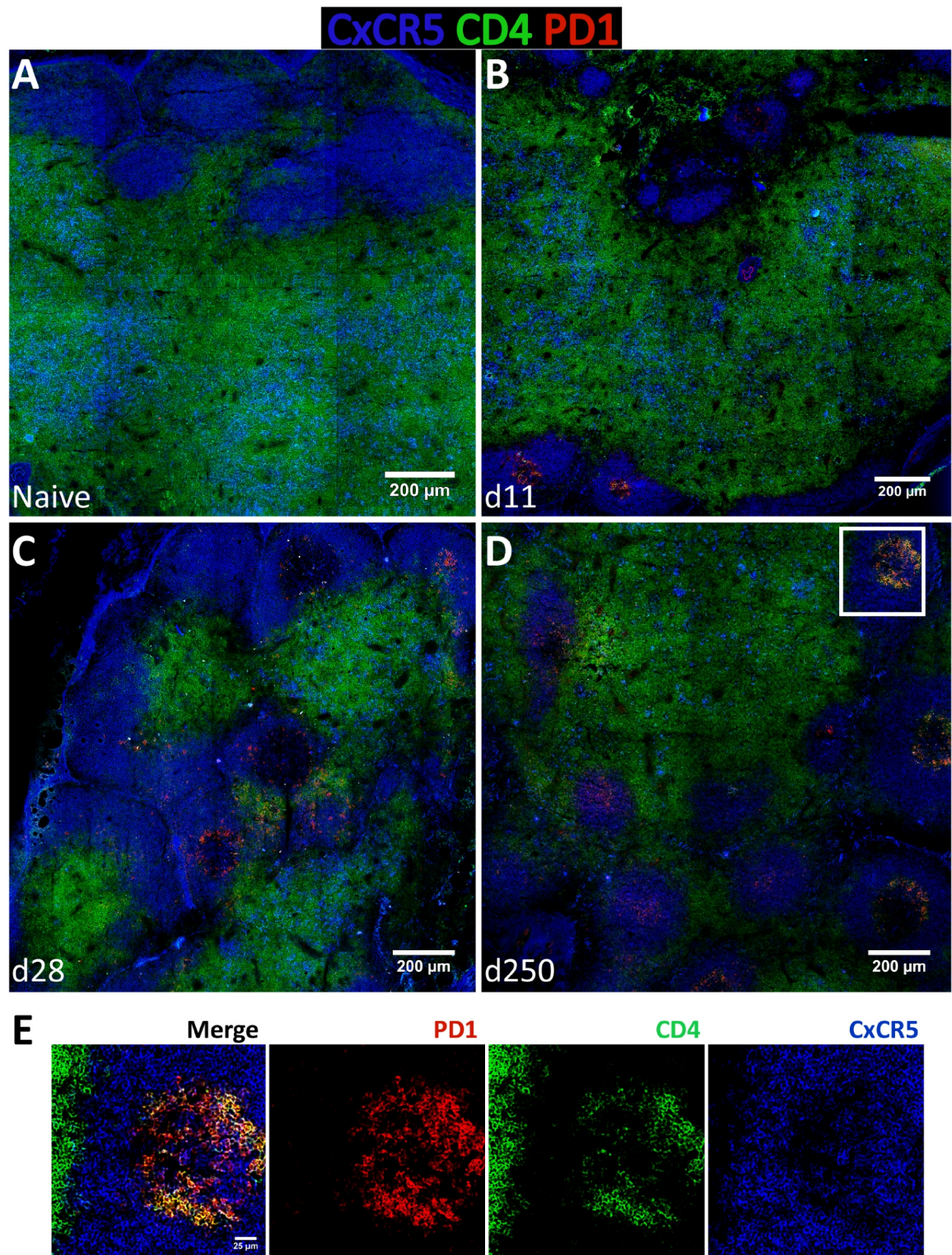
Supporting Figure 6. Dynamics of lymph node germinal center development in macaques infected with *Leishmania infantum*. (A-D) Lymph node sections were stained with antibodies against Ki-67 (white), IgD (green), CD3 (blue) and CD20 (red) and imaged by confocal microscopy. Shown are representative pictures of a naïve animal (A) and at 11 (B), 28 (C) and 250 (D) days after infection. (E) Lymph node tissue sections were stained with an antibody against Ki-67 by immunohistochemistry and germinal centers were quantified in naïve macaques (n=3), and at days 11 (n=2), 28 (n=2) and 250 (n=4) days after infection. Three distinct sections per animal were examined. Bars depict mean $\pm$ SEM. Statistical analysis was performed by one-way ANOVA, followed by a Bonferroni's post-hoc test.



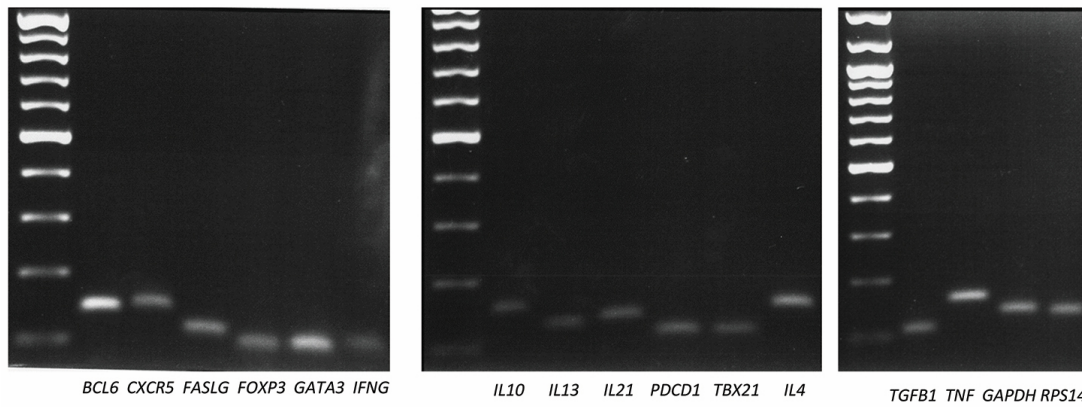
Supporting Figure 7. (A) Serum levels of IL-21 were quantified using a commercial ELISA assay. Each dot represents sera from an individual animal (B) The relative transcript levels of IL-21 in total spleen mononuclear cells, shown as fold change $\pm$ SEM (n=2-4) over non-infected samples, were determined by qPCR.



Supporting Figure 8. Dynamics of Tfh cell differentiation in the lymph nodes of *L. infantum*-infected rhesus macaques. **(A)** The relative transcript levels of *CXCR5*, *BCL6*, *PDCD1* and *IL21* in sorted lymph node $CD4$ T cells were determined by qPCR. Results are shown as fold change $\pm$ SEM over non-infected samples. **(B)** Representative density plots depicting the expression of *CXCR5* and *Bcl-6* (upper panels) or *CXCR5* and *PD-1* (lower panels) in lymph node $CD4$ T cells during the course of infection. **(C)** Expression (mean $\pm$ SEM) of *CXCR5*, *Bcl-6* and *PD-1* among splenic $CD4$ T cells during the course of infection. **(D)** Percentage (mean $\pm$ SEM) of expression of the double positive *CXCR5*⁺*Bcl-6*⁺ or *CXCR5*⁺*PD-1*⁺ populations and the triple positive *CXCR5*⁺*Bcl-6*⁺*PD-1*⁺ population among lymph node $CD4$ T cells. Statistical analysis was performed by one-way ANOVA, followed by a Bonferroni's post-hoc test.



Supporting Figure 9. Follicular helper T cell imaging in lymph nodes during *L. infantum* infection of rhesus macaques. (A-D) Lymph node tissue sections were stained with antibodies against CXCR5 (blue), CD4 (green) and PD-1 (red) and imaged by confocal microscopy. Shown are representative pictures of a naïve animal (A) and at 11 (B), 28 (C) and 250 (D) days after infection. (E) Inset from figure S8D as defined by the white square.



Supporting Figure 10. QPCR products were separated in a 2% agarose gel. The 100 bp DNA markers are shown alongside the bands.

Supporting Table 1. Information related to the antibodies used in flow cytometry and tissue immunofluorescence studies.

Antibody	Clone	Manufacturer	Dilution
Flow cytometry			
PE-CD62L	SK11	BD Biosciences	1/50
PE-CD4	L200	BD Biosciences	1/50
PerCP-CD4	L200	BD Biosciences	1/50
APC-CD45RA	T6D11	Miltenyi Biotec	1/50
ECD-CD45RA	2H4LDH11LDB9	Beckman Coulter	1/50
APC-CD95	DX2	BD Biosciences	1/50
PE-CF594-CD20	2H7	BD Biosciences	1/50
PerCP-CD3	SP34-2	BD Biosciences	1/50
FITC-CD27	M-T271	BD Biosciences	1/50
PE-CD21	B-ly4	BD Biosciences	1/50
APC -PD-1	eBio-J105	eBioscience	1/50
FITC-CxCR5	710D82.1	NHP Reagent Resource	1/50
PE-CF594-BCI-6	K112-91	BD Biosciences	1/25
Tissue Immunofluorescence			
Purified CxCR5	710D82.1	NHP Reagent Resource	1/20
AF488-CD4	OKT4	Biolegend	1/20
AF647-PD-1	EH12.2H7	Biolegend	1/20
Purified Ki-67	MIB-1	DAKO	1/50
FITC-IgD	IADB6	Southern Biotech	1/50
PE-CF594-CD3	SP34-2	BD Biosciences	1/50
eFluor660-CD20	L26	eBioscience	1/50

Supporting Table 2. Sequence, PCR product size and accession number of the primers used in this study.

Gene	Forward primer (5' – 3')	Reverse primer (5' – 3')	Product length (bps)	ENSEMBLE Accession IDs	
				Gene	Transcript
<i>BCL6</i>	AGCAAGGCATTGGTGAAG	GCTCACAACATGACAACATC	138	ENSMMUG00000009198	ENSMMUT00000012862
<i>CXCR5</i>	GACCTGTTCTTGAATTTGAC	GAGGAAGATGAGGCTGTAGG	144	ENSMMUG00000009316	ENSMMUT00000013019
<i>FASLG</i>	CTACCAGCCAGAAGCATACAG	GGGCTTGCCTGTAAATG	110	ENSMMUG00000007285	ENSMMUT00000010180
<i>FOXP3</i>	CAGCTGGTGCTGGAGAAG	TCGCTCACCACAGATGAAG	91	ENSMMUG00000008624	ENSMMUT00000012056
<i>GATA3</i>	CTTCGCTACCCAGGTGAC	CGACGACTCTGGAATTCTG	95	ENSMMUG00000006944	ENSMMUT00000009704
<i>IFNG</i>	AAAGAAACGGGATGACTTTG	GACAGTTCAGCCATCACTTG	105	ENSMMUG000000019225	ENSMMUT00000007007
<i>IL10</i>	ATGAAGGATCAGCTGGACAAC	GATGTCTGGGTCGTGGTTC	146	ENSMMUG000000023569	ENSMMUT00000003151
<i>IL13</i>	GTCAACATCACCCAGAACC	CAGGGACTGTCTCGTTCAG	115	ENSMMUG000000003131	ENSMMUT00000004436
<i>IL21</i>	CAAGATCGCCACATGATTAG	TGACCACTCACAGTTGTCTC	128	ENSMMUG000000003346	ENSMMUT00000004728
<i>PDCD1</i>	GACAGCGGCACCTACCTC	CTTCTGCCCTTCTCTCTGTC	102	ENSMMUG000000008592	ENSMMUT00000012015
<i>TBX21</i>	GAGTCCATGTACGCATCTG	TTGGGTAGGAGAGGAGAGTAG	97	ENSMMUG000000007098	ENSMMUT00000009906
<i>IL4</i>	CTGCCTCCAAGAACAAC	TGCTTGTGCTGTGAAAC	129	ENSMMUG000000003133	ENSMMUT00000004438
<i>TGFB1</i>	ACCCACAACGAAATCTATGAC	CAGGTTCAAGTACTGCTTCTC	96	ENSMMUG000000008142	ENSMMUT00000001376
<i>TNF</i>	ATGTTGTAGCAAAACCCTCAAG	TGAAGAGGACCTGGGAGTAG	149	ENSMMUG000000008845	ENSMMUT00000002362
<i>GAPDH</i>	GACCTGTTCTTGAATTTGAC	GAGGAAGATGAGGCTGTAGG	113	ENSMMUG000000018679	ENSMMUT00000008204
<i>RPS14</i>	AAGCAGATGGTTCCTTCATC	TAGCCTCTCATCTGCGTATTC	116	ENSMMUG000000002848	ENSMMUT00000000826

Supporting Material and Methods. Detailed description of the protocols employed for quantification of serum analytes.

Serum IL-21 was quantified using a sandwich ELISA kit (eBiosciences), following manufacturer's instructions.

FasL in the serum was quantified by a solid-phase immunoassay (MBL), according to manufacturer's instructions. This assay uses anti-FasL mAbs (clones 4H9 and 4A5). Peroxidase substrate was used to quantify FasL and the absorbance measured at 450 nm. Quantification of serum albumin, total protein, alanine transaminase (ALT), c-reactive protein (CRP), γ -glutamyl transpeptidase (Gamma-GT), total bilirubin (TBil), complement components C3 and C4 and total serum IgM and IgG were all performed on an AutoAnalyzer (PRESTIGE® 24i, PZ Cormay S.A.). Briefly, to quantify albumin, a colored complex was formed with bromocresol green, in an acidic medium and absorbance of the complex measured at 630 nm. Total protein titration was based on the biuret reaction and color intensity measured at 546 and 700 nm. ALT quantification was performed with a modified method in which ALT catalyzes the reversible transfer of an amino group from

alanine to α -ketoglutarate, forming glutamate and pyruvate. The pyruvate produced is reduced to lactate by lactate dehydrogenase (LDH) and NADH. The rate of decrease in concentration of NADH, measured photometrically at 340 nm, is proportional to the catalytic concentration of ALT present in the sample. In CRP determination, an antigen-antibody reaction occurs between CRP in a sample and anti-CRP antibody which has been sensitized to latex particles. This agglutination is detected as an absorbance change at 572 nm. Gamma-GT determination was performed by a kinetic method, in which the enzyme catalyzes the transfer of the γ -glutamyl group from γ -glutamyl-p-nitroanilide to the acceptor glycylglycine. The rate of 2-nitro-5-aminobenzoic acid formation, measured photometrically at 405 nm, is proportional to the catalytic concentration of GGT present in the sample. C3 measurement was performed by a quantitative turbidimetric test by comparison from a calibrator of known C3 concentration. Anti-C3 antibodies form insoluble complexes with C3 present in the samples, which cause an absorbance change at 340 nm. A similar method was used for C4 quantification, using anti-C4 antibodies. Quantification of total serum IgG and IgM was performed employing a previously validated turbidimetric method (Skoug *et al.* 1988). Briefly, anti-human IgG or IgM antibodies, which cross-react with non-human primates, form insoluble complexes with the IgG or IgM present in the samples, which causes an absorbance change at 600 nm (Carru *et al.* 2004). Briefly, 100 μ L of 5 % TCA (Sigma) were added to 100 μ L of serum standards (D-(+)-Neopterin; Sigma) or samples and vortexed for 10s. The samples were centrifuged at 10000 rpm for 5 min and 50 μ L of the supernatant was diluted with 200 μ L of bidistilled water. Samples (30 μ L) were injected in a UFLC Prominence Shimadzu (USA). Separation was carried out in a Ascentis® C18 reversed-phase column (5 μ m $\times$ 15 cm $\times$ 4.6 mm), using water:acetonitrile (99:1 v/v) as the mobile phase and a flow rate of 1.5 mL/min. Neopterin was detected at a Shimadzu RF-10AXL fluorescence detector by its native fluorescence (353 nm excitation, 438 nm of emission).

Article 2.

Longitudinal analysis defines the kinetics of CD8 T cell differentiation and chronic exhaustion in rhesus macaques infected with *Leishmania infantum*.

(To be submitted)

Here, we extend the analysis made in the previous article to a longitudinal description of the CD8 T cell response in the blood and secondary lymphoid organs during the course of *L. infantum* infection of rhesus macaques. Our analysis included immunophenotyping for markers of CD8 T cell differentiation, cytotoxicity and exhaustion, CD8 T cell cytokine gene expression and tissue localization to obtain a global picture of the CD8 response during infection.

Main findings

- Splenic and lymph node CD8 T cells acquire terminal effector and effector memory phenotypes after *L. infantum* infection. However, downregulation of surface CD28 and upregulation of CD95 indicates that splenic CD8 T cells progress towards a more terminal state of differentiation;
- At chronic phase of *L. infantum* infection of rhesus macaques, splenic CD8 T cells exhibit significantly decreased expression of Th1 cytokines, as compare with the acute phase;
- T-bet induction in splenic CD8 T cells during the acute phase of infection is associated with the expression of granzyme B. At the chronic phase, loss of the T-bet-expressing splenic CD8 population is concomitant with granzyme B downregulation.
- EOMES is similarly induced in splenic CD8 T cells during the acute phase, but, unlike T-bet, its expression does not appear associated with granzyme B and remains elevated at the chronic phase.
- The functional impairment of splenic CD8 T cells at the chronic phase of infection was concomitant with the elevated expression of inhibitory receptors, particularly LAG-3.

Longitudinal analysis defines the kinetics of CD8 T cell differentiation and chronic exhaustion in rhesus macaques infected with *Leishmania infantum*.

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Abstract

The development of an effective vaccine for visceral leishmaniasis requires a detailed knowledge of the mechanisms underlying both functional and dysfunctional T cell responses. CD8 T cells play an important role in controlling the parasite, but the timing of acquisition of effector and memory functions remains largely unknown. We employed an experimental model of rhesus macaques infected with *L. infantum* to follow the CD8 T cell response during the course of infection. Our analysis revealed that the expansion and differentiation of the splenic CD8 T cell pool were associated with the induction of T-bet and EOMES. These two transcription factors appear to regulate distinct functions in the responding CD8 population. While T-bet was strongly associated with the expression of granzyme B, EOMES levels persisted elevated even after loss of expression of cytotoxic molecule. Interestingly, the loss of function of splenic CD8 T cells in the chronic phase of infection was associated with the upregulation of inhibitory T cell receptors, particularly LAG-3, suggesting the occurrence of T cell exhaustion. Our study provides new insights into the orchestration of CD8 T cells responses during visceral *Leishmania* infection that could be helpful in the design of future vaccines or immunotherapies aimed at maintaining a functional pool of this important lymphocyte subset.

Introduction

Visceral leishmaniasis (VL), caused by the sand fly-transmitted protozoan parasites *Leishmania infantum* or *L. donovani*, is a chronic disease that afflicts some of the poorest regions of the world. Despite substantial efforts, no human vaccine is available to prevent VL (Kedzierski 2010). Difficulties in translating data from animal models and limited knowledge on the immune mechanisms underlying T cell memory development are regarded as the major obstacles in successful vaccine design for *Leishmania* infections (Kedzierski 2010).

Upon infection, naïve CD8 T cells are primed by dendritic cells (DCs) in secondary lymphoid organs initiating a differentiation program characterized by remarkable proliferation of the antigen-specific pool. At the peak of the response, the expanded population is highly heterogeneous, ranging from short-lived effectors, also known as cytotoxic T lymphocytes (CTLs), to memory precursors that are predisposed to survive past the contraction phase and confer long-term protection against reinfection (Thaventhiran *et al.* 2013). The CTL is a sophisticated cell type endowed with the ability to migrate to virtually every corner of the body and kill infected cells via mechanisms relying on perforin/granzyme or death ligands. CTLs are also major producers of cytokines such as INF- γ or TNF, hence being an integral part of a type 1 immune response that follows an intracellular pathogen infection (Kaech *et al.* 2012).

The size and composition of the responding CD8 population during a primo-infection is dependent on several factors. These include the duration of the TCR-MHC interactions, the magnitude and quality of co-stimulatory signaling and the presence of pro-inflammatory cytokines secreted by DCs such as type I IFNs or IL-12 (Lanzavecchia *et al.* 2002, Kaech *et al.* 2012). As such, CD8 T cells must integrate this diversity of signals in coping with the decision of becoming a short-lived CTL or a memory T cell (Arens *et al.* 2010).

Studies in the past decade unveiled a crucial role for two T-box transcription factors, T-bet and Eomesodermin (EOMES), in regulating the differentiation of effector and memory CD8 T cells (Rutishauser *et al.* 2010). In recently activated CD8 T cells, EOMES and T-bet cooperate by inducing the expression of IFN- γ , granzyme B or perforin (Intlekofer *et al.* 2005), hence promoting an effector identity in CD8 T cells. However, and despite cooperating at several levels, the expression of T-bet and EOMES among the responding CD8 population appears to be somewhat reciprocal with T-bet levels being highest in short-lived CTLs while EOMES is enriched in the memory pool (Banerjee *et al.* 2010, Rao *et al.* 2010).

In contrast with infections that are resolved acutely, several pathogens are capable of chronic persistence in the host, hence subjecting T lymphocytes to continuous stimulation and impacting on the self-renewal capacity of memory CD8 T cells (Virgin *et al.* 2009,

Rodrigues *et al.* 2014a). In the long term, chronic infections induce a gradual loss-of-function in the bulk of the responding CD8 population; a phenomenon known as T cell exhaustion (Wherry 2011). On their path towards terminal exhaustion, CD8 T cells progressively accumulate surface expression of inhibitory receptors such as PD-1 or LAG-3 (Wherry 2011). Furthermore, transcriptional profiling studies indicate that exhausted T cells are in a differentiation state distinct from effector and memory T cells (Doering *et al.* 2012).

To date, the role played by CD8 T cells during *Leishmania* infections remains a matter of dispute, as both host-protective and pathological CD8 responses have been reported (Stager *et al.* 2012). Nevertheless, convincing evidence demonstrates that CD8 T cells are important in the formation and maintenance of granulomas that control parasites in the liver (Stager *et al.* 2012). Less is known about the participation of CD8 T cells in the immune response in the spleen, the main site of parasite persistence during chronic VL (Kaye *et al.* 2011a). Recent studies in murine models and human patients evidenced a progressive loss of function and acquired expression of inhibitory receptors of splenic CD8 T cells that are suggestive of exhaustion (Joshi *et al.* 2009, Gautam *et al.* 2014). Strikingly, we still miss a detailed analysis of the kinetics of CD8 T cell differentiation during experimental VL that takes into account all the recently identified players that regulate the differentiation of CD8 T cell into effectors or memory cells.

We have recently introduced a non-human primate (NHP) model of VL to study the immune mechanisms associated with parasite control or dissemination in a physiological relevant host (Rodrigues *et al.* 2014b). Here, we provide a detailed phenotypic and functional analysis of the CD8 T cell dynamics during the course of *L. infantum* infection in rhesus macaques. Our data revealed the prominent expansion of the splenic CD8 T cell pool after infection, which was associated with the acquisition of terminal effector and effector memory phenotypes. Expression of T-bet in splenic CD8 T cells paralleled the acquisition of effector functions, being highest at one month after infection and decreasing at the chronic phase while EOMES expression persisted elevated even after the loss of effector molecules. Finally, our data highlights that the loss of CD8 T cell effector function at the chronic phase is associated with persistent expression of the inhibitory receptor LAG-3. Globally, our study provides new insights into the immunobiology of CD8 T cells during VL that could be helpful in the design of future therapeutic interventions aimed at improving the durability of the CD8 T cell response in an infectious context.

Results

Kinetics of CD8 T cell expansion and differentiation in the blood and secondary lymphoid organs during *L. infantum* infection of rhesus macaques

Previously, we reported that rhesus macaques infected with *L. infantum* were able to contain the parasites in visceral compartments (liver and spleen) during the first weeks after inoculation. However, progression to the chronic phase was associated with a general dissemination of the parasite in the blood, bone marrow and lymph nodes (Rodrigues *et al.* 2014b). Here, we examined the same animals for a longitudinal analysis of the CD8 T cell response in the blood and secondary lymphoid organs during the acute and chronic phase of *L. infantum* infection.

The percentage of CD8 T cells in the blood or lymph nodes did not change significantly during the course of our experiments (Fig. 1A-B). In contrast, the splenic CD8 T cell pool expanded significantly after infection, from 16.9 ± 3.5 % of the total splenocytes in naïve animals to 44.9 ± 7.1 % at day 28 post-infection (pi); a frequency that persisted elevated at the chronic phase (day 250 pi) (Fig. 1C). In absolute numbers, it represented a 4-fold increase in the size of the splenic CD8 T cell pool at days 28 and 250 pi, as compared with non-infected animals (Fig. 1D).

The majority of blood CD8 T cells presented a differentiated phenotype, even in non-infected animals, as noted by the low expression of CD62L (Fig. 1E-F). Hence, effector memory (CD62L⁻ CD45RA⁺) or terminally differentiated (CD62L⁻ CD45RA⁺) CD8 T cells predominated in the blood, (Fig. 1E-F). The relative frequencies of these subsets remained relatively constant throughout the experiments, despite slight fluctuations (Fig. 1E-F). In contrast, the pool of CD8 T cells in lymph nodes and in the spleen underwent major phenotypic changes after infection (Fig. 1G-J). The percentage of naïve (CD62L⁺ CD45RA⁺) CD8 T cells decreased substantially in both anatomical locations. In lymph nodes, cells with an effector memory phenotype predominated, particularly at the chronic phase of infection (Fig. 1G-H), while in the spleen, terminally differentiated CD8 T cells become the most abundant subset at one month after infection and towards the chronic phase (Fig. 1I-J). Interestingly, the percentage of central memory (CD62L⁺ CD45RA⁻) CD8 T cells did not suffer significant variations throughout the infection in any of the anatomical sites evaluated, accounting for around or less than 10% of the total CD8 T cells.

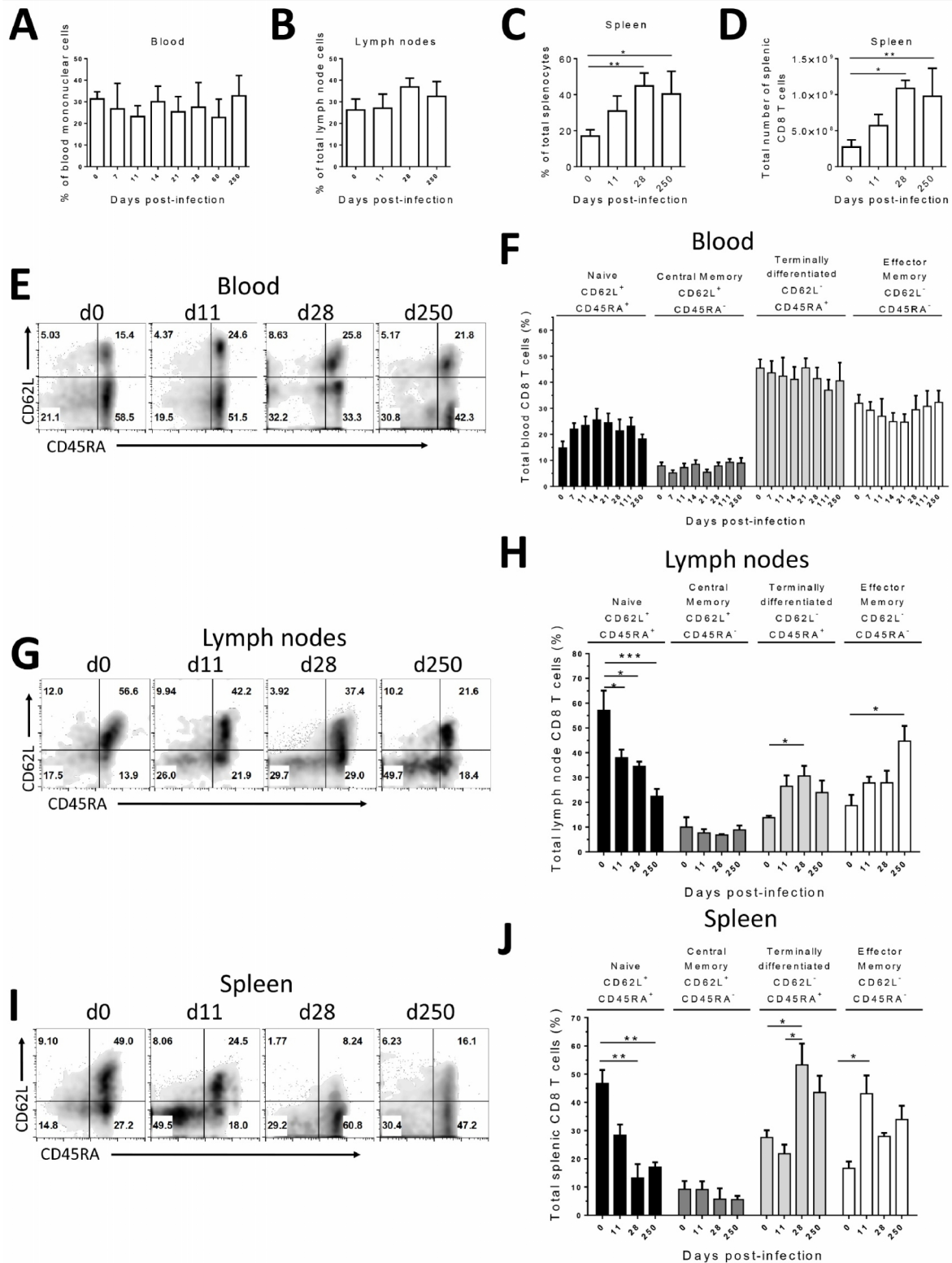


Figure 1. Splenic and lymph node CD8 T cells acquire terminal effector and effector memory phenotypes after *L. infantum* infection. (A–C) Percentage of CD3⁺ CD8⁺ CD20[−] cells during the course of infection, as determined by flow cytometry, in the blood (A), lymph nodes (B) and spleen (C). (D) Total number of splenic CD3⁺ CD8⁺ CD20[−] cells during infection. (E–J) The percentages of naïve (CD62L⁺ CD45RA⁺), central memory (CD62L⁺ CD45RA[−]), effector memory (CD62L[−] CD45RA[−]) and terminal effector (CD62L[−] CD45RA⁺) cells among CD3⁺ CD8⁺ CD20[−] cells were determined by flow cytometry. (E, G and I) Representative density plots of the distinct CD8 T cell subsets at the indicated time points after infection in the blood (E), lymph nodes (G) and spleen (I).

(F, H and J) Bar charts depict averaged data (mean $\pm$ SEM) for the blood (F), lymph nodes (H) and spleen (J). Plots present data from 4-11 (blood) or 2-4 (spleen, lymph node) macaques per time-point. Statistical analysis performed using one-way ANOVA followed by a Bonferroni's post-hoc test.

Expression of the costimulatory receptor CD28 is indicative of CD8 T cell proliferative potential and its loss correlates with terminal differentiation (Effros 2004). Phenotypic characterization revealed that about half of blood CD8 T cells expressed CD28 (Fig. 2A, top panels); a percentage that remains constant throughout infection and is in agreement with their effector or effector memory phenotype (Fig. 1E-F). Surprisingly, the vast majority (about 90%) of lymph node CD8 T cells maintained expression of CD28 during the course of infection (Fig. 2A, middle panels); a phenotype that was not predictable based on their patterns of CD62L and CD45RA expression (Fig. 1G-H). Contrasting with lymph nodes, splenic CD8 T cells progressively lost expression of CD28 (Fig. 2A, bottom panels), which is suggestive of a more terminal differentiation as compared with lymph node-residing CD8 T cells.

Next, we assessed surface expression of the death receptor CD95 (Fas), as it is also a marker associated with CD8 T cell differentiation and commonly employed in rhesus macaques (Arnoult *et al.* 2003, Jankovic *et al.* 2003). Accordingly, the bulk of blood CD8 T cells expressed CD95, whose percentage of expression did not change significantly throughout the experiments (Fig. 2B, top panels). In lymph nodes, the expression of CD95 in the CD8 T cell pool was lower than in the blood but manifested a non-statistical increasing trend after infection (Fig. 2B, middle panels). The percentage of splenic CD8 T cells expressing CD95 progressively increased after infection, being significantly augmented at days 28 and 250 pi (Fig. 2B, lower panels), hence displaying the inverse trend of CD28 expression as the cells progress towards more terminal differentiation states.

Fas (CD95)-mediated apoptosis is a crucial mechanism involved in reducing the size of the responding T cell population during the contraction phase of an immune response (Krammer *et al.* 2007). Some evidence also suggested that peripheral CD8 T cells may undergo death triggered by the death ligand TRAIL (Janssen *et al.* 2005). Given the increased expression of CD95 in splenic CD8 T cells after infection (Fig. 2B), we tested whether these cells were more prone to die after exogenous addition of the death ligands (Fig. 2. C-H). Splenic CD8 T cells became progressively more sensitive to FasL-induced cell death as the time after infection advanced, attaining a plateau at one month after infection, a time-point at which about half of the splenic CD8 T cell pool was susceptible to Fas-mediated cell death (Fig. 2G-H). In contrast, in the blood and lymph nodes, the percentage of annexin V⁺ CD8 T cells, after overnight incubation with soluble Fas ligand (CD95L), was, despite minor variations, constant during the experiments (Fig. 2C-F). Unlike

Fas ligand, TRAIL was unable to increase the apoptotic frequency of blood or lymphoid organ CD8 T cells over the spontaneous rate of cell death (Fig. 2G-H).

Overall, the differences observed in the phenotype and susceptibility to Fas-mediated cell death between the CD8 T cell pools in the spleen and lymph nodes suggests that the pattern of parasite colonization of each organ impacts on the differentiation of the CD8 T cell pool. In this sense, the more terminal state of differentiation exhibited by CD8 T cells is possibly a consequence of parasite persistence in this site.

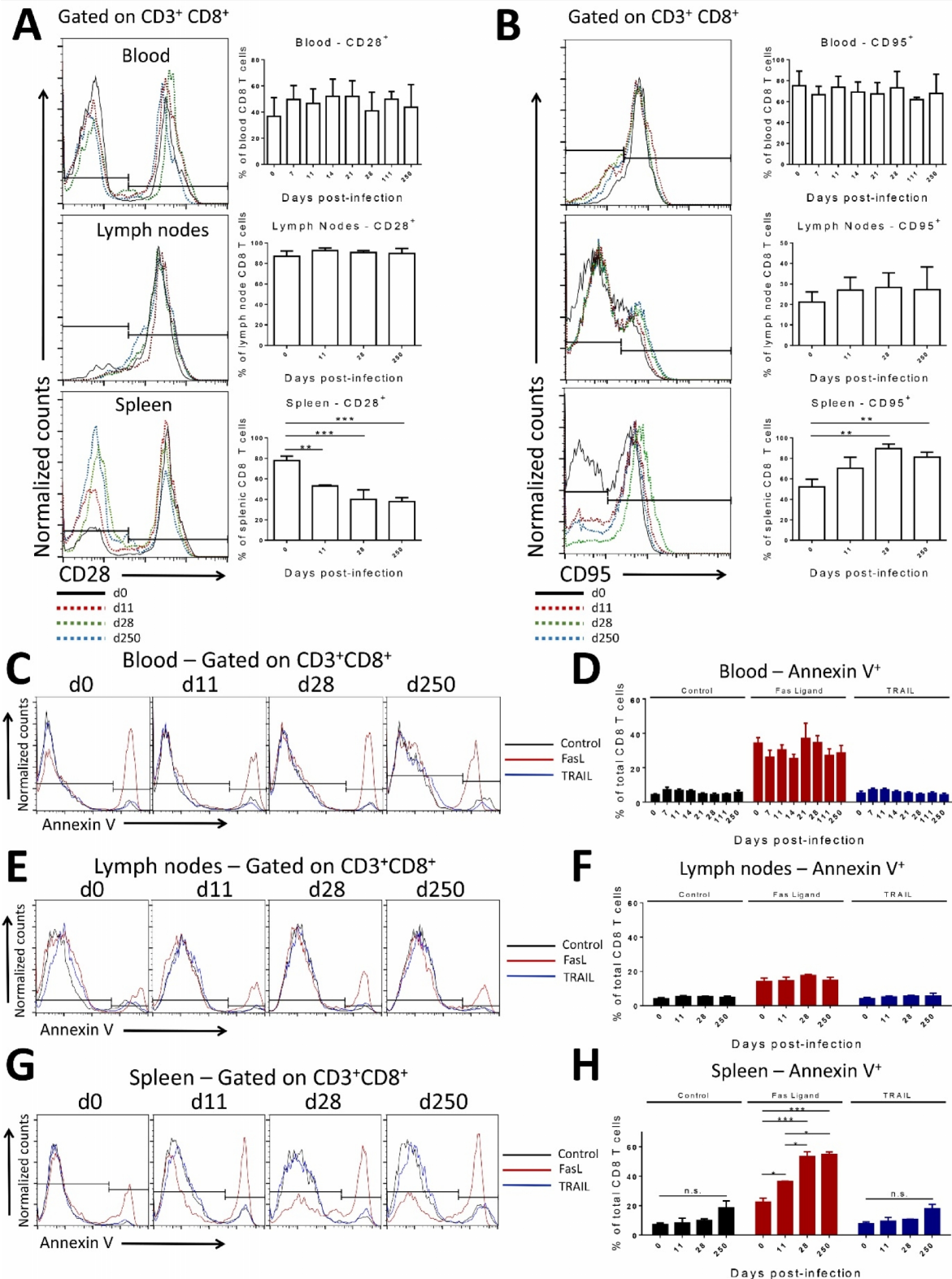


Figure 2. Splenic, but not lymph node, CD8 T cells experience terminal differentiation after *L. infantum* infection, associated with sensitivity to Fas-mediated apoptosis. **(A)** Kinetics of CD28 expression in CD8 T cells in the blood (top), lymph nodes (middle) and spleen (bottom). The left panels are representative histograms at the indicated time-points. The left panels depict averaged data (mean $\pm$ SEM). **(B)** CD95 expression in CD8 T cells during the course of infection in the blood (top), lymph nodes (middle) and spleen (bottom). The left panels depict representative histograms. In the right, the averaged data is shown (mean $\pm$ SEM). **(C-H)** PBMCs (C-D), lymph node cells (E-F) or SMCs (G-H) retrieved from infected macaques at the indicated time-points were

cultured overnight in the presence of Fas ligand (100 ng/mL), TRAIL (100 ng/mL) or vehicle (DMSO). The percentage of apoptotic CD8 T cells was determined by annexin V binding combined with staining for CD3 and CD8. (C, H and E) Representative histograms showing annexin V binding in the blood (C), lymph nodes (E) and spleen (G). (D, F and H) Pooled data showing the mean $\pm$ SEM of annexin V binding in the blood (D), lymph nodes (F) and the spleen (H). All plots depict data from 4-11 (blood) or 2-4 (spleen, lymph node) macaques per time-point. Statistical analysis performed using one-way ANOVA followed by a Bonferroni's post-hoc test.

Decreased expression of type I cytokines in splenic CD8 T cells in the chronic phase of *L. infantum* infection of rhesus macaques

Production of type I cytokines, such as IL-2, TNF or IFN- γ , is an important effector function of CD8 T cells (Zhang *et al.* 2011). In *L. infantum*-infected rhesus macaques, the transcript levels of *IFNG*, *IL2* and *TNF* in purified splenic CD8 T cells did not increase significantly during the acute phase of infection (Fig. 5A), in contrast with our previous observations in splenic CD4 T cells (Rodrigues *et al.* 2014b). Nevertheless, a decrease in the transcript levels of these cytokines was evident at the chronic phase of infection, with statistical differences detected for *TNF* and *IFNG* (Fig. 5A). In lymph nodes, cytokine expression by CD8 T cells was relatively constant throughout infection (Fig. 5B), though the *IL2* and *TNF* mRNAs manifested a tendency towards lower levels the chronic phase (Fig. 5C-D). Cytokine mRNA abundance was in general 2- to 8-fold lower in lymph node than in splenic CD8 T cells, depending on the time-point analyzed, as noted by their higher threshold cycle (Ct) values (Fig. 5A and C), indicating that lymph node CD8 T cells produce fewer cytokines than splenic CD8 T cells. Collectively, these results evidence an impaired capacity for CD8 T cells to produce type I cytokines in the spleen during the chronic phase of *L. infantum* infection of rhesus macaques.

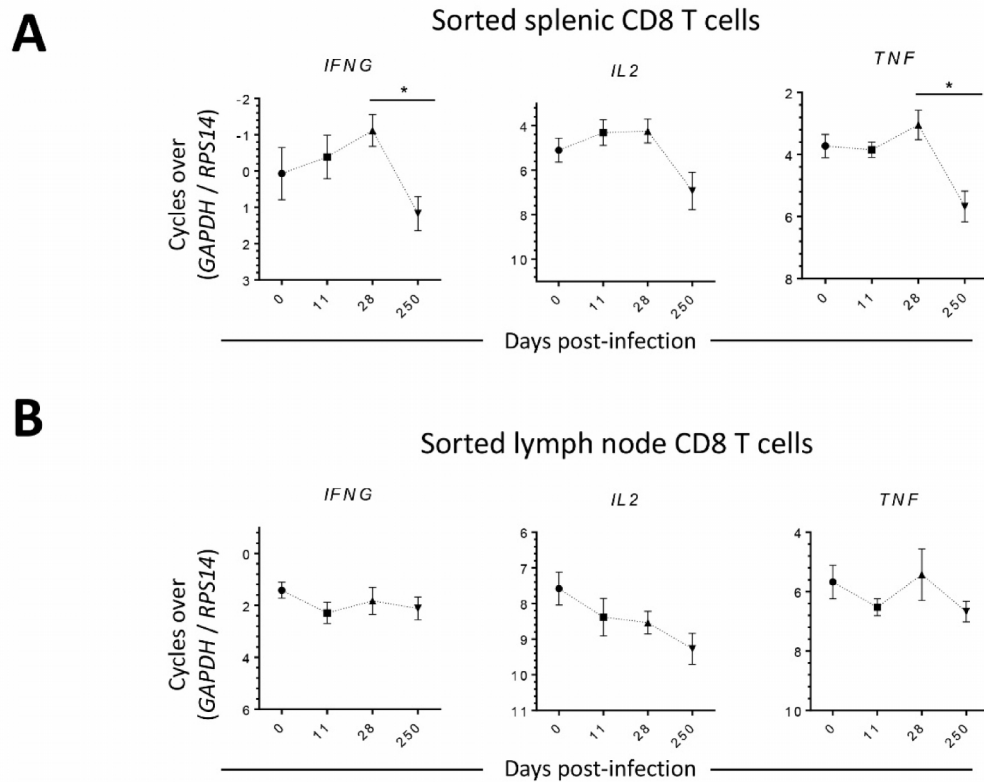


Figure 3. Cytokine gene expression by splenic CD8 T cells during the course of *Leishmania infantum* infection of rhesus macaques. **(A)** CD8 T cells were sorted from total splenocytes at various time-points after infection and cytokine gene expression was determined by quantitative real-time PCR. Results are shown as mean $\pm$ SEM of the number of cycles over the geometric mean of the two housekeeping genes (*GAPDH* and *RPS14*) required to detect *IFNG* (left), *IL2* (center) and *TNF* (right). **(B)** Cytokine mRNA levels in sorted lymph node CD8 T cells. Data from 2-4 macaques per time-point. Statistical analysis performed using one-way ANOVA followed by a Bonferroni's post-hoc test.

T-bet induction in splenic CD8 T cells during the acute phase of infection is associated with the expression of granzyme B

T-bet and EOMES are critical in coordinating the differentiation program of CD8 T lymphocytes from naïve to effector cells (Kaeck *et al.* 2012); however, their kinetics of expression during *Leishmania* infections has not been previously addressed. Hence, we explored the dynamics of T-bet and EOMES expression in splenic CD8 T cells during the course of *L. infantum* infection in rhesus macaques and its relationship with the expression of granzyme B.

About 20% of splenic CD8 T cells in non-infected macaques expressed granzyme B (Fig. 3A). This percentage progressively increased during the acute stage of infection, peaking at day 28 pi; a time-point at which more than 40% of the cells were positive for granzyme B (Fig. 3A). Interestingly, at the chronic phase of infection (day 250 pi), the fraction of cells expressing granzyme B decreased to levels closer to pre-infection.

The kinetics of T-bet expression in splenic CD8 T lymphocytes was similar to that of granzyme B, hence peaking at one month after infection and decreasing at the chronic phase (Fig. 3B). EOMES was similarly induced after infection but, in contrast with granzyme B and T-bet, its expression remained elevated at the chronic phase, as compared with non-infected animals (Fig. 3C).

Flow cytometric analysis revealed that, among the T-bet-expressing CD8 T cells, the vast majority also expressed granzyme B (Fig. 3D, F). Nevertheless, T-bet induction is not mandatory for expression of granzyme B, as a fraction of the granzyme B⁺ pool was not expressing T-bet (Fig. 3D, F). Contrasting with T-bet, the EOMES⁺ subset of CD8 T cells was not necessarily associated with granzyme B expression (Fig. 3E, G).

In lymph nodes, expression of granzyme B, T-bet and EOMES among the CD8 T cell population was much lower than in the spleen, and we could not detect any significant variations as a result of infection (Fig. S1). As such, despite their differentiated phenotype (Fig. 1H), lymph node CD8 T cells do not acquire expression of cytotoxic molecules after infection.

Globally, our data suggests that T-bet and EOMES play distinct roles during the differentiation of splenic CD8 T cells in *L. infantum*-infected macaques. The results presented suggest that T-bet is strongly associated with the acquisition of cytotoxic potential while EOMES expression persists high until the chronic phase and apparently is not a primary coordinator of granzyme B expression.

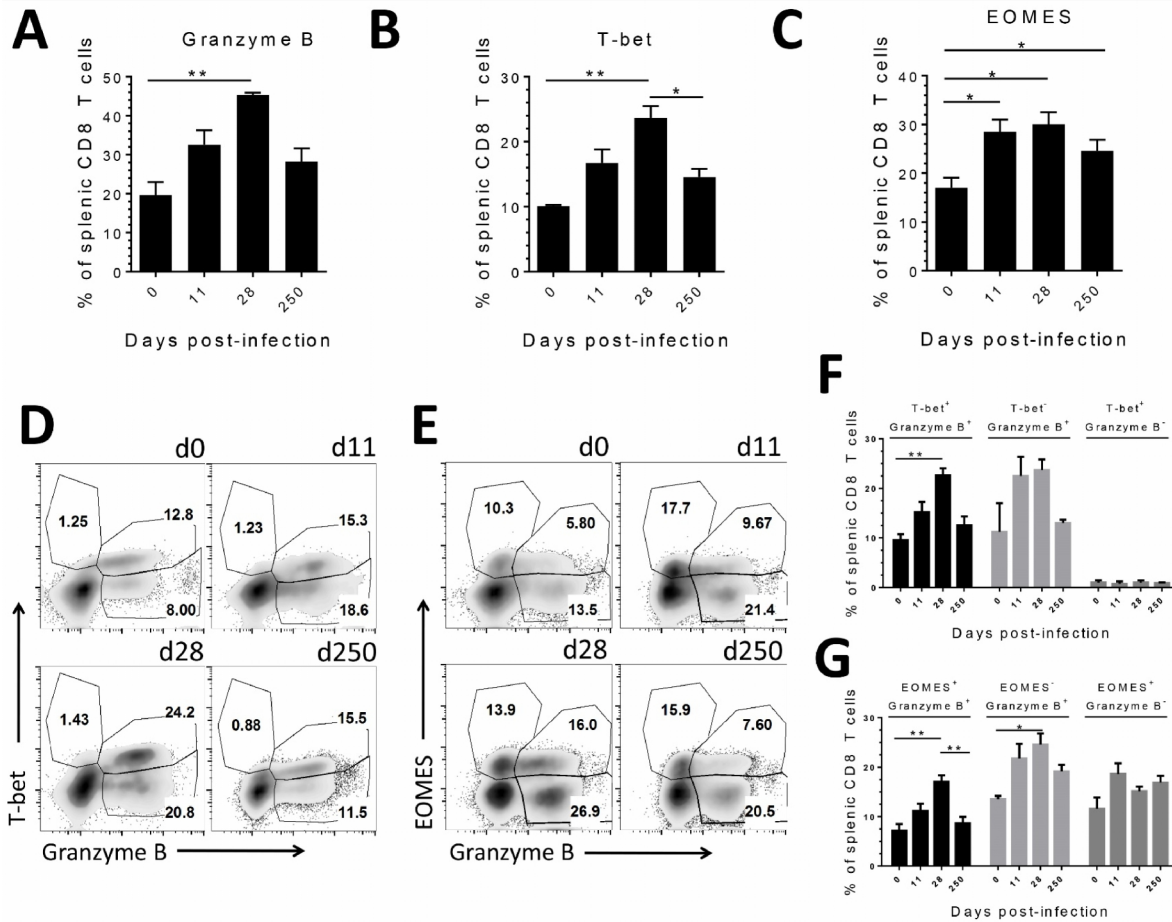


Figure 4. CD8 T cell expression of T-bet and granzyme B is tightly associated, peaking at one month pi and decreasing at the chronic phase, while EOMES expression persists elevated after the loss of cytotoxic molecule. (A-C) Flow cytometric determination of the percentage (mean $\pm$ SEM) of splenic CD8 T cells expressing Granzyme B (A), T-bet (B) and EOMES (C). (D-G) Quantification of granzyme co-expression with T-bet (D, F) or EOMES (E, G) among splenic CD8 T cells during the course of infection. (D-E) Representative T-bet/granzyme B (D) or EOMES/granzyme B (E) dotplots at the indicated time points after infection. (F-G) Graphs depict the average percentages (mean $\pm$ SEM) of the subsets defined in (D-E). Data was gathered from 2-4 macaques per time-point. Statistical analysis performed using one-way ANOVA followed by a Bonferroni's post-hoc test.

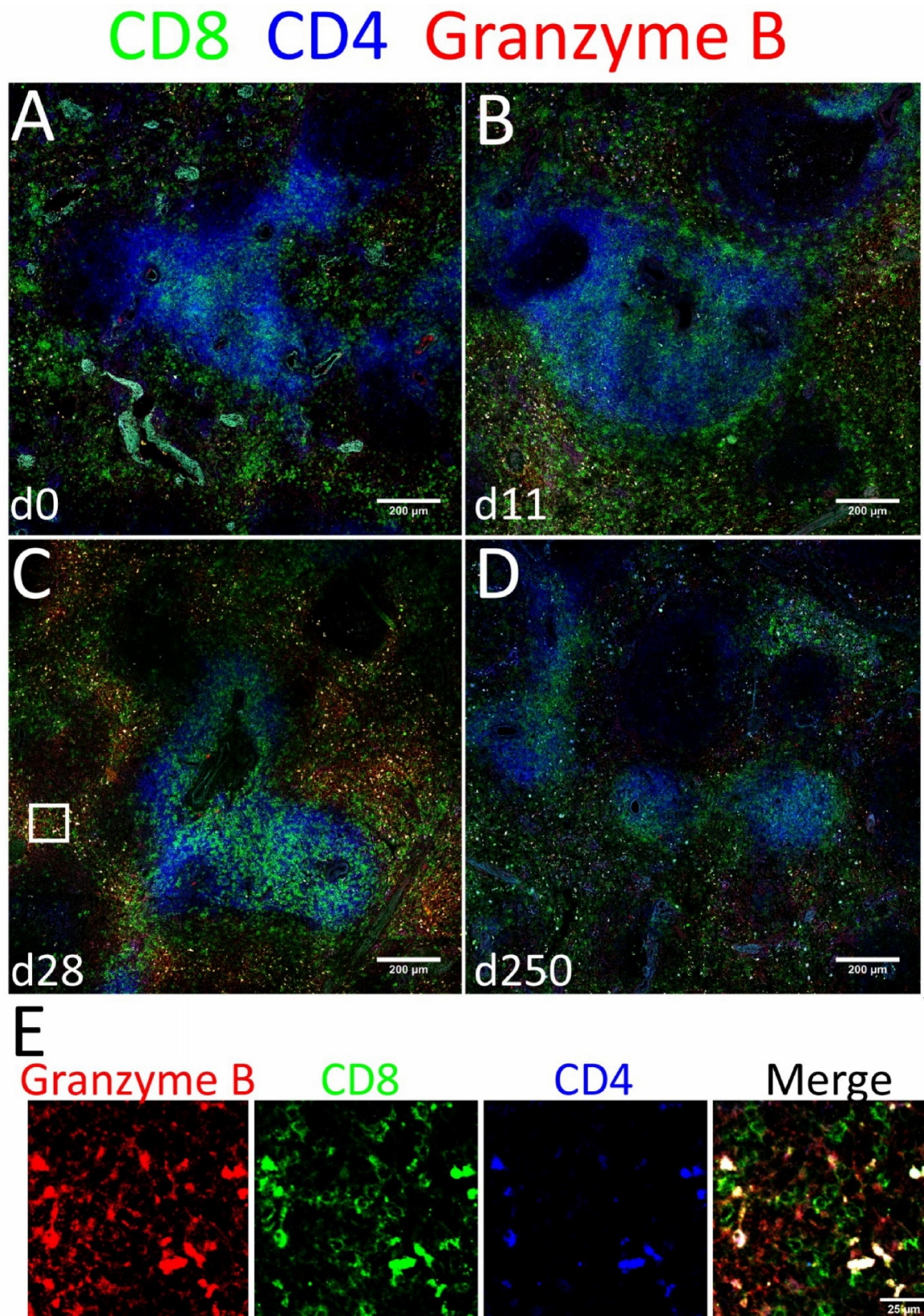


Figure 5. Granzyme B expression is strongly induced at day 28 after infection and localizes to the red pulp and marginal zone of the spleen. **(A-D)** Confocal tissue immunofluorescence of splenic sections retrieved from a naïve macaque (A) or infected with *L. infantum* at days 11 (B), 28 (C) and 250 (D) post-infection. CD4 (blue), CD8 (green), granzyme B (red). **(E)** Inset from figure 4C as defined by the white square.

Expression of inhibitory receptors in CD8 T cells is increased at the chronic phase of infection

Our data is clearly suggestive of CD8 T cell functional impairment at the chronic phase of infection, particularly the splenic pool (Fig. 3-5). Yet, these cells retain an effector or effector-memory phenotype (Fig. 1-2). These observations are consistent with the occurrence of T cell exhaustion (Wherry 2011), prompting us to analyze the expression of inhibitory receptors in CD8 T cells during the course of infection.

In the spleen, expression of PD-1 among the CD8 T cell pool manifested an increasing, though not significant, trend as the infection progressed towards the chronic phase (from $5.4 \pm 1.7\%$ in naïve animals to $10.1 \pm 3.5\%$ at day 250 post-infection) (Fig. 6A-B). A significant increase was found, however, in the expression of the inhibitory receptor LAG-3 in splenic CD8 T cells at days 28 and 250 pi, as compared with non-infected animals (from $21.7 \pm 3.2\%$ in naïve animals to 39.7 and 37.5% in macaques infected for 28 and 250 days, respectively) (Fig. 6A-B). In lymph nodes, we failed to detect any significant increase in the expression of the two inhibitory receptors analyzed (Fig. 6C-D). Nevertheless, LAG-3 expression tended to increase progressively as the infection advanced towards the chronic phase ($8.6 \pm 1.5\%$ before infection to 12.4 ± 1.8 at the chronic phase) (Fig. 6C-D).

Taken together, the data suggests that chronic CD8 T cell loss of function in the spleens of rhesus macaques infected with *L. infantum* is related with increased expression of inhibitory receptors, particularly LAG-3.

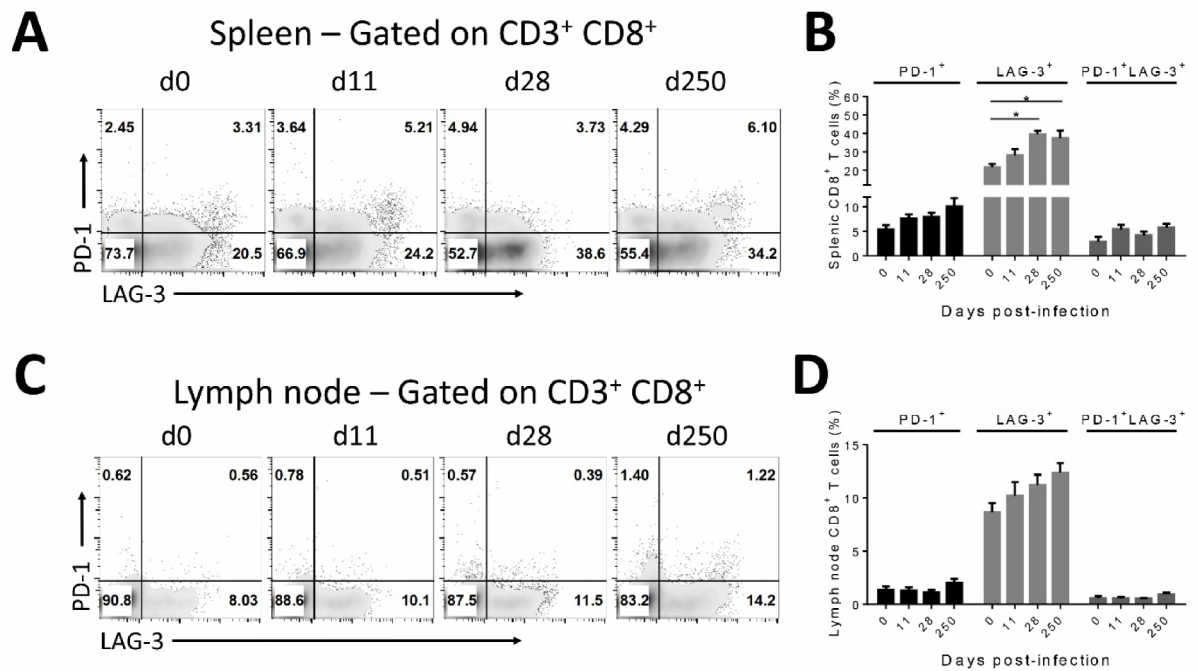


Figure 6. Loss of CD8 T cell effector function at the chronic phase is associated with upregulation of the inhibitory receptor LAG-3. **(A-B)** LAG-3 expression in splenic CD8 T cells during the course of infection; (A) shows representative dotplots of PD-1 and LAG-3 expression and (B) shows the pooled data (mean $\pm$ SEM). **(C-D)** LAG-3 expression in lymph node CD8 T cells during infection. The presented data corresponds to 2-4 macaques analyzed per time-point. Statistics were assessed using one-way ANOVA followed by a Bonferroni's post-hoc test.

Discussion

Few studies have specifically addressed the role of CD8 T cells during VL although some earlier work suggested that this lymphocyte subset is an integral part of the immune response required to control chronic visceral *Leishmania* infection (Joshi *et al.* 2009, Beattie *et al.* 2010a). Here, we presented the results of a descriptive analysis of the phenotypic and functional properties of CD8 T cells during experimental infection of rhesus macaques with *L. infantum*. Our data revealed a 4-fold expansion of the splenic CD8 T cell pool after infection, associated with the acquisition of an effector phenotype that persisted until the chronic phase of infection. Additionally, the expression of granzyme B and, to a lesser extent, type-1 cytokines was induced in splenic CD8 T cells, notably at one month after parasite inoculation. However, progression to the chronic phase of infection was associated with splenic CD8 T cell loss-of-function and increased expression of T cell inhibitory receptors, hence suggestive of functional exhaustion.

The impressive expansion of the splenic CD8 T cell pool is hardly surprising and is in agreement with observations in murine (Joshi *et al.* 2009), canine (Reis *et al.* 2014) and human VL (Taher *et al.* 2009), which are frequently associated with an inversion of the CD4/CD8 ratio (Taher *et al.* 2009, Reis *et al.* 2014). The amplification of the splenic CD8 T cell population occurred very early after parasite infection and was coincident with parasite containment in visceral organs such as the spleen and liver (Rodrigues *et al.* 2014b). Concurrent with their expansion, splenic CD8 T cells acquire an effector phenotype, characterized by downregulation of CD62L and CD28, upregulation of CD95. We further observed that splenic CD8 T cells become increasingly more susceptible to Fas-mediated cell death, hence supporting their differentiated status.

Accompanying these surface phenotype changes was the induction of T-bet and EOMES in splenic CD8 T cells. Yet, the two transcription factors appear to regulate separate functions in the responding CD8 T cell population. While T-bet was strongly associated with the expression of the cytotoxic molecule granzyme B, expression of EOMES appears to be regulated independently of the acquisition of cytotoxic potential. Over the past years, many studies have helped to elucidate the contribution of T-bet and EOMES in determining the fate of activated CD8 T cells (Kaeck *et al.* 2012). The prevailing model suggests that T-bet is induced by inflammation and promotes a full-fledged effector identity in CD8 T cells (Cox *et al.* 2011). In contrast, EOMES appears enriched in cells that will later form the memory pool (Arens *et al.* 2010). However, when pathogens are capable of chronic persistence in the host, the memory CD8 pool is subjected to continuous stimulation, impacting on the balance between T-bet and EOMES subsets and eventually leading to the collapse of the CD8-mediated immunity and T-cell exhaustion (Wherry 2011). In this scenario, models

elaborated based on studies with prototypical acute infections no longer apply. Recent work is starting to clarify the transcriptional network that is associated with T cell exhaustion (Wherry *et al.* 2007, Doering *et al.* 2012). In these studies, it was found that EOMES is expressed at high levels in exhausted CD8 T cells, while T-bet is reduced (Wherry *et al.* 2007, Paley *et al.* 2012), which is compatible with our findings. However, deletion of EOMES does not result in improved CD8 T cell responses or control of chronic viral infection (Paley *et al.* 2012). As such, it seems that an intricate relationship, in which EOMES⁺ and T-bet⁺ CD8 T cell subsets have distinct but complementary functions, is necessary to maintain a pool of functionally competent CD8 T cells in the setting of a chronically persistent pathogen. While our study has started to unravel previously unexplored areas, certainly a lot is yet to be done to obtain a more precise picture about the regulation of EOMES and T-bet in CD8 T cells during VL.

Granzyme B expression was strongly induced in the spleen after infection, particularly at day 28 pi. Its expression localized to the marginal zone/ red pulp, which is in agreement with previous reports (Choy *et al.* 2003). Furthermore, these are the areas of parasite residency within the spleen, as *Leishmania* protozoa were shown to be preferentially taken up by red pulp and marginal zone macrophage populations (Engwerda *et al.* 2004a). Granzyme B expression localized in areas populated by CD8 T cells, but other cell types also seem to contribute to the pool of granzyme-B-expressing cells. Given the descriptive nature of our study, it is not possible to conclude whether a granzyme B-mediated cytotoxic action of CD8 T cells is protective against the parasite or instead promotes tissue pathology as reported in mouse models of cutaneous leishmaniasis (Novais *et al.* 2013).

Recent studies in canine and human VL reported the occurrence of immune exhaustion in splenic CD8 T cells associated with an increased surface expression of PD-1 (Esch *et al.* 2013, Gautam *et al.* 2014). In rhesus macaques infected with *L. infantum*, PD-1 expression was not significantly augmented at the time of CD8 T cell loss of function, although our analysis exhibited an increasing trend of PD-1 expression in the total splenic CD8 T cell population. A significant increase was found for the inhibitory receptor LAG-3 in splenic CD8 T cells. LAG-3 expression is also a marker of T cell differentiation (Grosso *et al.* 2009), which is in agreement with our observation that LAG-3 expression in splenic CD8 T cells was already increased at day 28, a time-point at which the cells do not demonstrate functional exhaustion. These observations suggest that by itself LAG-3 expression is not sufficient to drive exhaustion in CD8⁺ T cells. Instead, it is the persistent expression of LAG-3, possibly complemented by induction of additional inhibitory receptors at the chronic phase that progressively erodes the functionality of CD8 T cells (Grosso *et al.* 2009).

LAG-3 is a homologue of the CD4 molecule, binding to MHC class II with high affinity, which suggests that it may be important in controlling T cell reactivity (Triebel *et al.* 1990, Baixeras

et al. 1992). Some reports indicate that LAG3 activates DCs through MHC class II (Demeure *et al.* 2001, Casati *et al.* 2008). Others, in contrast, demonstrated that LAG-3⁺ lymphocytes present at sites of inflammation may impair the differentiation of monocytes, thus limiting the magnitude of the ongoing T-cell immune responses (Buisson *et al.* 2005, Liang *et al.* 2008).

LAG-3 expression has not been previously evaluated as an exhaustion marker of CD8 T cells during VL. However, during *Plasmodium* infection in mice, the exhaustion of splenic CD8 cells is associated with upregulation of both PD-1 and LAG-3 and only a therapy blocking signaling through both receptors, but not each individually, could restore the CD8 functionality (Butler *et al.* 2012). Similarly, dual anti-LAG-3/anti-PD-1 antibody treatment was able to clear mice of established tumors (Matsuzaki *et al.* 2010, Woo *et al.* 2012), to enhance viral response *in vivo* (Blackburn *et al.* 2009), and to participate in controlling autoimmunity (Okazaki *et al.* 2011). In this sense, it would be of particular interest for future studies to evaluate whether blocking signaling through LAG-3, PD-1 or both can restore the effector function of CD8 T cells in VL.

In lymph nodes, CD8 T cells exhibited some, but not all, of the phenotypic changes indicative of differentiation that we analyzed. However, these cells do not seem to acquire extensive effector function as judged by their low expression of granzyme B and cytokines. This partial differentiation of lymph node CD8 T cells is evident as early as day 11 after infection, a time-point at which we did not detect parasites in lymph nodes (Rodrigues *et al.* 2014b). These cells were possibly primed by the residual parasite population present in the lymph nodes during the acute phase. In alternative, they are primed in the spleen and migrate to lymph nodes, as suggested by observations in mouse models of VL (Bunn *et al.* 2014).

An insufficient knowledge on the mechanisms governing T cell memory formation and the use of experimental models that do not accurately mimic human disease are regarded as the major limitations in achieving a successful vaccine for VL (Kaye *et al.* 2011b). Here, we presented an ensemble of data delineating the differentiation, acquisition of effector function and ultimate exhaustion of CD8 T cells in a model of rhesus macaques infected with *L. infantum*. Our work confirms the suitability of the rhesus macaque as a faithful model for VL and may represent a starting point for future studies exploring previously unaddressed issues concerning this important lymphocyte subset during *Leishmania* infections.

Methods

Animals, parasites and infections

This study employed 11 young adult rhesus macaques (*Macaca mulatta*) of Chinese origin. Animals were seronegative for STLTV-1 (Simian T Leukemia Virus type-1), SRV-1 (type D retrovirus), herpes-B viruses and SIVmac. A group was left as non-infected control (n=3) and the remaining animals were inoculated intravenously via the saphenous vein with 2×10^7 stationary-phase *L. infantum* promastigotes (clone MHOM/MA/67/ITMAP-263) per kg of body weight. Subgroups of infected animals were sacrificed at distinct time points after infection (n=2 for days 11 and 28 pi and n=4 for day 250 pi). Peripheral blood, lymph nodes (a pool of axillary and inguinal LNs) and spleen were recovered for cellular analysis. Blood sampling was performed at additional time points after infection.

Immunophenotyping

Peripheral blood was collected to EDTA-coated tubes and fresh cell suspensions were prepared from macaque spleen and lymph nodes. Cells were stained with a panel of monoclonal antibodies provided in Supplementary Table 1 (Table S1). Blood and spleen erythrocytes were lysed in a hypotonic solution and 50000 events corresponding to mononuclear cells were acquired in a Cytomics FC500 (Beckman Coulter) and further analyzed using FlowJo software (Tree Star, Inc.).

Intracellular staining for transcription factors and granzyme B

Splenic mononuclear cells (SMCs) were isolated by density gradient centrifugation using LymphoPrep (PAA Laboratories). Cryopreserved splenic mononuclear cells and lymph node cells were used to assess the expression of transcription factors and granzyme B. Briefly, after thawing, cells were stained for surface CD3 and CD8, fixed and permeabilized using the FoxP3 staining buffer set (eBiosciences), following the manufacturer instructions and stained intracellularly for the transcription factors T-bet and EOMES and the cytotoxic molecule granzyme B.

Ex-vivo apoptosis

PBMCs, SMCs and lymph node cells were cultured overnight in complete media in the presence of Fas ligand (100 ng/ml), TRAIL (100 ng/mL) or vehicle (DMSO). The percentage of apoptotic CD8 T cells was determined by flow cytometry as described previously (Laforge *et al.* 2011). Briefly, cells are stained with FITC-labeled annexin-V combined with staining for CD8 and CD3. Thirty thousand events corresponding to CD3⁺ cells were acquired in the flow cytometer and analyzed in FlowJo.

Quantitative-PCR

Around 500,000 CD8 T cells from SMCs or total lymph node cell suspensions were sorted using a FACS Aria II cell sorter (BD Biosciences), lysed in RLT buffer (RNeasy Micro Kit, QIAGEN) and stored at -80°C until further use. RNA was purified and reverse transcribed using the AffinityScript QPCR cDNA synthesis kit (Stratagene). Gene expression was analyzed by qPCR, with 100 ng of cDNA as starting material. The thermal profile consisted of a hold of 15 min at 95°C, followed by 40 cycles of denaturation (95°C, 15 sec), annealing (60°C, 30 sec) and extension (72°C, 30 sec). Two macaque reference genes, *GAPDH* (GACCTGTTCTTGAATTTGAC / GAGGAAGATGAGGCTGTAGG, forward and reverse primers, respectively) and *RPS14* (AAGCAGATGGTTCCTTCATC / TAGCCTCTCATCTGCGTATTC) were employed to normalize sample input. The results were expressed as the number of cycles over the geometric mean of the two reference genes required to detect the mRNA of interest. Macaque-specific primers were designed using the AutoPrime software. *IL2* (CAAGAAGGCCACAGAATTG / CCAGAGGTTTGAGTTCTTCTTC), *TNF* (ATGTTGTAGCAAACCCTCAAG / TGAAGAGGACCTGGGAGTAG); *IFNG* (AAAGAAACGGGATGACTTTG / GACAGTTCAGCCATCACTTG).

Immunofluorescence confocal microscopy of splenic sections

The spleens were sectioned (7.5 µm thickness) in a frozen cryostat and stored unfixed at -80 °C. After rehydration, sections were fixed in 4% PFA (15 minutes at room temperature) followed by acetone (20 minutes at -20 °C). Slides were saturated in blocking solution (5% normal goat serum, 0.3% triton X-100) for 1 hour at RT. Fluorochrome-conjugated antibodies were diluted in antibody dilution buffer (1% BSA, 0.3% triton X-100) and incubated overnight with tissue sections at 4 °C. After washing, slides were mounted with antifade mounting medium. Sections were imaged in a Zeiss LSM 710 confocal microscope and tiled images acquired with a 20X objective and stitched using the Image J stitching plugin (Preibisch *et al.* 2009). Images were further analyzed and processed using Image J and Adobe Photoshop. Table S1 provides information on the antibodies used for immunofluorescence.

Statistical analysis

Statistics were performed with the GraphPad Prism 5 software. Data is presented as means ± SEM. A one-way analysis of variance (ANOVA) followed by a Bonferroni's post hoc test was used for comparison between the different time points after infection.

Ethics Statement

All the animal experiments described in the present study were conducted at the MIRcen platform (Fontenay-aux-Roses, France) in agreement with the European Union guidelines for the handling of laboratory animals (http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm). The animal care and use protocol was issued by the IACUC/ethics committee (MIRcen, CAJ–10–30).

Acknowledgments

This work was supported by an ANR (Agence Nationale de la Recherche) grant (LeishApo) and a Partenariat Hubert Curien (PHC) (program Volubilis, MA/11/262) to JE. The research leading to these results has also received funding from the European Community's Seventh Framework Programme under grant agreement No.602773 (Project KINDRED). VR is supported by a doctoral fellowship from FCT (Fundação para a Ciência e Tecnologia); code SFRH/BD/64064/2009. RS is supported by Programa Ciência – financed by Programa Operacional Potencial Humano POPH – QREN– Tipologia 4.2 – Promoção do Emprego Científico, co-funded by Fundo Social Europeu and National funding from Ministry of Science, Technology and Higher Education (MCTES). We thank François Lachapelle and Christophe Joubert for their help at the non-primate center MIRCEN platform, CEA/INSERM, Fontenay-aux-Roses. We also thank Catarina Leitão (IBMC, Porto, Portugal) for all the technical help with the FACS Aria II cell sorter.

Supporting information

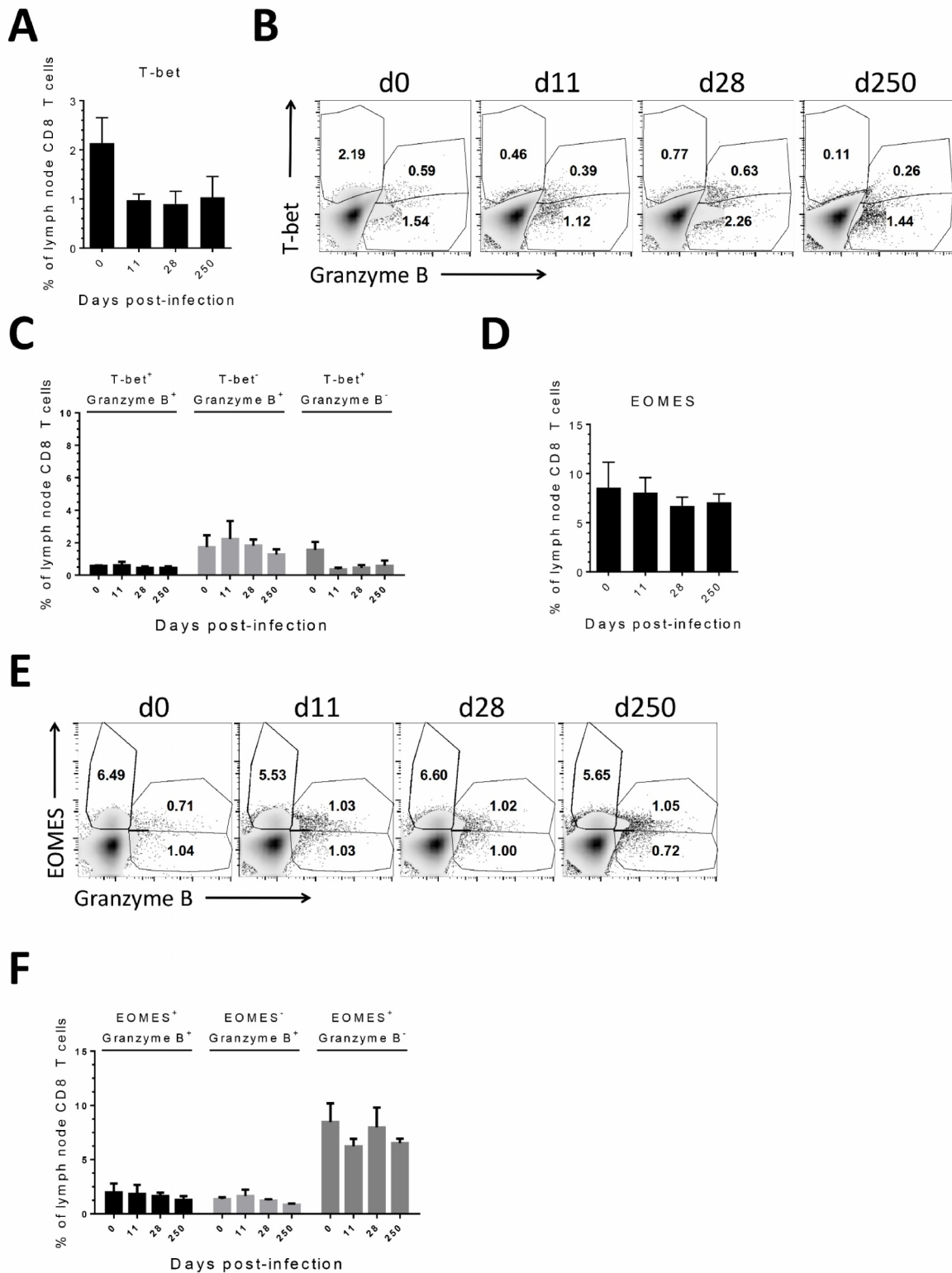


Figure S1. Expression of T-bet, EOMES and granzyme B markers is not significantly induced in lymph nodes during *L. infantum* infection of rhesus macaques. **(A)** Flow cytometric quantification of the percentage (mean $\pm$ SEM) of lymph node CD8 T cells expressing T-bet. **(B-C)** Quantification of granzyme co-expression with T-bet among lymph node CD8 T cells during the course of infection; **(B)** Representative T-bet/granzyme B dotplots at the indicated time points; **(C)** Graphs depict the average percentages (mean $\pm$ SEM) of the subsets defined in

(B). (D) Determination of the percentage (mean $\pm$ SEM) of lymph node CD8 T cells expressing EOMES. (E-F) Quantification of granzyme co-expression with EOMES among lymph node CD8 T cells. Data was gathered from or 2-4 macaques per time-point. Statistical analysis performed using one-way ANOVA followed by a Bonferroni's post-hoc test.

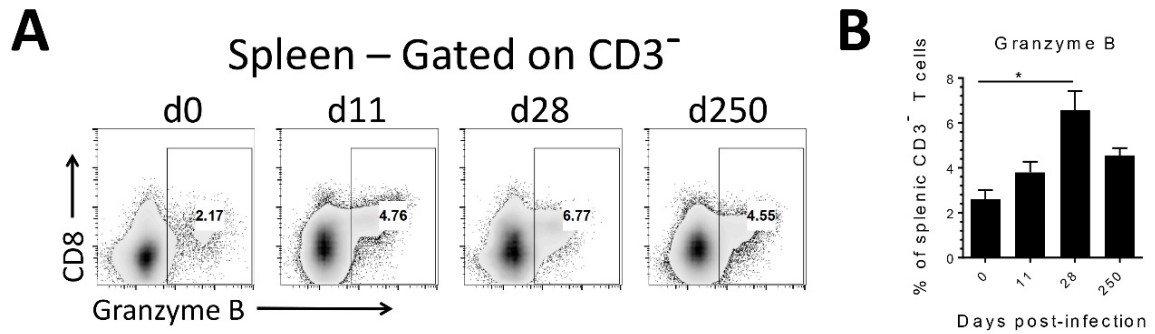


Figure S2. Granzyme B expression by CD3 negative cells during the course of infection. (A-B) Flow cytometric quantification of granzyme B expression in splenic CD3⁻ cells; (A) shows representative dotplots of granzyme B expression and (B) shows the pooled data (mean $\pm$ SEM). Data corresponds to 2-4 macaques analyzed per time-point. Statistics were assessed using one-way ANOVA followed by a Bonferroni's post-hoc test.

Table S1. Information relative to the antibodies used in flow cytometry and confocal microscopy studies.

Antibody	Clone	Manufacturer	Dilution
Flow cytometry			
FITC-CD8	BW135/80	Miltenyi Biotec	1/50
PerCP-CD3	SP34-2	BD Biosciences	1/50
PE-CD62L	SK11	BD Biosciences	1/50
APC-CD45RA	T6D11	Miltenyi Biotec	1/50
PE-CD28	CD28.2	BD Biosciences	1/50
APC-CD95	DX2	BD Biosciences	1/50
APCH7-CD20	2H7	BD Biosciences	1/50
PE-Granzyme B	GB11	Invitrogen	1/50
PECy7-CD8	RPA-T8	BD Biosciences	1/50
PECF594-CD3	SP34.2	BD Biosciences	1/50
FITC-Lag-3	Polyclonal	R&D Systems	1/50
APC-PD-1	eBio-J105	eBioscience	1/50
eFluor660-EOMES	WD1928	eBioscience	1/50
eFluor660-T-bet	eBio4B10	eBioscience	1/50
Tissue Immunofluorescence			
AF488-CD4	OKT4	Biolegend	1/20
BV605-CD8	RPA-T8	Biolegend	1/20
APC-Granzyme B	GB11	Invitrogen	1/20

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Chapter IV | Discussion and Perspectives

1. Macaques as models for the study of visceral leishmaniasis

1.1. Model validity

Apart from their use in the pre-clinical evaluation of drug and vaccine candidates, non-human primates have been underexplored as models for fundamental studies on the regulation of immunity during VL. In stark contrast, fundamental research with many other infectious agents has profited much from studies in primate models over the past decades (Gardner *et al.* 2008).

A few previous studies demonstrated that rhesus macaques infected with *L. infantum* develop a clinical condition that shares many similarities with human VL (Porrozzi *et al.* 2006, Grimaldi *et al.* 2014). Porrozzi and colleagues (2006) reported a very rapid onset of clinical disease after infection, with some animals becoming severely cachectic as early as 9 weeks after infection. In contrast, we did not observe a clear disease manifestation over the course of our 8-month experiments. Differences in strain virulence may partially explain the contrasting outcomes. However, it is important to refer that these previous reports used hamster-derived *L. infantum* amastigotes for macaque infections (Porrozzi *et al.* 2006, Grimaldi *et al.* 2014) while our work employed stationary-phase promastigotes grown in axenic culture. Each strategy has its own advantages and drawbacks. Hamster-derived amastigotes are extremely virulent and commonly used to induce rapid clinical disease in other animal models (Garg *et al.* 2006, Pescher *et al.* 2011). In contrast, a large proportion of a promastigote inoculum is rapidly cleared by innate immunity within a few days after infection (Engwerda *et al.* 2004a). By calculating the total number of parasites in infected organs, we estimate that 98.5% of the initial inoculum was already cleared by day 11 after infection, in our model (data not shown). As such, a promastigote inoculum is drastically reduced in size in the first days after infection, therefore, requiring a longer time to replicate and reach a population able to cause clinical symptoms. This slow progression is in agreement with observations in humans in which the asymptomatic phase usually lasts for several months (Singh *et al.* 2014). In contrast, hamster-derived amastigotes are able to withstand extensive innate killing, hence accelerating the onset of the clinical disease. It is thus acceptable to suggest that a model based on the injection of hamster-derived amastigotes is probably the best option for studies aiming to evaluate the performance of a drug candidate, given that clinical disease is achieved quickly. In contrast, infecting macaques with promastigotes is probably a better approach for fundamental studies on the immune response during VL.

Despite the absence of overt clinical disease, which also reflects the early end-point used in our experiments, the occurrence of anemia, hypergammaglobulinemia, the increase in serum alanine transaminase and splenic cellularity are reminiscent of VL in humans.

An important issue concerning most experimental animal models of VL is the size of the inoculum and the route of parasite administration. Visceral leishmaniasis is a vector-borne disease. In the wild, sandflies deliver an inoculum of a few hundred parasites to the dermis of the host. Ideally, an experimental model should employ sandflies for parasite delivery. However, the use of sandflies is necessarily associated with a number of limitations related with the standardization of the inoculum and the need for specialized facilities to breed and maintain sandfly colonies. The use of intradermal inoculation and addition of salivary components to the inoculum were proposed as proxies of natural infection (Ahmed *et al.* 2003, Gomes *et al.* 2008). As previously referred, there is evidence from mice and hamsters that the use of a more natural parasite dose and route of infection may alter the kinetics of colonization of the viscera and disease progression, as compared with the typical intravenous delivery of high parasite dose (Ahmed *et al.* 2003, Aslan *et al.* 2013). The best argument for the use of the intravenous high-dose is to ensure a homogeneous course of infection across all experimental animals. This aspect is particularly relevant with models relying on outbred animals, such as rhesus macaques, in which a high variability across individuals is expected. For that reason, we have employed the high-dose intravenous route in our studies, but it is important to bear in mind that some observations probably deviate from natural infection.

1.2. Organ-specific immunity during experimental VL in macaques

Our study provided the first kinetic analysis of the parasite load in infected tissues during the course of VL in rhesus macaques. Importantly, unlike the self-contained infection observed in mice, macaques exhibit a progressively higher parasite burden in blood and tissues as the chronic phase of infection advances. Nevertheless, and similar to the mouse model, in rhesus macaques, *L. infantum* colonizes the host in an organ-specific manner, albeit with very different tissue patterns of colonization. By combining the quantification of the parasite load with a longitudinal analysis of the phenotype and molecular signature of immune populations, we could define patterns of immunity related with parasite control versus parasite growth and dissemination. A rough overview of the immune response reveals the emergence of type I immunity in the first weeks after inoculation that is concomitant with parasite containment in visceral organs. As the infection advanced

towards late stages, a suppressive environment appeared and was associated with parasite growth and systemic dissemination of the infection.

Blood

In the blood, parasitemia decreased steadily over the first weeks after inoculation. Such observation probably reflects splenic and hepatic sequestration and is in agreement with observations in experimental infections in mice and dogs (Engwerda *et al.* 2000). Furthermore, given that the inoculum consisted of a heterogeneous population from a stationary promastigote culture, a significant proportion of the injected parasites is probably susceptible to complement-mediated lysis, particularly the pre-metacyclic forms (Franke *et al.* 1985). After the initial clearance phase, parasitemia reached almost undetectable levels by one month post-infection but eventually rebounded at the latter stages of the experiments. The presence of circulating parasites in chronic disease suggests that the macaques were capable of transmitting the parasite and complete its life cycle; a possibility that would require exposing the animals to a competent vector for confirmation.

Surface phenotype analysis of the differentiation status of blood CD4 and CD8 T cells revealed a significant proportion of effector and effector-memory cells in non-infected animals, particularly in the CD8 compartment. During our 8-month course experimental infections, no significant changes occurred in the distribution of the blood CD4 and CD8 T cells across the naïve, central-memory, effector-memory, and terminal-effector phenotypes. Such occurrence may reflect the dynamic nature of the pool of blood T cells, which is in continuous turn-over. Furthermore, we observed a significant variability among animals, which may preclude the detection of minor differences. In human patients, some studies reported a lower frequency of memory CD4 and CD8 T cells circulating in the blood during active disease (Hailu *et al.* 2005, Clarencio *et al.* 2009). However, this decrease appears to occur only at advanced stages of the disease, when the patients are already lymphopenic (Hailu *et al.* 2005).

Spleen

The spleen is the major site of parasite persistence during chronic VL. In susceptible mice infected with visceral *Leishmania* for a long time, the splenic parasite load eventually reaches a plateau and is maintained at a relatively constant level throughout the life of the animal (Engwerda *et al.* 2004a). Despite the high variability among individual animals, which is probably a consequence of the use of outbred animals, our data indicates that in macaques the splenic parasite burden progressively increases over time. These

observations are reminiscent of VL in humans and similar to observations in the hamster model (Melby *et al.* 2001).

The splenic pools of CD4 and CD8 T cells expanded several-fold during the first weeks after infection; a phenomenon commonly observed in other VL models and human patients, and a significant contributor for the occurrence of splenomegaly (Nylen *et al.* 2007a, Joshi *et al.* 2009, Taher *et al.* 2009). In macaques, splenomegaly was not clear, but the drastic increase in splenic cellularity resulted mainly from the expansion of CD4 and CD8 T cells. In this context, studies in the mouse model of VL suggest that splenomegaly is a host response to accommodate the progressive increase in size of the splenic T cell pool during chronic VL (Polley *et al.* 2005a).

Our longitudinal immunophenotyping analysis revealed that splenic T cells adopted terminal-effector and effector-memory phenotypes in the first weeks after infection, in agreement with observations in mice (Gomes-Pereira *et al.* 2004, Kaye *et al.* 2004, Joshi *et al.* 2009). The expansion and differentiation of CD4 and CD8 T cells are probably related with the abundance of parasite antigen that results from extensive innate killing during the first weeks. However, some degree of bystander activation of non-specific T cells is also expected due to the inflammatory environment that characterized the early days/weeks after parasite inoculation. In mice infected with *L. donovani*, bystander activation of CD4 and CD8 T cells has been reported (Polley *et al.* 2005a, Polley *et al.* 2005b).

In the long-term, the pool of splenic T cells maintained a terminal-effector and effector-memory phenotype, as observed in chronic VL patients (Cenini *et al.* 1993). Further analysis revealed increased expression of the death receptor Fas (CD95) in splenic CD4 and CD8 T cells, which translated to a pro-apoptotic tendency when these cells were cultured *ex vivo* with Fas ligand. However, arguing against a role for Fas-mediated apoptosis, we found that the serum levels of Fas ligand did not increase as a result of infection. As such, while T cells die *ex vivo* when exposed to FasL, this process might not be happening *in vivo*. However, binding of soluble FasL is not the sole mechanism for the activation of the Fas apoptotic pathway. Instead, most T cells are thought to die by a mechanism involving the engagement of Fas by membrane FasL expressed in a neighboring cell; a process known as contact fratricide (Krammer *et al.* 2007). Supporting this notion, VL patients exhibit augmented Fas and FasL expression in splenic lymphocytes (Eidsmo *et al.* 2002). In mice, splenic T cells are similarly more susceptible to Fas-mediated apoptosis *ex vivo*, but infection of *Fas*-deficient mice does not decrease the level of T cell apoptosis *in vivo* (Alexander *et al.* 2001). Possibly, in the absence of Fas, other mechanisms may compensate to avoid excessive accumulation of activated T cell clones (Green 2008). In this context, it would be interesting to investigate whether the apoptosis of splenic T cells

during chronic VL results from the deprivation of growth factors; a process mediated by the pro-apoptotic protein Bim (Krammer *et al.* 2007). Globally, the current data indicates that, during chronic VL, T cells undergo apoptotic demise via Fas, and perhaps Bim,-dependent mechanisms while parasite persistence continuously primes naïve T cells into the effector pool. In essence, the splenic T cell pool during chronic VL is a dynamic population of relatively short-lived effector or effector memory cells that undergo a continuous turn-over. Transition from the acute to the chronic phase of infection was associated with definite changes in the molecular signature of splenic CD4 T cells. In the acute phase, splenic CD4 T cells displayed a Th1 profile, while the increase in *IL10* in the chronic phase is indicative of a regulatory profile. The switch from an inflammatory towards a regulatory signature in splenic CD4 T cells is reminiscent of experimental VL in mice (Stanley *et al.* 2007), albeit with a slower progression of the disease course in macaques. Our gene expression analysis also supported the view that conventional CD4 T cells are the primary producers of IL-10 during the chronic phase of infection in the spleen. This notion was reinforced by the fact that the levels of the *FOXP3* transcript in splenic CD4 T cells do not undergo significant alterations during the course of infection, hence arguing against a role for regulatory CD4 T cells (Tregs) as important producers of pathologic IL-10. We could not, however, detect the presence of IFN- γ /IL-10 double producer CD4 T cells by flow cytometry (data not shown). In mouse models of VL, these double producers have been described as the most relevant source of disease-promoting IL-10 (Stager *et al.* 2006, Owens *et al.* 2012, Resende *et al.* 2013). Nevertheless, the presence of these cells in the spleen of human VL patients has not been confirmed to date.

Expression of IL-10 by Th1 cells is a widespread phenomenon that ensures a tight control over excessive activation that may cause pathology (Saraiva *et al.* 2010, Cope *et al.* 2011). During infections with the apicomplexans *Plasmodium* or *Toxoplasma*, the emergence of IL-10⁺ IFN- γ ⁺ CD4 T cells is required to limit excessive pathology (Jankovic *et al.* 2007, Freitas do Rosario *et al.* 2012). Even during VL some evidence suggests that IL-10 may be host protective; particularly, in regulating the response in the liver. Indeed, the extensive hepatic necrosis accompanying *L. donovani* infection in *TNF*-deficient mice may result from a concomitant defect in IL-10 induction (Stanley *et al.* 2007). Likewise, the severe hepatic pathology that follows *L. donovani* infection in *IL27R*^{-/-} mice involves CD4 T cells and may result from curtailed IL-10 induction (Rosas *et al.* 2006a).

In mice, the role of DCs in promoting the regulatory switch in CD4 T cells was recently uncovered (Owens *et al.* 2012, Resende *et al.* 2013). One study with splenic samples from human patients suggested a role for IL-27 produced by myeloid cells in promoting IL-10 production in T cells (Ansari *et al.* 2011). In the work presented in this thesis, the dynamics

of DCs and macrophages were not explored, but our ongoing work includes a longitudinal analysis of the phenotype and pattern of cytokine production by these populations.

The progressive loss of the normal architecture of the lymphoid structures of the spleen is one of the most striking pathological features of experimental and human VL (Kaye *et al.* 2004). At eight months of *L. infantum* infection of rhesus macaques, the boundaries between B cell follicles and T cell areas appear to be less sharply-defined, and the white pulp evidences some disorganization, albeit less severe as compared with studies in the mouse model (Engwerda *et al.* 2002). Possibly, these milder histopathological features in macaques as compared with mice or humans reflect the early end-point employed. Therefore, future studies, employing a longer time-frame experiments, will be useful to elucidate whether the loss of integrity of the splenic architecture reaches the same degree of severity in macaques as observed in mice or humans. In this context, a major issue is the existence of important differences in the structure of the white pulp between rodents and primates, particularly in the organization of the marginal zone (Mebius *et al.* 2005). As such, observations in mice may not directly translate to humans and the macaque model might prove more informative and relevant.

Lymph nodes

The kinetics of parasite colonization of peripheral lymph nodes contrasted with the patterns observed in the spleen. Given the low tropism evidenced by *L. infantum* for peripheral lymph nodes at the early stages of infection, the parasite load observed in these sites at the chronic phase is quite impressive. Our approach involved only three sampling points after infection, as a result of the limited number of animals that are necessarily associated with macaque studies. As such, we could not obtain a detailed picture of the kinetics of parasite colonization of the lymph nodes. One possibility is that a small number of parasites colonizes lymph nodes shortly after infection and persists in these sites as a residual population throughout the acute phase. As an immunosuppressive environment emerges towards the chronic phase, these parasites become able to replicate locally. Alternatively, the increase in parasite load in lymph nodes at the chronic phase may result from the migration of infected cells from the blood. Supporting this hypothesis is the increase in parasitemia at later stages. In humans, peripheral lymph node involvement during VL is highly variable across endemic regions. For instance, while rare in the Indian sub-continent, generalized lymphadenopathy is common in VL patients from Sudan (Zijlstra *et al.* 2001). Examination of the lymph nodes from fatal VL cases revealed a drastic lymphocyte depletion and infiltration of plasma cells and parasitized macrophages (Veress *et al.* 1977).

Our longitudinal immunophenotyping analysis showed little change in the differentiation phenotype of lymph node CD4 T cells throughout infection. We cannot exclude, however, some local differentiation of CD4 T cells that, due to its small magnitude and the variability among experimental animals, did not translate to a significant pattern of differentiation of effector and memory cells. In mouse models based on intravenous parasite injection, peripheral lymph nodes are rarely studied and thought to have minor contributions to the ongoing response. Perhaps more informative, in hamsters chronically infected with *L. donovani*, T cells from the lymph nodes demonstrate severe anergy, as denoted by impaired proliferation in response to common T cell mitogens (Banerjee *et al.* 2011). The anergic behavior of lymph node T cells resulted from myeloid cell-derived TGF- β (Banerjee *et al.* 2011). Although we have not analyzed cytokine production by macrophages and DCs, our data revealed an environment enriched in TGF- β and IL-10 at the chronic phase. While speculative, the data available from our study and others fits a model in which blood-circulating infected cells migrate to the lymph nodes and, by secreting suppressive cytokines, preclude the initiation of a robust T cell response. This phenomenon is probably more relevant in the chronic phase, when the suppressive environment is also established in the spleen, and more infected cells circulate in the blood.

While lymph node CD4 T cells did not differentiate significantly during infection, the CD8 T cell pool shifted towards a majority of effector and effector-memory cells. Remarkably, this occurred very early after infection; a time at which parasites were hardly detectable in lymph nodes. It is possible that a small amount of parasite antigen present in lymph nodes during the acute phase is sufficient to prime a large number of CD8 T cells towards effector or effector-memory phenotypes. However, more likely, these cells were primed elsewhere, for instance in the spleen, and subsequently migrated to the lymph nodes through the high endothelial venules (HEVs). In the steady state, only naïve or central-memory T cells are capable of crossing HEVs, due to the requirement for the expression of CD62L and CCR7 for attachment to and migration across HEVs (Girard *et al.* 1995). However, in an inflamed state, traffic through HEVs becomes less selective, and the entry of effector in addition to naïve and memory lymphocytes is allowed (Girard *et al.* 2012). This hypothesis, however, would predict that effector CD4 T cells should similarly arise in lymph nodes after infection, but this appears not to be the case. Possibly, effector CD4 T cells are held in infection sites such as the spleen and less able to traffic towards additional locations. In any case, interpretations of our data are highly speculative at this stage. As such, future studies should explore the patterns of lymphocyte circulation, containment or egress in distinct organs during VL. One recent study in mice found that blocking lymphocyte egress from the spleen precludes the detection of proliferating parasite-specific CD4 T cells in peripheral lymph

nodes, supporting the notion that, during VL, most T cells are primed in the spleen and subsequently migrate to additional sites (Bunn *et al.* 2014). Unfortunately, CD8 T cells were not evaluated in this study, which impedes a comparison between the magnitude lymph node colonization between the two T cell subsets.

Liver

In mice, parasites are virtually cleared from the liver by the eighth week after infection, concomitant with granuloma maturation (Engwerda *et al.* 2004a). In contrast, in the macaque model, infected animals were not able to control hepatic parasite growth, and the highest burden was observed at the chronic stage of infection. Concomitantly, the serum levels of alanine aminotransferase were also elevated, indicating the occurrence of hepatocellular damage accompanying the higher hepatic parasite burden at the chronic phase. We did not explore further the immune response in the liver, to understand the mechanisms underlying this failure to control parasite load. Our ongoing work aims to explore the dynamics of hepatic immune cells during infection. Furthermore, we also plan to examine liver sections by a combination of routine colorations and tissue immunofluorescence to assess the formation, maturation and cellular constitution of liver granulomas, as well as other pathological occurrences. In the hamster model of *L. donovani* infection, parasite replication in the liver is similarly not controlled (Melby *et al.* 2001). Microscopic examination revealed that granulomas develop in the livers of infected hamsters, but appear to contain fewer lymphocytes, as compared with mice (Mangoud *et al.* 1997).

Bone Marrow

In many infectious settings, there is increased demand for immune cell production in the bone marrow (Takizawa *et al.* 2012). Such requirement is particularly evident for short-lived cells, such as neutrophils and indeed many pathogens cause a transient increase in granulopoiesis (Glatman Zaretsky *et al.* 2014). Furthermore, some pathogens, such as visceral *Leishmania*, may colonize the bone marrow and directly interact with hematopoietic stem cells or lineage precursors, impacting on the normal physiology of the bone marrow (Takizawa *et al.* 2012).

In *L. infantum*-infected macaques, the bone marrow presented a high parasite density at the chronic phase of infection; an observation also reported in mice (Cunha *et al.* 2013). Furthermore, neutrophilia was evident during the acute phase. Relatively few studies have explored the consequences of bone marrow colonization by visceral *Leishmania*. In mice, *L. donovani* infection causes a transient mobilization of progenitor cells from the bone

marrow to the blood (Cotterell *et al.* 2000a). Furthermore, the parasite lodges in bone marrow macrophages, stimulating the secretion of colony-stimulating factors that influence local hematopoiesis, by selectively promoting myelopoiesis (Cotterell *et al.* 2000b, Cotterell *et al.* 2000a). In human VL patients, the marrow presents hypercellularity with increased numbers of lymphocytes, plasma cells and macrophages harboring many parasites. Formation of lymphoid follicles and granulomas is also a common occurrence (Kumar *et al.* 2007, Dhingra *et al.* 2010). Thus, in the view of these prominent changes in the morphology and function of the bone marrow caused by infection, future studies, employing rhesus macaques, should aim to dissect the full relevance of the parasite-mediated disturbance of the normal physiology of the bone marrow.

2. Antibodies, B cells and Tfh cells in non-human primate visceral leishmaniasis

Experimental work performed over the past decades led to a prevailing view that considers B cells and antibodies of minimal importance in the protective immunity during VL. Indeed, high levels of serum antibody are characteristic of VL patients, correlate positively with disease severity and decrease upon drug cure (da Matta *et al.* 2000, Caldas *et al.* 2005, Miles *et al.* 2005). Furthermore, polyclonal B cell activation and hypergammaglobulinemia during VL have pathological consequences for the host, such as renal pathology (Dutra *et al.* 1985, Deak *et al.* 2010). Also, B cell-deficient mice display increased resistance against visceral *Leishmania* infection (Smelt *et al.* 2000, Bankoti *et al.* 2012).

Hypergammaglobulinemia appeared early and persisted throughout the course of *L. infantum* infection of rhesus macaques. The mechanisms leading to hypergammaglobulinemia during VL remain poorly understood. Parasite molecules that act as T cell-independent polyclonal B cell activators are thought to contribute to the process (Bohme *et al.* 1986, Cordeiro-Da-Silva *et al.* 2001, Silvestre *et al.* 2006, Silvestre *et al.* 2009b, Deak *et al.* 2010). B cells express several TLRs and, upon TLR engagement, differentiate into plasmablasts and secrete large amounts of antibody (Newell *et al.* 2010, Singh *et al.* 2012a). In this sense, it would be interesting to evaluate whether these B cell polyclonal activators exert their functions via TLRs. In rhesus macaques, we observed an early activation of splenic B cells that possibly accounts for the early appearance of hypergammaglobulinemia. Due to the lack of appropriate markers to detect plasma cells and plasmablasts in macaques we could not demonstrate their differentiation. Nevertheless, our data indicates that shortly after infection, B cells are activated, possibly by parasite factors, and start to secrete immunoglobulins, leading to a rapid establishment of hypergammaglobulinemia. In the long-term, chronic B cell activation and hypergammaglobulinemia may be maintained by inflammatory cytokines such as TNF, IL-6 or IL-1 β (Rieckmann *et al.* 1991, Weimer *et al.* 1998), as well as IL-10, which is a potent B cell growth factor (Rousset *et al.* 1992).

Despite the evidence of strong activation, the splenic B cell pool was maintained at a relatively constant size throughout the experimental period. Such observation may result from the downregulation of the B cell marker CD20 upon differentiation of B cells into plasmablasts (Amezcuca Vesely *et al.* 2012). Additionally, splenic B cells expressed high levels of CD95 (data not shown), which is in agreement with their pro-apoptotic nature and rapid turn-over (Amezcuca Vesely *et al.* 2012).

The splenic B cell compartment during *L. infantum* infection evidenced the progressive expansion of a population with an immature / atypical phenotype (CD21⁺CD27⁻). Unfortunately, there are no previous longitudinal studies exploring the differentiation of B cells in experimental VL. Nevertheless, similar to *Leishmania*, HIV infection also causes several defects in the B cell compartment, including polyclonal B cell activation and hypergammaglobulinemia (Moir *et al.* 2009). In AIDS patients, CD21⁺CD27⁻ B cells are similarly expanded and consist of a heterogeneous population constituted by immature / transitional B cells that also express CD10 and exhausted B cells that lack CD10 (Malaspina *et al.* 2006, Moir *et al.* 2008, Kardava *et al.* 2014). Unfortunately, the CD10 marker is not useful in macaques, and we excluded it from our analysis (Demberg *et al.* 2012). Nevertheless, it is reasonable to speculate that the expanded CD27⁺CD21⁻ B cell population in the spleen of *L. infantum*-infected macaques is constituted by immature cells, possibly recruited to the spleen from the bone marrow to meet the demands posed by the ongoing infection. Given that these immature B cells appear strongly activated during the course of infection, they should provide an important contribution to polyclonal hypergammaglobulinemia. Additionally, exhausted B cells may also contribute to the CD27⁺CD21⁻ pool and probably arise due to the chronic stimulation of B cells. Not much is known about exhausted B cells, but in HIV infection they exhibited impaired proliferative ability and augmented expression of inhibitory receptors (Moir *et al.* 2008). As such, it would be useful to obtain a detailed phenotypic and functional analysis of the B cell compartment of VL patients.

The elevated serum levels of total IgG in macaques during infection were not paralleled by similarly persistent high levels of parasite-reactive antibody. Instead, specific IgG was induced after infection, peaked at one month but decreased at the chronic phase. Some considerations should be taken into account while interpreting our data. We quantified the serum levels of parasite-specific IgG using a total extract from *L. infantum* amastigotes grown in axenic culture. However, axenic amastigotes are not perfectly similar to intracellular amastigotes with respect to their proteome (Pescher *et al.* 2011). As such, antibodies raised against antigens expressed by intracellular amastigotes but absent in axenic ones are not detected in these assays. The obvious alternative would be to produce a total parasite extract from intracellular amastigotes. However, these are difficult to isolate in large quantities, and parasites may partially differentiate to the promastigote form during the isolation procedure.

A second point meriting some clarification was the observation that the levels of *L. infantum*-specific IgG during the chronic phase of infection returned to values similar to pre-infection.

At first glance, these results may indicate a total loss of parasite-specific IgG at the chronic phase of infection. Such result would be at odds with findings in human VL patients in which, despite the occurrence of polyclonal hypergammaglobulinemia, there are always detectable levels of parasite-specific IgG. An alternative explanation comes from the observation that, even after extensive optimization of our ELISA assay, there was always a significant signal from non-infected animals, indicating the existence of naturally-occurring antibodies in the serum of rhesus macaques. Indeed, natural antibodies against *Leishmania* have been found in normal sera from many vertebrates (Schmunis *et al.* 1970, Buxbaum 2013). As such, our data indicates that the pool parasite-specific IgG in the chronic phase gives a signal of similar magnitude to the pool present before infection. As ELISA does not give any information on the identity of the antibodies, we cannot discern how different the specific IgG repertoire at the chronic phase is from the one present before infection. Such information would require a proteomic/serological methodology (Forgber *et al.* 2006).

Despite these limitations, our data is suggestive of a close association between the production of specific IgG and the differentiation of memory B cells and Tfh cells. However, and remarkably, despite the increasing parasite load in the spleen at the chronic phase of infection, there was a marked decrease in the levels of parasite-specific IgG, accompanied by the loss of memory B cells and Tfh cells. Understanding the mechanisms underlying the loss of these immune populations is crucial if we aim to devise therapies or vaccines aimed at preserving the production of specific antibodies.

The fact that many B cells appear activated shortly after infection, possibly in a T cell-independent manner, may indicate that few are able to follow the follicular pathway, interact with T cells and form a GC. Such scenario would explain the compromised numbers of Tfh cells in the spleen and their incomplete differentiation, as B cells are crucial for Tfh differentiation (Crotty 2011). However, even during chronic infection, naïve B cells (CD21⁺ CD27⁻) represent the majority of the splenic B cell pool, indicating that, despite the widespread B cell activation, there is an efficient replenishment during infection. Furthermore, GCs developed as a consequence of infection, indicating that at least some activated B cells are able to follow the follicular pathway. As such, there should be complementary mechanisms explaining the failure to maintain GCs, Tfh cells and memory B cells in the long term.

Effector CD4 T cell subsets are characterized by elevated expression of master transcription factors that, besides specifying the key characteristics of each subset, also actively repress features of alternative helper lineages (Zhu *et al.* 2010). For instance, T-bet, the Th1 master regulator, and Bcl-6, the key transcription factor for Tfh differentiation

are known to regulate the activities of each other via physical interactions (Oestreich *et al.* 2012). Current evidence supports a model in which the balance between T-bet and Bcl-6 co-expression determines the identity of an effector CD4 T cell, as either a Th1 or a Tfh cell (Oestreich *et al.* 2011). Further work unveiled a complex relationship between the Tfh and Th1 lineages. When primed *in vitro* in the presence of IL-12, CD4 T cells initially upregulate features of both Th1 and Tfh cells, including Bcl-6 and T-bet. However, continuous IL-12 signaling induces high levels of T-bet and IFN- γ that effectively repress Tfh characteristics to stably establish the Th1 lineage (Nakayamada *et al.* 2011). In this context, it is reasonable to speculate that the failure to maintain a sustained Tfh response during VL results from the strong Th1-polarizing environment present. Indeed, the gene expression data indicates a strong induction of T-bet and Th1-associated factors in splenic CD4 T cells during *L. infantum* infection in macaques. The observations we made in *L. infantum*-infected macaques are remarkably similar with those made on mice infected with the intracellular apicomplexan *Toxoplasma gondii* (Nakayamada *et al.* 2011). As with *Leishmania*, control of *T. gondii* infection requires the development of polarized Th1 responses (Zhu *et al.* 2012). In *T. gondii*-infected mice, both Th1 and Tfh cells can be detected in the spleen at early stages post-infection. However, at later stages the Tfh response declines markedly, and Th1 cells become largely dominant. Ablating the gene encoding T-bet (*TBX21*) resulted in sustained levels of Tfh cells, enhanced GC formation and increased specific humoral responses (Nakayamada *et al.* 2011).

Efficient regulation of the function of helper CD4 T cells by Tregs requires the co-option by the latter of selective markers of differentiation of the former (Oestreich *et al.* 2012). Recent studies have identified a GC-resident CD4 T cell population expressing FoxP3 and a number of Tfh-associated markers, including Bcl-6, PD-1 or CXCR5 (Chung *et al.* 2011, Linterman *et al.* 2011, Wollenberg *et al.* 2011). These cells, which were named T follicular regulatory cells (Tfr cells) represent about 10% of the follicle-associated CD4 T cells and were shown to repress Tfh function via mechanisms that have not yet been elucidated (Chung *et al.* 2011, Linterman *et al.* 2011, Wollenberg *et al.* 2011). Also, it is not entirely clear whether Tfr cells inhibit GC reactions by suppressing Tfh cells or GC B cells (Ramiscal *et al.* 2013). Tfr cells have not been studied in detail in an infectious context; nevertheless, in HIV/SIV infections the Tfr population does not appear to expand (Petrovas *et al.* 2012, Cubas *et al.* 2013), even in the context of a pathogenic accumulation of Tfh cells (Petrovas *et al.* 2012). Although the prevailing view argues against a major role for FoxP3⁺ CD4 T cells in mediating the immune-suppression during VL, a contribution of Tfr cells to the premature termination of GC reactions cannot be excluded.

One last mechanism that may contribute to the poor specific humoral response during chronic VL is the loss of lymphoid architecture of the spleen. Indeed, VL appears to affect the organization of many of the structures of the spleen, which is thought to preclude normal immune cell trafficking and negatively affect the immune response (Kaye *et al.* 2004). Particularly relevant in the context of humoral responses is the loss of follicular dendritic cells during VL (Smelt *et al.* 1997). FDCs form reticular networks in follicles and GCs retaining antigen at their surface as immune complexes. Furthermore, FDCs produce chemokines, cytokines and adhesion molecules that are crucial for follicle organization and GC maintenance (Victora *et al.* 2012). As such, FDCs are crucial players during GC reactions and, their loss is expected to underlie the defects in the humoral response during VL. In murine VL, the breakdown of FDC networks is evident by eight weeks post-infection and is accompanied by the loss of GCs. Additionally, FDC loss correlates with the parasite load and can be partially reversed by drug treatment (Smelt *et al.* 1997). The mechanisms leading to FDC depletion during VL remain unknown, but may involve factors secreted by the parasitized macrophages that infiltrate the follicles (Stanley *et al.* 2007). In macaques chronically-infected with *L. infantum*, remodeling of the splenic architecture was milder, as compared with humans or mice (Kaye *et al.* 2004). Although we have not analyzed FDCs, GC numbers were reduced, and they appeared less structured. As such, future studies in macaques employing longer infections should explore whether the reduced GC responses are associated with the loss of FDC networks. Furthermore, dissecting the mechanism responsible for FDC loss would be of great value if we aim to find strategies able to preserve this important cell type.

During chronic VL, amastigotes are exposed to antibodies when they egress from heavily infected macrophages to infect new ones. The view that antibodies are detrimental to the host is supported by the observation that, when ingested by macrophages, IgG-opsonized amastigotes promote IL-10 secretion and inhibit IL-12 production (Miles *et al.* 2005, Buxbaum 2008, Thomas *et al.* 2008). Mechanistically, IgG-opsonized amastigotes bind to Fcγ receptors on the surface of macrophages and activate the ERK pathway leading to initiation of *IL10* transcription (Yang *et al.* 2007, Thomas *et al.* 2008). Recent work has identified a glycoinositol phospholipid (GIPL) as the dominant amastigote surface antigen recognized by serum IgG in mice and humans (Buxbaum 2013). However, ligation of FcγRs on the surface of macrophages and DCs may lead to pro- or anti-inflammatory outcomes, depending on the identity of the Fc receptors activated, IgG subclass, cell type or the density of antibody present in the immune complex (Gallo *et al.* 2010, Guilliams *et al.* 2014). For instance, one study demonstrated that parasite-specific IgG is required for efficient *L. major*

uptake and IL-12 production by DCs, suggesting that FcγR-mediated uptake has contrasting outcomes in DCs and macrophages (Woelbing *et al.* 2006). Also, in a model of co-infection of *L. amazonensis* and *L. major* in mice, it was shown that resolution of the lesion required specific antibody that was able to enhance the microbicidal mechanisms in the macrophage, by promoting NADPH-oxidase assembly and ROS production (Gibson-Corley 2010, Gibson-Corley *et al.* 2012). As such, the role of antibodies and Fc receptors is more complex than previously appreciated, with the outcome of FcγR ligation being clearly context-dependent.

Furthermore, the role of antibodies as regulators of the inflammatory response is not necessarily detrimental to the host. While B cell-deficient mice resolve *L. donovani* infection more rapidly than WT mice, such resistance comes at the cost of increased hepatic pathology. However, administration of immune serum to infected B cell-deficient mice alleviates pathology without decreasing the efficiency of hepatic parasite clearance (Smelt *et al.* 2000).

In line with these arguments, there is now compelling evidence suggesting that antibody-mediated immunity plays important protective roles during infections by pathogens classically regarded as being controlled exclusively by cell-mediated immunity (Casadevall *et al.* 2006). For instance, the efforts to develop an efficient T cell-based vaccine against HIV have been hindered by disappoint results in human trials (McMichael *et al.* 2014). Recently, a major research focus has been the design of vaccines capable of eliciting anti-HIV broadly-neutralizing antibodies, similar to those present in patients that maintain low viremia for a long time (Kwong *et al.* 2013). Likewise, sustained evidence now supports a protective role for antibodies against infection by *Mycobacterium tuberculosis* (Achkar *et al.* 2013). This is particularly relevant for leishmaniasis research, because, as *Leishmania*, *M. tuberculosis* survives and replicates inside macrophages, and the prevailing dogma has classically discarded humoral immunity as relevant for host defense (Achkar *et al.* 2013). Even conceding that parasite opsonisation by specific IgG is deleterious for the host, there are alternative mechanisms through which antibodies may contribute to the protective response against *Leishmania*; for instance by neutralizing parasite virulence factors. An illustrative example comes from the intracellular bacteria *Listeria monocytogenes*. A monoclonal antibody against listeriolysin, the pore-forming toxin of *L. monocytogenes*, was capable of blocking bacterial replication inside macrophages and provided resistance to infection in mice (Edelson *et al.* 1999, Edelson *et al.* 2001). Antibodies against *Leishmania* virulence factors, such as the metalloprotease gp63, have been detected in the sera of VL patients (Shreffler *et al.* 1993), but it is not clear whether they are capable of neutralization or play any protective function. As such, it is imperative to identify antibodies with

neutralizing capacity and to evaluate whether their administration is capable of modifying the course of the disease to the benefit of the host. However, it is not clear that VL patients can produce such antibodies. For instance, it takes at least two years of infection for HIV-infected patients to develop broadly-neutralizing antibodies against the virus (Kwong *et al.* 2013). Analysis of the sequence of these antibodies revealed that they were highly mutated relative to their germline precursors (Klein *et al.* 2013). As such, these potent neutralizing antibodies appear to result from extensive affinity maturation, possibly resulting from several passages through germinal centers for each B cell clone (Locci *et al.* 2013). Contrary to *Leishmania*, HIV is a rapidly-mutating agent, which certainly increases the time necessary for the generation of broadly-neutralizing antibodies (Kwong *et al.* 2013). Nevertheless, given the destruction of FDC networks and loss of GCs during VL, it may well be possible that high-affinity antibodies do not have the time to develop. It would be very informative, in this respect, to assess the number of mutations from the germline precursors in specific IgG from VL patients. Also, measuring the affinity of the antibodies produced against particular antigens at several time-points during the course of experimental VL would give valuable information on whether the process of affinity maturation is compromised.

Rather than considering the role of antibodies solely as pathological or irrelevant, it is perhaps wiser to acknowledge that these molecules may play both protective and non-protective roles during VL. As such, vaccine design against VL should include stimulation of humoral immunity, taking care to maximize the protective functions of antibodies and minimize the pathologic ones.

3. CD8 T cells in macaque visceral leishmaniasis: differentiation and exhaustion

Evidence collected over the past decades supports an important protective role for CD8 T cells during infections by visceral *Leishmania* (Stern *et al.* 1988, Kaye *et al.* 1992, Tsagozis *et al.* 2003, Murray 2005, Polley *et al.* 2006, Joshi *et al.* 2009). How CD8 T cells mediate protection during VL is an issue not entirely elucidated as these cells have been far less studied than CD4 T lymphocytes. Type I cytokines, such as IFN- γ or TNF, secreted by CD8 T cells are known to complement CD4 T cell responses in the spleen and liver during VL (Mary *et al.* 1999, Polley *et al.* 2005a, Beattie *et al.* 2010a). Less clear is whether CD8 T cell-mediated cytotoxicity plays any protective role. Some studies in mice demonstrate that parasite-specific cytotoxic T lymphocytes (CTLs) generated during infection are capable of killing infected cells *ex vivo* (Tsagozis *et al.* 2003, Tsagozis *et al.* 2005, Costa Lima *et al.* 2014), via mechanisms relying on the perforin/granzyme and Fas/FasL pathways (Tsagozis *et al.* 2003). However, it is not clear whether the parasite is killed concomitantly with the demise of the host cell. *In vitro* experiments indicate that parasites remain viable after CD8-mediated host cell lysis, but are eventually killed after infecting neighboring macrophages pre-activated with CD8-derived IFN- γ (Smith *et al.* 1991). These observations suggest that the cytotoxic and cytokine-secreting functions of CTLs collaborate for efficient parasite killing, but such mechanism has so far not been demonstrated *in vivo*. Also, the fact that *Leishmania*-infected cells are resistant to several pro-apoptotic stimuli indicates that the parasite actively inhibits death pathways and suggests that any mechanism leading to apoptosis of an infected cell is presumably protective for the host (Rodrigues *et al.* 2012). Our data in macaques demonstrates that splenic CD8 T cells produce elevated levels of granzyme B and to a lower extent Th1 cytokines during the first weeks after *L. infantum* infection, which coincides with parasite containment in visceral organs. Also, tissue immunofluorescence confirmed the strong expression of granzyme B in the first weeks and its localization to the red pulp / marginal zone of the spleen.

The role of CD8 T cell effector function has been studied with more detail in infections with cutaneous *Leishmania* species (Stager *et al.* 2012). In mouse models of CL and human patients, the current paradigm indicates that CD8 T cells producing IFN- γ contribute to the protective response against the parasite while CD8-mediated cytotoxicity leads to tissue pathology and promotes skin ulceration (Muller *et al.* 1994, Belkaid *et al.* 2002b, Faria *et al.* 2009, Novais *et al.* 2013, Santos Cda *et al.* 2013). Whether a similar dichotomy in the effector functions of CD8 T cells is operative in VL remains unknown and is a matter of great interest in future studies.

Despite the induction of effector CD8 molecules during the acute phase of *L. infantum* infection of rhesus macaques, analysis at the chronic phase revealed a significant loss of CD8 T cell effector markers. The patterns of type I cytokine and cytolytic molecule expression we observed in macaques are very similar to those found in mouse models of VL, although the loss of CD8 T cell effector function appears to occur faster in mice (Tsagozis *et al.* 2003, Joshi *et al.* 2009). Indeed, after strong induction in splenic CD8 T cells during the first four weeks of infection, the expression of IL-2, IFN- γ , TNF and granzyme B returns to pre-infection levels by the fifth week post-inoculation in mice (Tsagozis *et al.* 2003, Tsagozis *et al.* 2005, Joshi *et al.* 2009). Furthermore, and similar to our observations in macaques, in the mouse model, splenic CD8 T cells adopted effector and effector memory phenotypes that were maintained in the long course (Tsagozis *et al.* 2003, Joshi *et al.* 2009). We extended these observations by showing that the expression of granzyme B in CD8 T cells during the acute phase of infection is associated with T-bet expression. Nevertheless, T-bet was not an essential requirement for granzyme B production by CD8 T cells, in agreement with previous findings (Pearce *et al.* 2003). While EOMES was similarly induced with the differentiation of CD8 T cells, its expression was maintained at relatively elevated levels in the chronic phase, even after the apparent exhaustion of splenic CD8 T cells. While EOMES expression has been proposed as critical for induction of granzyme B (Pearce *et al.* 2003), our data in macaques argues against such crucial requirement as we observed a substantial percentage of EOMES⁺ granzyme B⁺ CD8 T cells.

During chronic infections, CD8 T cells are continuously stimulated, which progressively decreases the size of the responding memory pool. In the long term, such persistent activation leads to a collapse of CD8 immunity, evidenced by T cell exhaustion and apoptosis (Wherry 2011). Careful analysis of the CD8 T cell pool during lymphocytic choriomeningitis virus (LCMV) infection in mice revealed that T-bet and EOMES are co-expressed in effector-memory CD8 T cells during the acute phase of infection. In contrast, in the chronic phase, the two transcription factors were reciprocally expressed, with EOMES^{hi} CD8 T cells largely outnumbering T-bet^{hi} cells (Paley *et al.* 2012). Adding complexity to this story, during the chronic phase of the LCMV mouse model, T-bet^{hi} CD8 T cells were able to proliferate upon activation and converted into EOMES^{hi} cells, which expressed PD-1 and were exhausted. Viral persistence and continuous stimulation eventually led to the depletion of the T-bet^{hi} pool resulting in total collapse of CD8 immunity (Paley *et al.* 2012). These observations underscore the complex relationship between T-bet and EOMES-expressing CD8 T cell subsets during chronic infections. Our data in *L. infantum*-infected macaques, while not as detailed as in chronic viral models, is

nevertheless suggestive of a progressive attrition of splenic T-bet⁺ CD8 T cells, which may underlie the functional exhaustion of the CD8 T cell pool during VL. As such, further phenotypic and functional characterization of memory CD8 T cells during chronic VL is required, in particular, if we aim to understand the relative contributions of short-lived terminally differentiated and longer-lived CD8 T cells to the protective response against the parasite.

Apart from the persistently high viral loads, additional driving forces are known to promote exhaustion in T cells in chronic viral infection. These include the action of suppressive cytokines and regulatory populations, such as Tregs (Wherry 2011). Some of these factors are similarly induced during chronic VL and may lie at the genesis of CD8 T exhaustion or contribute to its maintenance in the long-term (Gigley *et al.* 2012). Concerning *L. donovani* / *infantum* infections, IL-10 and TGF- β are soluble factors that likely contribute to the induction and maintenance of the exhausted state in T cells, as reported in viral and *Toxoplasma* infections (Brooks *et al.* 2008, Tinoco *et al.* 2009, Bhadra *et al.* 2014).

Exhaustion of CD8 T cells may as well result of insufficient or inappropriate CD4 T cell help (Wherry 2011). In chronic viral infections, CD4 T cell-derived IL-21 is crucial for the maintenance of the pool of multifunctional effector/memory CD8 T cells that control viral load (Elsaesser *et al.* 2009, Frohlich *et al.* 2009). Given the prominent alterations of CD4 T cell function observed during the course of VL, it would be of interest to investigate whether CD8 T cell exhaustion results from qualitative or quantitative decrease of CD4 T cell help. Recent studies based on microarray data and network analysis are starting to unveil the transcriptional program that characterizes exhausted CD8 T cells (Wherry *et al.* 2007, Doering *et al.* 2012). These studies highlighted the notion that exhausted T cells are at least as different from effector and memory T cells as these are from each other (Wherry 2011). In exhausted CD8 T cells, several transcription factors are expressed in high levels, including the B lymphocyte-induced maturation protein-1 (BLIMP-1), the nuclear factor of activated T cells-2 (NFAT-2), the basic leucine zipper transcription factor ATF-like (BATF) or EOMES (Wherry *et al.* 2007, Shin *et al.* 2009, Quigley *et al.* 2010, Paley *et al.* 2012), while others, such as T-bet, appear diminished (Paley *et al.* 2012). However, these transcriptional signatures refer to models of chronic viral infections and may differ from VL, in which exhaustion may be driven by alternative forces. As such, it would be of great interest to isolate exhausted CD8 T cells from experimental VL models and VL patients and dissect in detail the transcription program that operates to maintain the exhausted state. Such information will be valuable if we aim to design therapies aimed at re-invigorating the CD8 T cell response.

4. Future directions

The work presented in this thesis confirmed the suitability of the rhesus macaque as a valuable model for the study of visceral leishmaniasis. We provided the first detailed description of the progression of the immune response from the acute to the chronic phase of VL in a host model that shares ample similarities with humans. Our study highlighted the intricacies of the host-pathogen interaction during VL. In the view of a pathogen that cannot be eradicated acutely, the immune system demonstrates a remarkable plasticity by switching towards a more tolerant approach. The parasite, in turn, explores these regulatory mechanisms to increase its population in the host and augment the chances of transmission.

Our study was necessarily exploratory, due to the lack of previous immunological data on macaque models of VL. In this context, it may lay the ground for more frequent employment of rhesus macaques in studies aiming to understand the fundamental mechanisms regulating immunity during VL. While our experiments were descriptive in nature, research in infectious diseases employing macaques is not limited to longitudinal descriptions. Technological progress towards the generation of transgenic macaques has been slow-paced, although some exciting developments were made in a transgenic macaque model for the study of Huntington's disease (Yang *et al.* 2008). Nevertheless, even considering that technical difficulties may eventually be overcome, it is extremely unlikely that transgenic macaques will be as widely used as their murine counterparts. Such implausibility mainly derives from the ethical issues that will arise and the repressive legislation that will likely follow (Suran *et al.* 2009). Also, breeding takes much longer in macaques as compared to mice, which poses a problem of difficult resolution, given the pace at which biomedical research currently advances. Fortunately, alternative approaches to manipulate the macaque immune system are currently available and in use. As illustrative examples, antibody-mediated depletion of immune populations has been performed with high efficiency in macaques. Also, recombinant cytokines or growth factors have been produced and administered to monkeys with the aim of modulating the immune response (Evans *et al.* 2013).

Obviously, the classical limitations associated with the use of non-human primates in biomedical research will continue to exist and must be kept in mind. In particular, studies involving macaques usually employ outbred animals, which may confound data interpretation due to the contribution of the host genetics on the phenotype variance. For a number of reasons related with animal welfare, inbreeding is not recommended in non-

human primates, although measures may be employed to ensure some genetic homogeneity among experimental animals (Kanthaswamy *et al.* 2010).

With respect to studies on T cell immunity, one of the most ground-breaking advances in the last decades was the development of tools capable of detecting antigen-specific T cells. In particular, the development of multimeric MHC/peptide complexes (MHC tetramers) allowed the quantification of specific T cells with higher accuracy, as compared with the classical methods based on cell proliferation or cytokine production (Altman *et al.* 1996, Murali-Krishna *et al.* 1998). In this context, a limitation of our study was the fact that we analyzed the bulk of CD4 and CD8 T cell populations. As such, significant changes in the parasite-specific T cell pool, may be diluted and pass unappreciated if the object of analysis is the total population present in a given organ. This issue becomes even more problematic in macaques, due to the variability between animals. MHC tetramers specific for *Leishmania* antigens have already been developed and employed in the mouse model (Stetson *et al.* 2002, Pagan *et al.* 2013), but none has so far been engineered using macaque MHC alleles. These, however, have been produced for SIV peptides, in the context of AIDS research (Velu *et al.* 2009) and the NIH Tetramer Core facility produces MHC tetramers on demand for the detection of T cells specific for virtually any antigen. Thus, a particular recommendation emerging from our work is that future research should focus on the analysis of specific adaptive responses. With these tools in hand, researchers on the leishmaniasis field should consider the macaque model as a powerful tool for the re-evaluation of observations made initially in mice or even as a starting model for hypothesis testing.

Chapter V | Publications in related fields

Microrreview

Modulation of mammalian apoptotic pathways by intracellular protozoan parasites

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Summary

During intracellular parasitic infections, pathogens and host cells take part in a complex web of events that are crucial for the outcome of the infection. Modulation of host cell apoptosis by pathogens attracted the attention of scientists during the last decade. Apoptosis is an efficient mechanism used by the host to control infection and limit pathogen multiplication and dissemination. In order to ensure completion of their complex life cycles and to guarantee transmission between different hosts, intracellular parasites have developed mechanisms to block apoptosis and sustain the viability of their host cells. Here, we review how some of the most prominent intracellular protozoan parasites modulate the main mammalian apoptotic pathways by emphasizing the advances from the last decade, which have begun to dissect this dynamic and complex interaction.

Introduction

In the complex interplay between an intracellular pathogen and its host, members of the main pathogens classes have been shown to manipulate the survival of host cells, either by promoting or inhibiting it for their own advantage.

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Indeed, infection by an intracellular parasite might represent a stress signal sufficient to initiate an apoptotic process in the host, thus providing an altruistic mechanism to eliminate infected cells and limit parasite dissemination. As pathogens and hosts live and evolve together, it is not surprising that the former had developed strategies to circumvent this apoptotic immunity of the host. Additionally, most protozoan parasites are auxotrophic in regard to several essential metabolites, which further increase their dependency on host cell viability. Under certain instances protozoan parasites may even promote apoptosis in the infected cell to facilitate their release during the transition from one stage of the infection to the next.

In this review, we intend to summarize the main findings on the modulation of host cell apoptosis by intracellular protozoan parasites as a central component of their pathogenicity. We will focus on the kinetoplastids *Leishmania* spp. and *Trypanosoma cruzi* and the apicomplexans *Toxoplasma gondii*, *Theileria* spp. and *Plasmodium* spp., which all cause elevated morbidity and mortality in humans and other mammals. We recapitulate the mechanisms through which intracellular protozoa modulate the apoptotic cascades of infected cells, either by promoting or inhibiting the apoptotic process according to their own needs at a given stage of the infection.

Apoptosis

Apoptosis is a well-described form of programmed cell death that plays a critical role in embryonic development, in maintaining cellular homeostasis in the adult organism, and in eliminating neoplastic, damaged or infected cells.

Two main routes lead to the apoptotic process: the extrinsic and the intrinsic pathways. In the extrinsic pathway, the cell is instructed to die by a signal originated at the surface in the form of death receptor engagement. Upon stimulation by their respective ligands, such as TNF- α (tumour necrosis factor- α) or Fas ligand (FasL), a signalling cascade is initiated allowing the recruitment of adaptor proteins that promote the formation of the DISC (death-inducing signalling complex), which is responsible for the activation of the initiator caspase-8 or caspase-10.

The Bcl-2 (B cell lymphoma-2) protein family plays a central role in the intrinsic apoptotic pathway by controlling the integrity of the outer mitochondrial membrane (OMM). Anti-apoptotic Bcl-2-family proteins such as Bcl-2, Bcl-xL (B-cell lymphoma-extra-large) and Mcl-1 (myeloid cell leukaemia 1) inhibit apoptosis by directly binding and blocking the pro-apoptotic Bcl-2-family proteins. The latter can be sub-divided into two groups: the effector proteins Bax (Bcl-2-associated $\times$ protein) and Bak (Bcl-2 antagonist killer 1) that, upon activation, homo-oligomerize in the OMM causing mitochondrial outer membrane permeabilization (MOMP), and the BH3-only proteins, such as Bim (Bcl-2-interacting mediator of cell death), Bid (BH3 interacting-domain death agonist), Bad (Bcl-2 antagonist of cell death) or Puma (p53-upregulated modulator of apoptosis) whose function is to relay the apoptotic signal to the effectors by acting as sensors of the death stimulus originating from several cellular processes (Chipuk *et al.*, 2010). Upon Bax and Bak activation, the OMM becomes permeabilized, releasing several apoptogenic factors from the mitochondria, the most important of which is cytochrome c. Once in the cytosol, cytochrome c binds APAF-1 (apoptotic protease activating factor-1) and induces activation of initiator caspase-9 in a large caspase-activating complex known as apoptosome. Once active, caspase-9 is able to induce the activation of effector caspases, such as caspase-3 or caspase-7.

Cells generally require the continuous presence of surviving signals, such as growth factors and cytokines, otherwise falling into a degenerative process known as death by neglect. Thus, an intracellular pathogen might take advantage of this requirement to control the life span of the host cell to its own advantage. Under normal conditions, the PI3-K (phosphatidylinositol 3-kinase)/PKB (protein kinase B) pathway and the MAPK (mitogen-activated protein kinase) pathway, among others, constitutively promote the phosphorylation of Bcl-2-family members such as Bad, Bax, Bim and others, promoting their cytoplasmic arrest or degradation and conferring a pro-survival state to the cell. In contrast, growth factor or cytokine deprivation leads to stabilization of pro-apoptotic members such as Bim. Additionally, deprivation conditions stimulate the activity of several transcription factors such as FOXO-3A (Forkhead box-O3A), c-Jun and p53 that direct the transcription of pro-apoptotic mediators (Chipuk *et al.*, 2010). In contrary, the NF- κ B (nuclear factor κ B) that constitutes a family of ubiquitous transcription factors regulates the expression of several anti-apoptotic molecules including Bcl-2, Bcl-xL, cIAPs (cellular inhibitors of apoptosis) and cFLIP (cellular FLICE-like inhibitory protein) (Perkins, 2007).

Over the last 10 years, MOMP and lysosomal membrane permeabilization (LMP) have been identified as

major events triggering programmed cell death. Thus, in a number of models, lysosomal destabilization and the ensuing efflux of cathepsins play early and important roles in cell death (Guicciardi *et al.*, 2004). This process is partly mediated by the activation of a caspase-dependent pathway causing the proteolytic activation of Bid and Bax. Most importantly, LMP results in cell death, independently of caspase activation (Bidere *et al.*, 2003; Laforge *et al.*, 2007).

Mitochondria are not only indispensable elements in the cell by providing the apoptogenic factors but are powerful organelles that regulate ATP and reactive oxygen species production, biosynthetic intermediates. The mitochondrion is not inert but undergoes reorganization of its structure in response to environmental factors to meet the cellular energy demands. The morphology of mitochondrial network depends on the balance between fusion and fission events, two synchronous-acting opposing processes. Thus, mitochondrial fragmentation due to either an excess of fission or a defect in fusion have been proposed to participate in facilitating the release of mitochondrial apoptogenic factors (Arnoult *et al.*, 2005; Estaquier and Arnoult, 2007). Additionally, compiling evidence suggests a role for Bcl-2 family members as regulators of mitochondrial fission and fusion in a manner independent of their role in apoptosis (Sheridan and Martin, 2010). Thus, new aspects in the cascade of events leading to cell death have emerged in the last decade involving mitochondrial dynamics.

Modulating the apoptotic pathways in the infected cell

Leishmania

Leishmania (L.) spp. are the causative agents of leishmaniasis, a group of diseases with clinical manifestations ranging from ulcerative skin lesions (cutaneous leishmaniasis) to disseminated visceral infection (visceral leishmaniasis or kala-azar).

In the early stages of *Leishmania* infection, neutrophils are the first leucocytes to arrive at the site of infection to phagocyte the parasite (Peters *et al.*, 2008). *Leishmania* is able to inhibit the spontaneous apoptosis of short-lived neutrophils, increasing their life span and providing a safe place for the parasites during the first days of the infection. Although the mechanism by which the parasite inhibits neutrophil apoptosis remains to be elucidated, it nevertheless involves a reduction in caspase-3 activity (Aga *et al.*, 2002).

The ability of *L. major* to suppress apoptosis is not dependent on the host cell genetic background. Bone marrow-derived macrophages (BMMs) from both resistant, C57Bl/6, and susceptible, BALB/c, mice showed

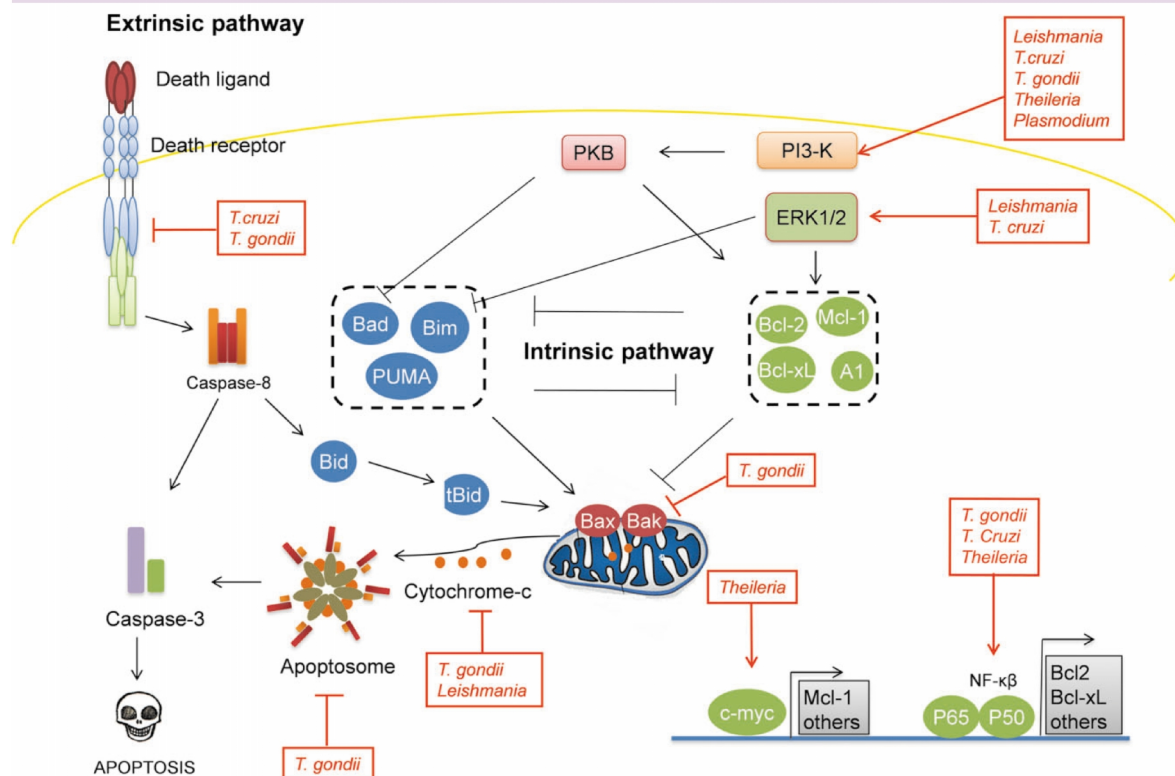


Fig. 1. An overview of the mammalian apoptotic pathways and the points of interference by intracellular protozoa. In the extrinsic pathway, death receptor triggering by death ligands leads to processing and activation of the initiator caspase-8, which then activates either directly the effector caspase-3 (type I cells) or cleaves the BH3 only protein Bid (type II cells) connecting the extrinsic and the intrinsic pathways. In the intrinsic pathway, diverse cellular insults result in the activation or upregulation of the BH3 only proteins of the Bcl-2 family, which then lead to MOMP by activating Bax and Bak, or by inhibiting the antiapoptotic Bcl-2 proteins. MOMP results in the release of cytochrome c from the mitochondria leading to the assembly of the apoptosome which results in activation of caspase-9. Once active, caspase-9 processes and activates effector caspase-3. Intracellular protozoa can interfere at several levels in the apoptotic cascade modulating cell survival. As Orpheus and his lyre who resisted the terrible Sirens by interfering with fatal songs, parasites have developed strategy to circumvent the apoptotic cascade.

delayed cytochrome c release from the mitochondria induced by M-CSF (macrophage-colony-stimulating factor) deprivation after *L. major* infection (Akarid *et al.*, 2004) (Fig. 1). The same study showed that the transcription of anti-apoptotic genes mediated by NF-κB was not necessary for the *L. major*-mediated repression of apoptosis. Ruhland *et al.* reported that *Leishmania*-mediated resistance of Raw 264.7 cells to apoptosis was dependent on PI3-K/PKB signalling, which resulted in Bad phosphorylation and sequestration (Fig. 1). This is independent of the NF-κB or p38 MAPK pathways (Ruhland *et al.*, 2007).

Infection with *L. donovani* also protected BMMs from M-CSF deprivation. Interestingly, the same effect was obtained by treating macrophages with the promastigote major surface molecule, LPG (lipophosphoglycan) (Moore and Matlashewski, 1994). Further work supported a possible role for LPG in the *Leishmania*-mediated inhibition of apoptosis in U-937 cells through PKC-δ (protein kinase C delta) signalling inhibition (Lisi *et al.*, 2005). However, it

was recently suggested that total phosphoglycans and not only LPG are responsible for inhibiting apoptosis (Donovan *et al.*, 2009). Possibly, *Leishmania* surface phosphoglycans are functionally redundant and the loss of one can be compensated by the action of the others. Furthermore, following differentiation of the promastigote to the intracellular amastigote form, phosphoglycans are nearly absent at the surface of the parasite (Naderer *et al.*, 2004), suggesting that their role in inhibiting apoptosis in the host cell is restricted to the early phase of the infection.

The transient transfection of a *Leishmania major* orthologue of macrophage inhibitory factor (MIF) was shown to protect Raw 264.7 cells from nitric oxide (NO)-induced apoptosis due to the activation of the ERK1/2 MAPK pathway and the reduction of the levels of Serine15-phosphorylated p53, which increase in response to NO treatment (Kamir *et al.*, 2008) (Fig. 1).

Leishmania infection in macrophages also leads to cleavage and inhibition of the mTOR (mammalian/

mechanistic Target of rapamycin) serine/threonine kinase resulting in a global repression of host translation (Jaramillo *et al.*, 2011). Apart from its role in controlling protein translation, mTOR has been associated with regulation of autophagy and in a cell type-dependent manner with both anti- and pro-apoptotic activities. As mTOR inhibition is a well-known autophagic stimulus and because autophagy acts in many instances as a stress response that avoids cell death, it is possible that autophagy induction in *Leishmania*-infected cells (Mitroulis *et al.*, 2009) may be the result of mTOR inhibition and represent an alternative anti-apoptotic strategy, although this has not been yet formally shown.

In conclusion, the factors allowing some *Leishmania* species to visceralize remain largely unknown. Whether modulation of host cell apoptosis may play a role in this process remains an open question. Inhibition of apoptosis in *Leishmania*-infected cells is achieved by manipulation of pro-survival pathways that do not involve NF- κ B activation, as the NF- κ B pathway is blocked in *Leishmania*-infected cells (Neves *et al.*, 2010). Possibly, the deleterious effects of the inflammatory response that results from its activation in macrophages and dendritic cells (the typical host cells for *Leishmania*) overrule the potential benefits for the parasite in activating NF- κ B.

Trypanosoma cruzi

The kinetoplastid *Trypanosoma cruzi* is the etiological agent of Chagas' disease. *T. cruzi* infection is associated with high levels of apoptosis in the host (Lopes *et al.*, 2007); however, the parasite also inhibits apoptosis in infected cells. In neuronal and glial cells, *T. cruzi* infection represses host cell apoptosis through the action of a secreted trans-sialidase, a parasite-derived protein that mimics host NGF (nerve growth factor) (Chuenkova *et al.*, 2001). *T. cruzi* trans-sialidase was shown to bind and activate the NGF receptor, TrkA (Neurotrophic tyrosine kinase receptor type 1) triggering the PI3-K/PKB pathway, resulting in increased Bcl-2 expression (Chuenkova and PereiraPerrin, 2004). After internalization of the parasite, the trans-sialidase protein is phosphorylated by PKB and in turn mediates the activation of PKB resulting in the downregulation of Bax, caspase-9 and the FOXO-3A transcription factor. This results in protection of Schwann cells from apoptosis induced by H₂O₂ and TNF- α /TGF- β (transforming growth factor β) treatment providing a mechanism for PKB activation at later times of the infection (Chuenkova and PereiraPerrin, 2009) (Fig. 1).

Trypanosoma cruzi infection also promoted survival of mouse neonatal cardiomyocytes cultured under serum deprivation conditions, an effect at least in part mediated by cruzipain, a cysteine protease secreted by *T. cruzi* (Aoki *et al.*, 2004). The mechanism involved an upregula-

tion of arginase-2 that paralleled a decrease in NO levels (Aoki *et al.*, 2004). Cruzipain also mediated by the activation of PI3-K/PKB and ERK pathways, leading to phosphorylation and cytoplasmic sequestration of Bad, upregulation of Bcl-2 and decreased caspase-3 activation (Aoki Mdel *et al.*, 2006). In another study, *T. cruzi*-mediated prevention of apoptosis in cardiac cells seems to be dependent on NF- κ B activation, but not of PI3-K/PKB (Petersen *et al.*, 2006). Overall, this suggests that different signalling pathways can be activated by the parasite at different stages of the infection (Fig. 1).

Trypanosoma cruzi blocks Fas/FasL induced apoptosis by increasing the levels of the c-FLIP protein (Hashimoto *et al.*, 2005). By disrupting the interaction between c-FLIP and Itch, an ubiquitin ligase that is involved in c-FLIP turnover, *T. cruzi* inhibits c-FLIP degradation, promoting its accumulation in infected cells (Murata *et al.*, 2008) (Fig. 1).

Toxoplasma gondii

Toxoplasma gondii is one of the most widespread intracellular pathogens, infecting virtually all types of nucleated cells and inducing remarkable modifications in host cells, including organelle reorganization, remodelling of the cytoskeleton around the parasitophorous vacuole. The parasite manipulates several host signalling pathways to confer an anti-apoptotic state to the infected cells.

In the extrinsic pathway, *T. gondii* inhibited Fas-mediated apoptosis by inducing an aberrant cleavage and degradation of the initiator caspase-8 (Vutova *et al.*, 2007). Regarding the mitochondrial pathway, a recent study showed that *T. gondii* prevents the activation and mitochondrial targeting of Bax. Importantly, this effect was achieved without changes in the total levels of Bax, Bak or the anti-apoptotic Bcl-2, suggesting that the parasite is not dependent on the transcription machinery of the host cell to inhibit apoptosis and may directly block Bax and Bak activation (Hippe *et al.*, 2009). In another report, *T. gondii* inhibited apoptosome assembly and caspase-9 activation in a cell-free reconstitution system, suggesting that parasite-derived molecules can directly interfere with cytochrome c-induced caspase activation (Keller *et al.*, 2006) (Fig. 1).

Other reports, however, suggest that *T. gondii* manipulates several signalling pathways to modify the balance of Bcl-2 family proteins towards a pro-survival state. In infected macrophages, *T. gondii* activated PI3-K/PKB signalling to protect the cells from apoptotic death (Kim and Denkers, 2006) but blocked activation of JNK MAPK in infected cells after UV exposure (Carmen *et al.*, 2006). Activation of NF- κ B in *T. gondii*-infected cells was accompanied by the induction of anti-apoptotic genes and protection from apoptosis was lost after infection of mouse

embryonic fibroblasts deficient for the p65 NF- κ B subunit (Payne *et al.*, 2003) (Fig. 1). Activation of NF- κ B correlates with localization of phosphorylated I κ B α at the parasitophorous vacuole membrane (PVM), and is independent of host IKK signalosomes, revealing the involvement of a *T. gondii*-derived I κ B kinase (TgIKK) present at the PVM in the activation of the NF- κ B pathway (Molestina and Sinai, 2005a). Further work showed that both host IKK and TgIKK contribute to sustained activation of NF- κ B during infection, with the host IKK providing the initial activation of the pathway in the early stage of the infection, while TgIKK contributes to sustain and modulate the response (Molestina and Sinai, 2005b). Nonetheless, the role of NF- κ B in *T. gondii* infection remains a point of controversy, as others suggest that NF- κ B translocation to the nucleus is inhibited during early stages of the infection, presumably to avoid the inflammatory response associated with NF- κ B activation (Shapira *et al.*, 2005). Additionally, strain subtype seems to influence NF- κ B activation. In a recent study, type II *T. gondii*-strain induces a higher level of NF- κ B activation as compared with type I and type III strains. Using a forward genetics approach, this study identified the GRA15 gene product, a dense granule protein, which is secreted to the host cell by type II strains and induces NF- κ B activation (Rosowski *et al.*, 2011).

Apicomplexan parasites possess specialized secretory organelles such as the micronemes, rhoptries and dense granules, which sequentially discharge proteins that play important functions during parasite invasion, PVM modification and control of host responses (Plattner and Soldati-Favre, 2008). Among apicomplexan secreted proteins, much attention has been captured by rhoptry proteins (ROP) for their effects in regulation of host transcription. Thus, ROP16, a serine–threonine kinase, was shown to phosphorylate and activate the transcription factors STAT3 (Signal Transducers and Activators of Transcription-3) and STAT6 (Saeij *et al.*, 2007) that are known to mediate the transcription of anti-apoptotic genes (Amin *et al.*, 2004). ROP18, another rhoptry kinase, was recently shown to phosphorylate host ATF6 (Activating Transcription Factor-6) leading to its proteasome-dependent degradation (Yamamoto *et al.*, 2011). ATF6 is a transcription factor involved in the unfolded protein response, and a mediator of endoplasmic reticulum stress-induced apoptosis (Szegezdi *et al.*, 2006). It is thus plausible that the ATF6 blockade in infected cells further represents another anti-apoptotic mechanism of *T. gondii*. Collectively, these recent findings on ROP proteins shed new light into how *T. gondii* modulates host responses, including apoptosis and have opened new lines of investigation.

Despite all the available evidence pointing out that *T. gondii* blocks the apoptotic process in the infected

cell, an alternative picture has been recently suggested. Death receptor ligation resulted in rapid parasite egress causing necrotic death of host cells due to the release of intracellular calcium as a consequence of caspase activation early in the process (Persson *et al.*, 2007). Despite the apparent contradiction between the results of this report and those of others, it is also possible that the outcome of death receptor ligation in infected cells varies according to the host cell, parasite strain or specific experimental conditions.

In conclusion, *T. gondii* employs several strategies to protect infected cells from apoptotic death (Fig. 1). These multiple activities became obvious in a recent study aiming to analyse the stability and turnover of several Bcl-2 family members after *T. gondii* infection (Carmen and Sinai, 2011). While the steady-state levels of the Bcl-2 protein remain unchanged upon infection, the turnover of Bax, Bad and Bid was found markedly increased in infected cells. The authors concluded that only multiple activities could account for the observed infection-induced perturbation on the stability of Bcl-2 family members (Carmen and Sinai, 2011). These multiple mechanisms may range from direct interference of the parasite with the core apoptotic machinery to modulation of survival pathways. Given its almost unrestricted cell range, the parasite probably requires distinct mechanisms to inhibit apoptosis in different cell types. It is therefore expected that future research will help to precise which pathways are modulated by *T. gondii* in distinct cell types. The significance of these studies would benefit from the use of primary cells as host cell models instead of the more common use of immortalized cell lines.

Theileria spp.

Theileria spp. infections are a cause of major losses in the cattle industry in Africa, Asia and regions of Southern Europe. The parasite infects host leucocytes, which thereafter are protected from apoptotic death, lose the ability to control their cell cycle and gain metastatic ability, a phenotype largely resembling cancer cells.

In *T. parva*-infected cells the IKK-signalosome is recruited to the parasite surface resulting in activation of the NF- κ B pathway (Heussler *et al.*, 2002; Schmuckli-Maurer *et al.*, 2010) and the continuous NF- κ B-mediated transcription of anti-apoptotic genes such as c-FLIP, cIAP and x-IAP (Kuenzi *et al.*, 2003) (Fig. 1). It is not clear how the parasite recruits the signalosome to its surface. Recent data showed that a parasite-derived protein, termed TpSCOP (*T. parva* schizont-derived cytoskeleton-binding protein), increases NF- κ B activity in infected cells suggesting that it might play a role in the formation of the template responsible for IKK-signalosome recruitment (Hayashida *et al.*, 2010).

Additional signalling pathways modulated by *Theileria* contribute to the prolonged survival of the infected lymphocytes. Activation of JNK signalling was shown to promote survival of *Theileria*-infected cells by a signalling pathway that leads to induction of AP-1 (activator protein 1) transcription factor. Blocking AP-1 activation by expression of a c-Jun dominant negative mutant rendered *Theileria*-infected B cells more susceptible to apoptosis due to decreased levels of anti-apoptotic Mcl-1 and cIAP (Lizundia *et al.*, 2006). Activation of PKA (protein kinase A) also protects *Theileria*-infected B cells from apoptosis, as its specific inhibition rendered cells more susceptible to apoptotic death (Guergnon *et al.*, 2006).

Theileria parva-infected B cells present increased levels of the transcription factor c-myc, which seems to be crucial for their anti-apoptotic state, as its specific inhibition resulted in rapid apoptotic death of the cells and decreased levels of Mcl-1 (Dessauge *et al.*, 2005) (Fig. 1). Cells infected by *Theileria* produce GM-CSF, which via an autocrine loop activates the JAK2 (Janus kinase 2)/STAT3 (signal transducers and activators of transcription-3) pathway inducing c-myc expression (Guergnon *et al.*, 2003; Dessauge *et al.*, 2005). Additionally, c-myc phosphorylation by the casein kinase II (CKII) in infected cells was shown to promote stabilization of the transcription factor and further contributing to increased levels of c-myc (Dessauge *et al.*, 2005).

Finally, a recent study showed that in cells infected by *T. annulata*, the p53 protein is sequestered in the cytoplasm in close association with the parasite membrane. Upon parasite elimination, p53 shuttles back to the nucleus resulting in increased transcription of APAF-1 and Bax. Nevertheless, the parasite-derived factor responsible for p53 arrest has not yet been identified (Haller *et al.*, 2010).

Plasmodium spp.

Plasmodium falciparum, the notorious etiological agent of malaria, infects primarily hepatocytes and erythrocytes and much of the pathology associated with malaria is due to severe anaemia as a result of the lysis of infected red blood cells.

After transmission by the *Anopheles* mosquito, *Plasmodium* sporozoites are rapidly transported to the liver, where they translocate through a number of hepatocytes. This process was shown to cause wounding in the traversed hepatocytes resulting in the release of hepatocyte growth factor (HGF) (Carrolo *et al.*, 2003). It has been suggested that HGF binds to the c-Met (c-mesenchymal-epithelial transition factor) on the surface of the hepatocytes, leading to PI3-K activation and protection of hepatocytes from apoptosis (Leiriao *et al.*, 2005) (Fig. 1). While HGF/c-MET signalling pro-

tects hepatocytes from apoptosis in early stages of *Plasmodium* infection, infected cells maintain an anti-apoptotic state but do not require PI3-K signalling, suggesting that yet unidentified mechanisms contribute to conserve the anti-apoptotic state (van de Sand *et al.*, 2005).

At the late liver stage, *Plasmodium berghei* merozoites actively induce a phenotype of cell death in the infected hepatocytes resulting in the release of membrane-bound vesicles filled with merozoites, termed merosomes (Sturm *et al.*, 2006). Notably, dying cells share some of the characteristics of apoptotic cells such as DNA condensation and loss of mitochondrial potential, but lack exposure of PS in the outer leaflet of the plasma membrane. A general cysteine protease inhibitor, but not a broad caspase inhibitor, abrogated the formation of the merosomes (Sturm *et al.*, 2006). Interestingly, *P. berghei* cysteine proteases of the serine repeat antigen (PbSERA) are strongly upregulated in the late liver stages and PbSERA3 translocates to the host cell at this stage, suggesting a crucial role in the induction of hepatocyte death (Schmidt-Christensen *et al.*, 2008). The absence of PS exposure in merozoite-filled merosomes may explain how they escape from the engulfment by the numerous Kupffer cells which survey the liver sinusoids.

During the blood stage of malaria infection, inside the erythrocyte, extensive modifications are induced in the host cell to accommodate *Plasmodium*'s nutrient requirements. Erythrocytes may undergo a type of programmed cell death termed eryptosis that is characterized by cell shrinkage, membrane blebbing and PS exposure and is stimulated by an increase in the cytosolic concentration of Ca^{2+} (Bratosin *et al.*, 2001). Among the alterations induced by *Plasmodium* in erythrocytes is the activation of ion channels in the cell membrane required for of nutrients and maintain osmotic equilibrium. These include Ca^{2+} permeable channels leading to Ca^{2+} entry into the erythrocyte and stimulation of eryptosis limiting the life span of the infected erythrocyte (Foller *et al.*, 2009). It has been shown that *Plasmodium* delays the execution of eryptosis by keeping the concentration of free Ca^{2+} in the cytosol of the erythrocyte low through sequestration (Huber *et al.*, 2005).

Concluding remarks and future perspectives

The last decade of research resulted in considerable advances in our understanding of the molecular mechanisms underlying the modulation of mammalian apoptotic pathways by intracellular parasites (Fig. 1). Apart from a few exceptions, the parasite-derived factors that are directly involved in the activation or deactivation of host cell apoptosis remain largely unknown. Although many studies have been conducted, the relevance of apoptosis modulation remains to be addressed in an *in vivo* context.

To date, few therapeutic strategies aiming to pharmacologically target apoptosis during parasitic infections have been tested *in vivo*. The dual ability showed by many parasites to both inhibit and promote apoptosis in host cells makes it difficult to predict whether this kind of intervention would prevent or exacerbate disease. Clearly, future studies should focus on the *in vivo* mechanisms of apoptosis in the context of these parasitic infections.

Although we restricted our discussion to five prominent protozoan parasites, others are also known to modulate the apoptotic process in the infected cell. The apicomplexan *Cryptosporidium parvum* infects host enterocytes where it resides in a peculiar intracellular but extracytoplasmic PV, sometimes denoted as an epicellular location. Interestingly, *C. parvum*-induced inhibition of host cell apoptosis was shown to depend on the IAP survivin (Liu *et al.*, 2008). This survivin-dependent mechanism of inhibition of host cell apoptosis represents a novel strategy of maintaining host cell viability, previously unappreciated in any other pathogen and that should deserve future attention.

There is paucity in the literature concerning the modulation of other forms of cell death by protozoan parasites besides apoptosis. In recent years great advances have been made in our understanding of the molecular mechanisms behind other types of cell death such as autophagic cell death, necroptosis or pyroptosis. Moreover, due to the role of lysosome destabilization in regulating apoptosis, it cannot be excluded that intracellular parasites do not interfere with LMP. Paradoxically, this point has not yet been addressed despite the fact that it is well known that such intracellular pathogens interfere with lysosome machinery (Jones and Hirsch, 1972; Mauel, 1982; Prina *et al.*, 1990; Andrade and Andrews, 2004).

It was recently shown that the prokaryote *Listeria monocytogenes* transiently disrupts mitochondrial dynamics and function at the onset of infection to interfere with cellular physiology. The interference resulted in increased mitochondrial fragmentation, decreased mitochondrial membrane potential and drop in respiration and cellular ATP. Interestingly, however, the bioenergetics crisis does not lead to apoptosis of the infected cells (Stavru *et al.*, 2011). No report has so far demonstrated the effect of intracellular protozoan parasites on host cell mitochondrial dynamics. The direct or indirect perturbation of mitochondria dynamics may play a crucial role on whether sustained viability of host cell is assured to preserve the pathogen replication niche or on the other hand trigger the apoptotic process to circumvent immune effector cells. It will be crucial to identify the host molecular mechanisms and the pathogen factors involved in such control.

With this knowledge in hand combined with the development of specific tools to study these other modes of cell death, it is expected that the upcoming years will bring

advances into how intracellular protozoa play with distinct cell death modalities.

Acknowledgements

This work was supported by an ANR grant and FCT grant No. PTDC/SAU-FCF/100749/2008. J.E. thanks the Canada Research Chair program for financial assistance. M.L. is supported by a fellowship from ANRS. V.R. is supported by fellowship from FCT code SFRH/BD/64064/2009. R.S. is supported by the FCT program Ciência 2008.

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Review

Impairment of T Cell Function in Parasitic Infections

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Abstract: In mammals subverted as hosts by protozoan parasites, the latter and/or the agonists they release are detected and processed by sensors displayed by many distinct immune cell lineages, in a tissue(s)-dependent context. Focusing on the T lymphocyte lineage, we review our present understanding on its transient or durable functional impairment over the course of the developmental program of the intracellular parasites *Leishmania* spp., *Plasmodium* spp., *Toxoplasma gondii*, and *Trypanosoma cruzi* in their mammalian hosts. Strategies employed by protozoa to down-regulate T lymphocyte function may act at the initial moment of naïve T cell priming, rendering T cells anergic or unresponsive throughout infection, or later, exhausting T cells due to antigen persistence. Furthermore, by exploiting host feedback mechanisms aimed at maintaining immune homeostasis, parasites can enhance T cell apoptosis. We will discuss how infections with prominent intracellular protozoan parasites lead to a general down-regulation of T cell function through T cell anergy and exhaustion, accompanied by apoptosis, and ultimately allowing pathogen persistence.

Introduction

Infections caused by the intracellular protozoa *Leishmania* spp., *Trypanosoma cruzi*, *Plasmodium* spp., and *Toxoplasma gondii* are associated with high morbidity and a heavy economic toll. These unicellular eukaryotes display complex life cycles whose successful completion relies on shuttling between different hosts. Particular selective pressures during host-pathogen coevolution shaped the developmental program of each parasite, giving rise to distinct clinical conditions (Box 1).

Protective immunity against parasitic infection is critically dependent on the development of a multifunctional T cell response that directly kills infected cells or induces phagocyte activation to destroy intracellular parasites [1–3]. As blood or tissue pathogens, their transmissibility to the insect vector or definitive host is low, and thus these pathogens devised strategies to dampen the T cell response and increase the time available for parasite transmission [4].

After breaching epithelial barriers, intracellular protozoa rapidly deploy strategies to resist innate mechanisms employed by infection site-recruited immune cells, such as macrophages or dendritic cells (DCs) [5,6]. These cells are also responsible for the transition between innate immunity and the onset of the adaptive response. As such, inhibiting the signals emanating from antigen-presenting-cells (APCs) represents an ingenious strategy to delay or hamper T cell responses [7], allowing rapid parasite replication and dissemination during the acute stage of infection. Nevertheless, adaptive immunity eventually develops and is generally associated with control of acute parasite infection [8–10]. Yet,

even in the presence of a robust T cell response, complete pathogen eradication is rarely achieved, signalling the onset of chronic infection, which may remain clinically silent throughout the host's life or give rise to complications several years after primary infection. Chronic parasite persistence has a profound impact on the effector capacity of T cells, inducing their gradual loss of function in a phenomenon known as T cell exhaustion [11].

Spanning both acute and chronic stages of infection is the programmed death of T cells, a homeostatic mechanism that ensures the elimination of most specific T cells after clearance of a foreign threat, yet allows the survival of a small number of memory cells capable of long-term, antigen-independent survival [12]. However, by interfering with the apoptotic T cell process, parasites may subvert the mechanisms of memory formation and reduce the numbers of specific T cells available to fight the pathogen in the long term [13].

Here, we review the current understanding of how intracellular protozoan parasites subvert the host T cell immunity during the full length of their developmental program within the mammalian host, through mechanisms that favour the occurrence of T cell anergy, exhaustion, and apoptosis.

Subverting the Signals Required for T Cell Activation Results in Delayed or Anergic T Cell Responses

T cell anergy was initially described as a state of non-responsiveness induced in vitro at the time of T cell stimulation, via T cell receptor (TCR), in the absence of a costimulatory signal [14]. Lack of costimulation results in defective nuclear recruitment of the transcription factors required to initiate *IL2* transcription [7]. In this sense, anergy is usually induced in T cells that bind their cognate peptide-MHC complexes displayed in the surface of

Citation: Rodrigues V, Cordeiro-da-Silva A, Laforge M, Ouaisi A, Akharid K, et al. (2014) Impairment of T Cell Function in Parasitic Infections. *PLoS Negl Trop Dis* 8(2): e2567. doi:10.1371/journal.pntd.0002567

Editor: Mauricio Martins Rodrigues, Federal University of São Paulo, Brazil

Published: February 13, 2014

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Funding: Work supported by an FCT (Fundação para a Ciência e Tecnologia) grant n° PTDC/SAU-FCF/100749/2008. JE thanks the Canada Research Chair program for financial assistance. JE and KA were supported by a Partenariat Hubert Curien (PHC) (program Volubilis, MA/11/262). VR is supported by a doctoral fellowship from FCT code SFRH/BD/64064/2009. RS is supported by the FCT program Ciência 2008. The research has received funding from the European Community's Seventh Framework Programme under grant agreement No. 602773 (Project KINDRED). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Box 1. Developmental Programs of Intracellular Parasitic Protozoa in the Mammalian Host and Associated Clinical Conditions

The kinetoplastids *Leishmania* spp. and *T. cruzi* and the apicomplexans *Plasmodium* spp. all rely on insect vectors for transmission to the mammalian host. After deposition in the dermis through the bites of infected sand flies, *Leishmania* parasites reside inside host phagocytes and, depending on the infecting species, can either cause localized cutaneous lesions (e.g., *L. major*) or visceral leishmaniasis (*L. donovani*, *L. infantum*), a chronic disease characterized by amastigote accumulation in visceral compartments such as the spleen or the liver.

T. cruzi metacyclic trypomastigotes are transmitted by the reduviid bug and cause an acute infection that lasts some weeks and is characterized by systemic infection of multiple host nucleated cells, within which the parasite persists in a cytoplasmic location. Development of adaptive immunity restricts parasite numbers and signals the beginning of chronic infection, which may persist for the life of the host. About two-thirds of the infected patients will never be afflicted by clinical disease during the chronic phase, while the remaining may develop chagasic cardiomyopathy or digestive complications such as megacolon or megaesophagus, usually 10 to 30 years after the initial infection.

Anopheles mosquitoes transmit *Plasmodium* sporozoites to the dermis of the host, initiating a developmental program that starts with parasite migration to the liver. The liver stage of infection is clinically silent but results in remarkable replication of the merozoite form inside hepatocytes. Merozoite egress from hepatocytes and infection of erythrocytes initiates the blood stage of infection and is responsible for the pathological sequelae that are typically associated with malaria, which include acidosis, anaemia, and cerebral malaria.

The apicomplexan *T. gondii* can infect humans through ingestion of undercooked meat containing viable tissue cysts or water contaminated with parasite oocysts. An early acute phase, which usually passes unnoticed or causes mild flu-like symptoms, is characterized by remarkable parasite dissemination in the body due to the virtually unlimited host cell range of the tachyzoite form. Strong pressure posed by adaptive immunity induces parasite differentiation to semidormant bradyzoites that form tissue cysts in the brain and muscle, initiating chronic infection that may last for the life of the individual. Complications arise in the case of acquired immunodeficiency and manifest as toxoplasmic encephalitis.

not fully mature APCs, hence unable to provide adequate costimulation [15]. In alternative, T cell anergy may be induced after expression of immunomodulatory molecules by APCs, including the ATP-catabolizing enzymes CD39 and CD73 or the tryptophan-metabolizing enzyme indoleamine-2,3-dioxygenase (IDO) [16]. These observations link the requirement of an inflammatory and/or immunogenic environment to efficient T cell activation (Table 1).

Even though the infection site and the anatomical location of acute replication differ among the pathogens discussed here (Box 1), intracellular parasites share similar mechanisms to disturb the functions of APCs and preclude effective T cell priming during the acute phase of infection. For instance, in mice and humans, acute

toxoplasmosis is associated with a transient, anergic-like suppression of T cell function [17–19]. In vitro studies unveiled the ability of *T. gondii* to down-modulate antigen presentation and costimulatory molecules in the infected APC [20–22]. Furthermore, a strong association between reduced DC-derived signals and decreased magnitude of the specific CD8 T cell response was observed after infection of mice with tachyzoites of the lethal RH strain, which is capable of remarkable dissemination through host tissues within the first few days after infection [19].

In murine *L. donovani* infection, parasites colonize the liver and quickly replicate, but hepatic infection self-resolves within one month [10]. A recent study found that LIGHT (a TNF superfamily ligand) signalling through the lymphotoxin-beta receptor (LT-βR) was detrimental to the onset of the specific CD4 T cell response, which may explain the rapid increase in hepatic parasite numbers during the first week after inoculation. An antibody therapy blocking LT-βR engagement by LIGHT resulted in increased TNF production by hepatic CD4 T cells, faster granuloma maturation and decreased hepatic parasite loads [23]. In contrast with the liver, the spleen is the site of chronic parasite persistence during murine visceral leishmaniasis. After *L. donovani* infection in mice, the expansion and activation of splenic CD8 T cells is delayed and lower in magnitude, when compared with a prototypical acute infection [24]. A similar delay in the onset of the splenic CD8 response was found during murine *T. cruzi* infection [25].

Increased expression of immunomodulatory molecules in APCs has also been noted in parasite infections [26–28]. Dendritic cells expressing ATP-metabolizing enzymes or IDO emerge in the local lymph nodes shortly after intradermal infection of mice with *L. amazonensis* and *L. major*, respectively. These DCs were poor inducers of T cell proliferation and, importantly, the T cell response could be restored by blocking the adenosine receptor A_{2B} or IDO [26,27]. Interestingly, loss of IDO activity was recently proposed as a biomarker for the restoration of the immune response in treated leishmaniasis patients [29].

The liver stage of *Plasmodium* infection is very silent, both clinically and immunologically, possibly due to a lack of recognition of the intrahepatic merozoite by innate immunity [30]. After deposition in the dermis, most sporozoites do not reach the blood stream but are instead conveyed to the local lymph nodes and digested inside DCs [31]. In mice inoculated with irradiated sporozoites, CD8 T cells, primed by DCs in the skin-draining lymph nodes, are able to migrate to the liver, recognize infected hepatocytes, and provide protection [32]. Similarly, mice given prophylactic chloroquine at the time of live sporozoite inoculation are protected against subsequent challenge [33]. The success of both immunization strategies seems to advent from the development of a robust intrahepatic CD8 T effector/memory response associated with high IFN-γ production [34]. Thus, efficient CD8 T cell priming can occur during *Plasmodium* infections, and it is important to gain further knowledge of the properties of the activated/matured APCs generated with these immunization strategies, with the aim of optimizing vaccine design.

Contrasting with hepatic infection, the blood stage of malaria is noticeably immunogenic and, conversely, immune evasion mechanisms mediated by *Plasmodium* become apparent [30]. Splenic DCs recognize and internalize *Plasmodium*-infected red blood cells (pRBCs) but fail to stimulate T cells [35,36]. This ability correlates with strain lethality [37] and is possibly caused by a systemic DC activation that occurs very early after inoculation (1 to 4 days), before the peak in parasitemia (days 4 to 7). As a consequence, presentation of parasite antigens is short-lived, as activated DCs

Table 1. T cell anergy versus T cell exhaustion.

Unresponsive state	Differentiation state affected	Driving forces	General characteristics of the unresponsive state
T cell anergy	Naïve/Effector	Impaired antigen presentation	Impaired activation and proliferation
		Reduced costimulation	Defective differentiation and effector function
		Expression of immunomodulatory molecules (IDO, CD73, CD39)	Apoptosis
		Regulatory cytokines (IL-10, TGF- β)	
T cell exhaustion	Effector	Antigen persistence, chronic activation	Progressive impairment of effector function
		Regulatory cytokines (IL-10, TGF- β)	Expression of inhibitory receptors (PD-1, TIM-3, LAG-3)
		Suboptimal priming (while in the naïve state)	Decreased expression of common γ chain cytokine receptors
			Apoptosis

doi:10.1371/journal.pntd.0002567.t001

become unable to phagocytose pRBCs, compromising T cell activation [38].

Parasite-derived molecules may directly inhibit T cell activation. Trans-sialidase, a glycoside hydrolase shed by *T. cruzi*, sialylates the surface of activated CD8 T cells, reducing their affinity to cognate peptide-MHC and decreasing their cytotoxicity [39]. Inhibition of trans-sialidase restored the CD8 T cell function and increased mice survival [39]. In addition, AgC10, a *T. cruzi* GPI-anchored mucin, binds to L-selectin in the surface of T cells and, by interfering with the phosphorylation of TCR-associated signal transducers, is capable of blocking *IL2* transcription, rendering T cells anergic during acute infection in mice [40].

Although anergy is viewed as a process regulating the initial phase of the T cell response, the emergence of regulatory T cells (Tregs) expressing immunomodulatory molecules, such as CTLA-4, IDO, or ATP-metabolizing enzymes, during parasite infection may contribute to sustaining T cell anergy during the chronic phase [41–44].

Chronic Parasite Infection Leads to Exhaustion of Specific T Cells

Immune exhaustion corresponds to a loss of effector function of antigen-experienced T cells that occurs in a progressive manner, starting with decreased proliferative ability, IL-2 production, and cytotoxic function, followed by an incapacity to produce IFN- γ and TNF- α , and culminating with physical deletion at terminal stages [11,45]. Exhausted T cells present high and sustained expression of inhibitory molecules such as programmed death-1 (PD-1), T-cell immunoglobulin, and mucin domain-containing protein-3 (TIM-3) and lymphocyte-activated gene-3 (LAG-3) [11] (Table 1).

T cell exhaustion associated with chronic infection was initially reported in viral models as specific CD8 T cells that failed to produce cytokines [46]. Recent work conjectured a similar pattern for chronic parasitic infections [47].

The occurrence of T cell exhaustion in viral models has been classically associated with concomitant high and persistent antigen levels [11]. In contrast, chronic parasite infections are characterized by lower pathogen burden, which is generally tissue-restricted, suggesting alternative driving forces in the induction of T cell exhaustion. For instance, acute *T. gondii* infection is usually controlled by the development of adaptive immunity, leading to parasite encystation and latency (Box 1). Nevertheless, this does not preclude subsequent exhaustion of CD8 T cells.

Indeed, infection of C57Bl/6 mice with *T. gondii* cysts of the ME49 strain causes death in 7 weeks, associated with parasite reactivation in the brain and concurrent with decreased numbers of brain-infiltrating CD8 T cells and their reduced production of IFN- γ and granzyme B, an indication of cellular exhaustion [48]. Increased PD-1 expression accompanied T cell exhaustion, and a treatment blocking the PD-1/PD-L1 pathway resulted in reinvigorated T cell function and prevented animal demise [48,49]. Recent data suggests that the CD40/CD40L axis plays a crucial role in the rescue of exhausted CD8 T cells in the context of α -PD-L1 therapy [1]. Importantly, reinvigoration of the CD8 T cell response through CD40L-CD40 signalling occurred not only in a CD8-intrinsic manner, but also by boosting CD4 helper cell function through induction of increased production of IL-21 [1], a cytokine previously shown to alleviate CD8 T cell exhaustion in viral models [50,51].

In murine *L. donovani* infection, splenic CD8 T cells exhibit exhaustion around 4–5 weeks after inoculation, with reduced production of IFN- γ , TNF, and granzyme B [24]. PD-1 expression in parasite-specific CD8 T cells and PD-L1 expression in splenic DCs paralleled the decrease in T cell function and blocking PD-1/PD-L1 interactions could reduce splenic parasite burden [24]. In cutaneous leishmaniasis caused by *L. mexicana*, expression of PD-1 in peripheral blood CD8 T cells correlates with lesion severity being found in patients with diffuse but not localized lesions [52].

In contrast, parasite-specific CD8 T cells do not undergo functional exhaustion after mice infection with *T. cruzi*. Furthermore, after drug cure, CD8 T cells adopted a central memory phenotype and protected against reinfection [53], a finding at odds with the view that exhausted T cells are dependent on antigen persistence and lost after antigen removal [11]. However, early studies in human patients affected by chronic Chagas disease evidenced a functional impairment of T cells that correlated with severity of cardiac pathology [54–56]. The reason for this dichotomy may lie in the much longer timeframe of human infection (1–2 years in mice versus decades in humans), presumably having a more severe impact on the functionality of T cells.

In line with findings in chronic viral infection [57–59], during parasitic disease, not only CD8 T cells are subjected to functional exhaustion. Parasite-specific splenic CD4 T cells up-regulate PD-1 and LAG-3 and become exhausted by day 30 after mice infection with *P. yoelii*-infected RBCs [60]. Simultaneous blockade of PD-1 and LAG-3 increased the numbers of multifunctional CD4 T cells

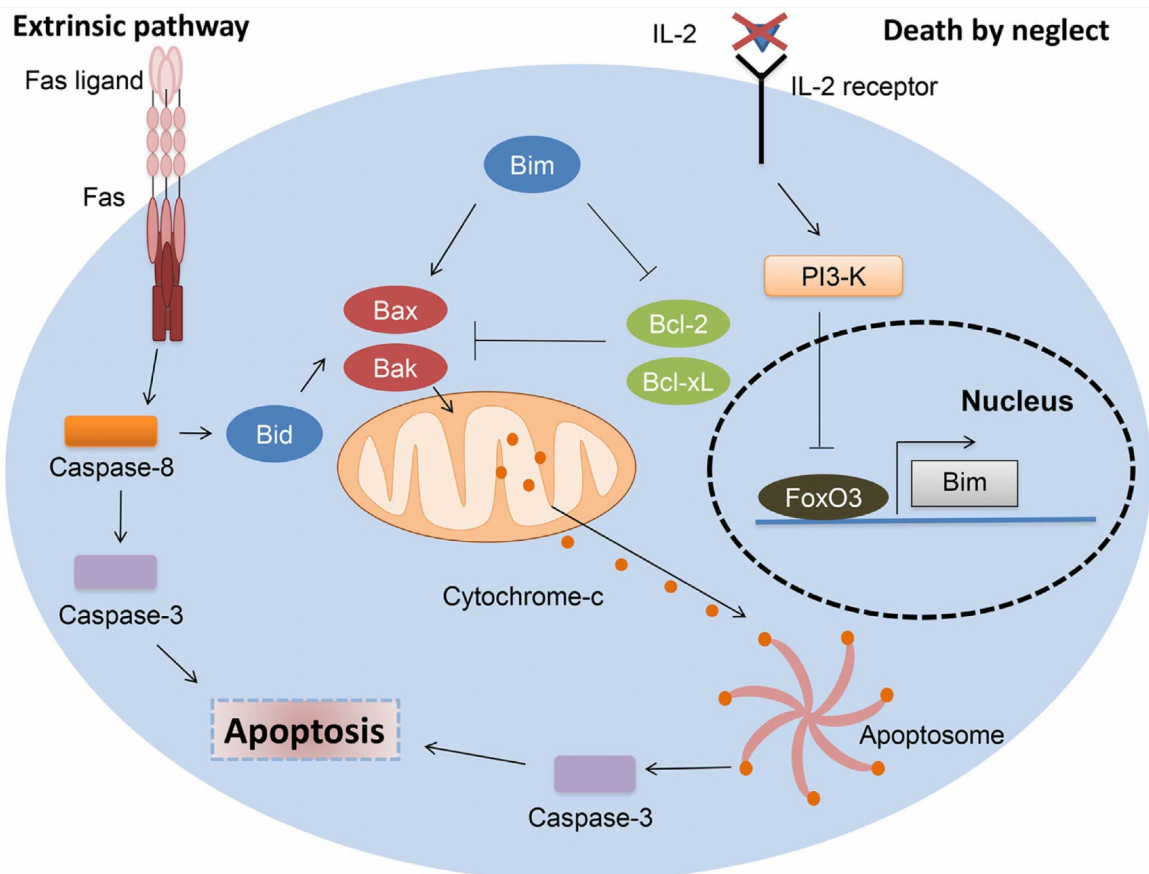


Figure 1. Mechanisms of T cell contraction after immune response resolution. T cell contraction after resolution of an immune response is usually accomplished through a combination of mitochondria- and death receptor-dependent mechanisms. As a result of T cell expansion, survival factors as IL-2 become scarce, and signalling through survival pathways, like the phosphoinositide 3-kinase (PI3-K)/Akt pathway, ceases, allowing FoxO3-dependent Bim induction. Bim promotes mitochondrial outer membrane permeabilization (MOMP) by relieving the inhibitory effect that antiapoptotic Bcl-2 and Bcl-xL exert on proapoptotic Bax and Bak. MOMP results in cytochrome-c release from the mitochondria, enabling activation of a supramolecular complex, the apoptosome that activates caspase-3. By processing numerous cellular substrates, activated caspase-3 ensures completion of the execution phase of apoptosis. T cell activation also induces Fas ligand expression in T cells, which, by engaging the death receptor Fas, enables caspase-8 activation at the death-inducing signalling complex (DISC). Caspase-8 then activates caspase-3. If the levels of caspase-8-activated caspase-3 are not sufficient to undertake apoptotic cell death, a mitochondrial amplification loop may occur through caspase-8-mediated Bid cleavage. This generates tBid, a proapoptotic Bcl-2 family member that promotes MOMP by activating Bax and Bak.

doi:10.1371/journal.pntd.0002567.g001

that produce IFN- γ , TNF, and IL-2 and accelerated parasite clearance [60]. In contrast, blockade of either PD-1 or LAG-3 alone had only modest effects on the recovery of functional CD4 T cells or decrease in parasitemia, suggesting that inhibitory receptors may play independent roles in the induction and/or maintenance of the exhausted state and that combined therapies might be more efficient in improving T cell fitness [61].

Importantly, caution has to be taken when classifying exhausted CD4 T cells based solely on the expression of PD-1. A recently described CD4 T cell subset, termed T follicular helper cells (Tfh), which is essential for B cell-mediated immunity, is characterized by the expression of PD-1 in association with the B cell follicle-homing chemokine receptor, CXCR5. In this context, it is interesting to note that α -PD-L1/ α -LAG-3 therapy dramatically increased *P. yoelli*-specific humoral responses and the numbers of

germinal centre B cells, presumably due to the accompanying increase in the number of Tfh cells. These findings suggest that therapeutic blockade of inhibitory receptors during chronic parasite infection may have beneficial effects that extend beyond the recovery of exhausted T cells. Finally, increased expression of PD-1 and LAG-3 has been recapitulated in T cells from human patients infected with *P. falciparum* [60,62].

It is worth mentioning, however, that despite their deleterious role in sustaining T cell exhaustion during chronic infections, inhibitory receptors protect host tissues during acute infection by dampening potentially pathogenic T cell responses. During acute *T. cruzi* infection in mice, ablating PD-1 signalling augments cardiac inflammation due to increased infiltration of activated CD4 and CD8 T cells [63]. Similarly, blocking the PD1/PD-L1 pathway during *P. berghei* ANKA infection promotes CD8 T cell

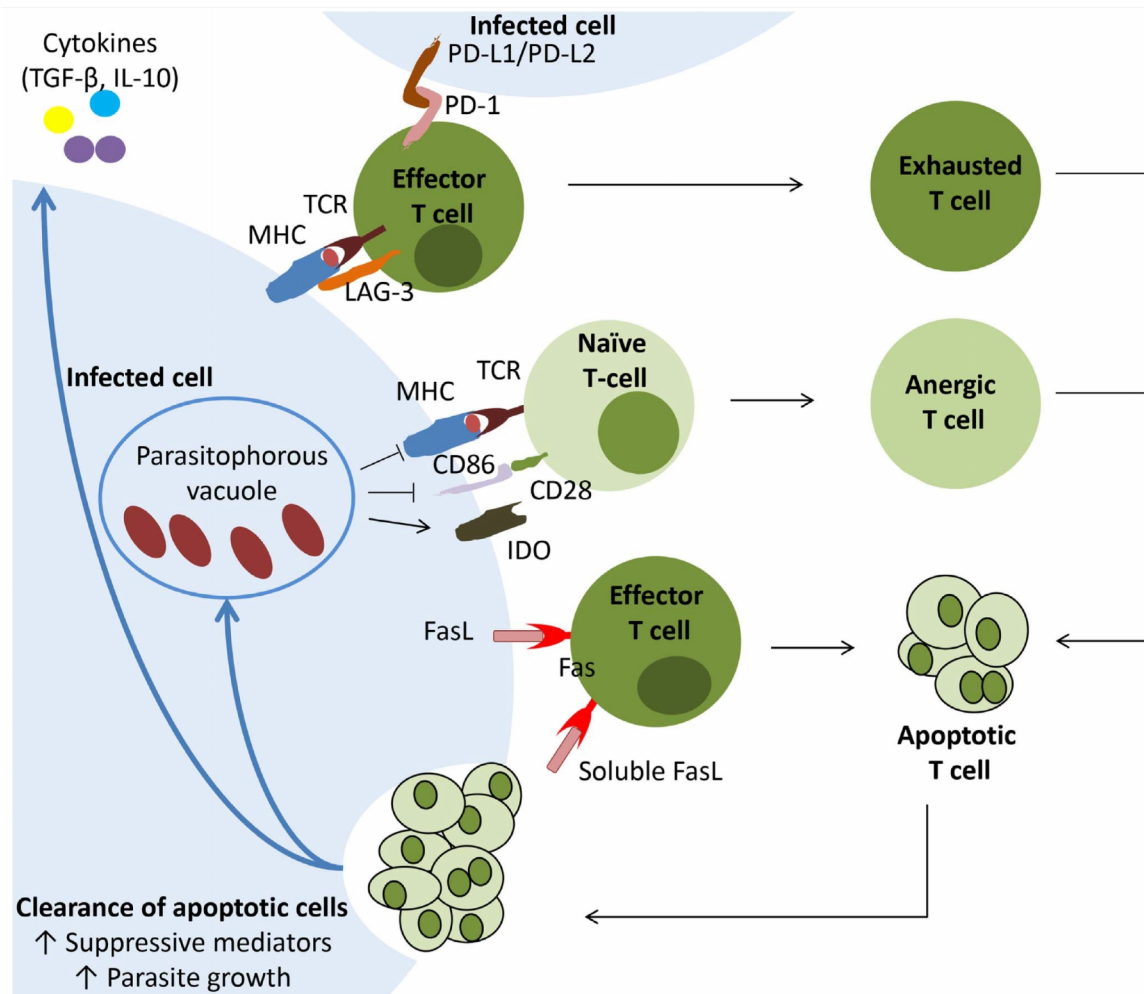


Figure 2. The uptake of apoptotic T lymphocytes by parasite-hosting phagocytes contributes to the remodelling of the parasite-hosting tissue as a bona fide protective niche. Increased rates of T cell apoptosis occur during parasite infection, mediated either by death receptor- or mitochondria-dependent mechanisms. Upon clearance, these apoptotic cells induce an alternative state of activation in phagocytes associated with production of suppressive mediators as TGF-β and IL-10, as well as promoting parasite growth. Suppressive cytokines act on effector T cells and, together with antigen persistence and inhibitory T cell receptors, induce exhaustion of these cells. Additionally, inhibition of antigen presentation and costimulation, acting along with suppressive cytokines or enzymes (as IDO, which catabolizes tryptophan), may render naïve T cells anergic and unresponsive throughout infection. Eventually effector, anergic, or exhausted T cells undergo programmed cell death, fuelling the pool of apoptotic corpses and aiding perpetuation of the suppressive state.
doi:10.1371/journal.pntd.0002567.g002

infiltration in the brain and augments the incidence of experimental cerebral malaria (ECM) in otherwise ECM-resistant Balb/c mice [61]. Thus, signalling through inhibitory receptors appears to be a homeostatic mechanism that regulates effector cell function at the peak of the response.

While T cell exhaustion is usually associated with chronic infection, a recent study in the *L. major* mouse model suggests that the fate of exhausted T cells may be influenced by the events at the acute phase. Infection of otherwise resistant C57Bl/6 mice with arginase-deficient (*arg^{-/-}*) *L. major* causes chronic persistence of cutaneous lesions associated with exhaustion of specific CD4 T cells from the draining lymph nodes. The appearance of exhausted CD4 T cells at the chronic phase appears to be a consequence of the

reduced primary CD4 response after infection with the transgenic/attenuated parasite [64]. Possibly, the curtailed acute response to *arg^{-/-}* parasites precludes effective parasite elimination, which subsequently fosters the exhaustion of effector CD4 T cells due to antigen persistence. Alternatively, it cannot be ruled out that *arg*-deficient parasites are less fit in inducing APC activation and maturation. This would preclude efficient T cell activation and favour anergy. Given that the transcriptional profiles of anergic and exhausted T cells partially overlap [65], it is plausible that a naïve T cell primed under suboptimal conditions is more prone to undergo functional exhaustion at later stages of infection, unveiling a possible relationship between the two states of T cell responsiveness.

Table 2. Impact of targeted inhibition of suppressive or apoptotic T cell pathways in the outcome of parasitic infection.

Suppressive pathway targeted	Parasite	Therapeutic approach	Infection outcome	Reference
T cell anergy	<i>Leishmania donovani</i>	Administration of an anti-LT β R mAb (blocks LIGHT binding to LT β R), started at the day of infection	Increased TNF production by hepatic CD4 T cells and promoted granuloma maturation and parasite clearance in the liver	[23]
	<i>Leishmania major</i>	Administration of an IDO inhibitor, initiated 14 days after infection	Increased CD4 T cell proliferation and reduced footpad swelling and parasite burden	[27]
	<i>Trypanosoma cruzi</i>	Treatment with inactive <i>T. cruzi</i> trans-sialidase, started at the day of infection	Treatment reverted the glycosylation status of CD8 T cells, decreased acute phase parasitemia and promoted mice survival	[39]
T cell exhaustion	<i>Toxoplasma gondii</i>	Anti-PD-L1 therapy, starting at 5 weeks post-infection	Augmented IFN- γ and granzyme B production by CD8 T cells and controlled <i>Toxoplasma</i> recrudescence	[48]
	<i>Leishmania donovani</i>	Anti-PD-L1 therapy, initiated at day 15 after infection	Rescued <i>L. donovani</i> -specific CD8 T cells from exhaustion with increased IFN- γ production and reduced splenic parasite burden	[24]
	<i>Plasmodium yoelii</i>	Anti-PD-L1 and anti-Lag3 therapy, starting at day 14 post-infection	Reinvigorated splenic CD4 and CD8 T cells, improved anti- <i>Plasmodium</i> humoral responses and decreased parasitemia	[60]
T cell apoptosis	<i>Trypanosoma cruzi</i>	Administration of zVAD (pan-caspase inhibitor), initiated at day 7 after infection	Reduced T cell apoptosis, promoted type 1 responses and reduced parasitemia	[77]
		Treatment with zLEHD (caspase-9 inhibitor), starting at 4 days post-infection	Protected mesenteric lymph node T cells from apoptosis and promoted their cytokine production	[78]
		Administration of an anti-FasL, starting at 11 days after infection	Therapy protected T cells from AICD, improved cytokine secretion and decreased parasitemia	[90]
		Therapy with zLETD (caspase-8 inhibitor), initiated 4 days after infection	Treatment inhibited T cell expansion and resulted in increased parasitemia	[95]
	<i>Plasmodium berghei</i>	Anti-IFN- γ treatment, daily, starting the first day after infection	Treatment prevented the deletion of parasite-specific CD4 T cells during acute phase of blood stage infection	[75]
		Anti-IFN- γ and anti-IL-12 treatment initiated 1 day before infection	Treatment promoted differentiation of long-lived memory CD4 T cells and decreased parasitemia	[75]

doi:10.1371/journal.pntd.0002567.t002

Immunoregulatory cytokines may also contribute to down-regulate T cell function by inducing or maintaining the states of exhaustion and anergy in protozoan infections [11,66]. IL-10 impairs monocyte and dendritic cell maturation, inducing their deaths [67], which in turn may affect T cell effector function [68]. In the context of parasite infections, IL-10 can play a protective role by limiting tissue damage, as suggested by the increased mortality observed in *T. gondii* and *P. chabaudi* infections after IL-10 signalling neutralization [69,70], or promote chronicity, as its neutralization during experimental visceral leishmaniasis improved CD4 T cell responses and led to disease resolution [71].

Finally, it is important to envision T cell exhaustion from a host-parasite co-evolution perspective. In this sense, T cell exhaustion may have evolved as a mechanism to avoid the immunopathology that would otherwise result from chronic pathogen persistence. Importantly, despite losing partial effector function, exhausted T cells may still apply some degree of immune

pressure on the pathogen to attain the dynamic equilibrium that characterizes chronic infection [11,72].

T Cell Apoptosis As a Pathological Component of Protozoan Infections

Apoptosis of T lymphocytes during the contraction phase of an immune response occurs through re-stimulation of activated T-cells in a process termed activation-induced cell death (AICD), or results from the absence of survival factors, known as activated T cell autonomous death (ACAD) or death by neglect [12]. AICD is usually accomplished through a death receptor-dependent mechanism. Upon activation, expression of death ligands, such as Fas ligand (FasL, CD95L) or TNF increases in T cells, allowing caspase-8 processing in the death-inducing signalling complex (DISC). In contrast, ACAD is mediated by the relative balance of the Bcl-2 family members [12]. In particular, the increased expression of the pro-apoptotic member Bim in T cells after cytokine deprivation relieves the inhibitory effect anti-apoptotic Bcl-2 and Bcl-xL exert on Bax

and Bak. This results in mitochondrial outer membrane permeabilization (MOMP) and apoptosome formation [12] (Figure 1).

Several studies demonstrate that T cell apoptosis occurs during natural or experimental infections with *Leishmania* spp. [73], *Toxoplasma gondii* [74], *Plasmodium* spp. [75], and *Trypanosoma cruzi* [76]. Furthermore, some studies showed evidence of augmented T cell responses and increased mouse survival after caspase inhibition during protozoan infection [77–79]. However, it is frequently hard to discern from published work whether T cell death is pathological or physiological [13,80]. This is particularly notorious through the acute phase of infection, during which a high turnover of T cells is expected and reflects the emergence and shutdown of the primary response. Yet, even during the early stages, regulation of apoptosis may impact the mechanisms of T cell memory formation and compromise immunity during chronic infection [81]. In this section, we examined the findings that support a pathological component for T cell apoptosis during protozoan infections, proceeding either through a death receptor or mitochondria-dependent mechanism.

Involvement of death receptors. The first hint that death receptor-mediated apoptosis of T cells exerts a negative impact in the immune response during parasite disease came from studies revealing augmented T cell responses in *Fas*, *Faslg* (FasL), or *TNF*-deficient mice after parasite infection [82–86]. These studies, however, also evidenced the importance of death receptor signalling in the clearance of inflammatory infiltrates. Supporting these results is the finding that serum levels of FasL are elevated in patients chronically infected with *P. falciparum* [87], *T. cruzi* [88], and *L. donovani* [89].

Further analysis defined the kinetics of Fas and FasL expression and T lymphocyte apoptotic death during infection. Splenic CD4 and CD8 T cells start to express CD95 around the second to third week after murine *T. cruzi* infection, which correlates with their death by AICD [76,90]. Treatment with an anti-FasL, but not anti-TNF or anti-TRAIL antibodies, could rescue both subsets from apoptosis, improving T cell effector functions and protecting mice from death [76,90].

Importantly, mice vaccinated with an adenoviral vector expressing two *T. cruzi*-dominant epitopes presented improved CD8 T cell functionality and decreased parasitemia after parasite challenge, a phenotype attributed to the lack of CD95 expression in parasite-specific CD8 T cells [76]. Recently, the RIG-I-like receptor LGP2 was shown to repress CD95 expression in activated CD8 T cells in a murine model of West Nile virus infection [91]. While a potential role for LGP2 in parasitic infections remains to be addressed, this data has implications for vaccine design and how it could fine-tune the immune response with the aim to hamper death receptor signalling and improve T cell survival. Also, the decreased levels of parasitemia in immunized and infected animals may explain the lack of CD95 expression in CD8 T cells, due to lower immune activation [76].

Nevertheless, previous work has shown accelerated mice mortality after *T. cruzi* infection in the absence of Fas signalling [92], possibly due to excessive renal inflammation [93] and altered cytokine patterns that favour the expansion of a non-protective Th2 response [90]. Additionally, a recent study has revealed that a polymorphism in the *Fas* promoter is associated with protection in childhood malaria [94]. The protective *Fas* allele was associated with higher expression of CD95 in PBMCs, which was interpreted as facilitating T lymphocyte death and decreased immunopathology. These examples further demonstrate the dual roles played by death receptors, in particular Fas, during infection. On the one hand, death receptor triggering may compromise T cell immunity,

but on the other hand, it prevents the pathogenic accumulation of activated T cell clones and limits tissue pathology.

Finally, some studies aimed to address the magnitude and functional properties of the T cell response after parasite infection of *Casp8* (caspase-8)-deficient mice or in the presence of caspase-8 inhibitors [77,95]. These, however, yielded conflicting results and should be interpreted in view of the known role of caspase-8 in the activation of NF- κ B after TCR triggering [96].

Death by neglect. The limitation of survival factors upon clonal expansion of activated T cells induces Bim expression in T cells, triggering the mitochondrial pathway of apoptosis, in a process known as death by neglect. By partnering with Fas-dependent mechanisms, Bim-mediated apoptosis ensures clearance of most effector T cell clones, yet allows the survival of a minute number of self-sustaining memory T cells [97].

Perhaps the clearest example of how Bim-mediated T cell apoptosis negatively affects the immune response during parasite infection comes from the *L. major* mouse model. While long-term immunity to *L. major* infection in resistant strains is thought to be dependent on the persistence of a small number of parasites in sheltered niches regulated by IL-10-producing effector or regulatory T cells [98,99], sterile cure could be achieved after *Bim* ablation [100]. These mice exhibited increased numbers of parasite-specific CD4 T cells that produced IFN- γ at the infection site and draining lymph node and were protected from reinfection, suggesting again that interfering with T cell death may boost vaccine efficiency.

Recent evidence suggests that heightened expression of inflammatory mediators during the acute stages of infections exacerbates the contraction phase of the immune response, compromising the establishment of T cell memory [81]. During acute blood-stage *Plasmodium* infection in mice, a significant proportion of parasite-specific T cells undergo apoptotic demise. These could be saved by blocking IFN- γ signalling, but not TNF or Fas [75]. In a recent study, a *Plasmodium*-encoded homologue of the macrophage migration inhibitory factor (PMIF) was shown to potentiate the

Five Key Papers in the Field

1. Vasconcelos JR, Bruna-Romero O, Araujo AF, Dominguez MR, Ersching J, et al. (2012) Pathogen-induced proapoptotic phenotype and high CD95 (Fas) expression accompany a suboptimal CD8+ T-cell response: reversal by adenoviral vaccine. *PLoS Pathog* 8: e1002699. doi:10.1371/journal.ppat.1002699
2. Joshi T, Rodriguez S, Perovic V, Cockburn IA, Stager S (2009) B7-H1 blockade increases survival of dysfunctional CD8(+) T cells and confers protection against *Leishmania donovani* infections. *PLoS Pathog* 5: e1000431. doi:10.1371/journal.ppat.1000431
3. Reckling S, Divanovic S, Karp CL, Wojciechowski S, Belkaid Y, et al. (2008) Proapoptotic Bcl-2 family member Bim promotes persistent infection and limits protective immunity. *Infect Immun* 76: 1179–1185.
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Key Learning Points

- The initial encounter between an intracellular protozoan parasite and the host's dedicated antigen-presenting cells is a multilayered interaction that often results in an inability of the latter to efficiently prime naïve antigen-specific T cells. This leads to delayed or anergic T cell responses, providing a window of time for parasite replication, dissemination, and sheltered establishment in the host.
- Chronic parasite persistence, acting along with immunosuppressive factors, profoundly affects the effector function of specific T cells, leading to their progressive loss of cytotoxic or helper activities in a phenomenon known as T cell exhaustion.
- By diverting T cell differentiation towards the formation of terminal effectors, intracellular parasites exacerbate the contraction phase of the T cell response, negatively influencing the establishment of a durable T cell memory and reducing the number of specific T cells available in the long-term.
- A delicate balance between T cell expansion and T cell death has to be attained in order to impose sufficient immune pressure on the parasite while also avoiding the immunopathology resulting from the accumulation of activated T cell clones.

inflammatory response during acute blood-stage infection in mice. As a result, the differentiation of splenic T cells is diverted towards formation of short-lived terminal effector cells that die in a Bim-dependent manner [101]. *PMIF* ablation or IL-12/IFN- γ neutralization instead promoted the differentiation of long-lived memory T cells and ameliorated protection after reinfection [101]. Thus, a large proportion of T cell deaths during protozoan infection might be the result of differentiation of terminal effector T cells. In this sense, parasites exploit a host homeostatic pathway to curtail the magnitude and duration of the T cell response.

Finally, it is important to recognize that this increased rate of apoptotic T cell death is not without immunological consequences. Phagocyte internalization of dying cells suppresses production of inflammatory mediators but, instead, promotes expression of TGF- β and IL-10 [102]. Due to their suppressive effects on APCs functions, apoptotic cells may contribute to induce or maintain anergy and exhaustion in T cells, helping to perpetuate a state of down-regulated T cell function (Figure 2). Furthermore, internal-

ization of apoptotic cells may actually fuel parasite growth inside macrophages, as observed for *T. cruzi* and *L. major* [103,104].

Concluding Remarks and Therapeutic Implications

The role of T cell immunity in controlling protozoan infection is clearly established, as demonstrated by parasite reactivation in the context of T cell immunodeficiency, such as co-infection with HIV. Intracellular parasites engage in complex and long-lasting interactions with the mammalian host during the full length of their developmental programs. Such intricate co-existence provides several targets for therapeutics to intersect the infection and ameliorate T cell function (Table 2).

Nevertheless, some caveats should be highlighted as we set the stage to design future experiments in this area. First, experimental routes of infection do not always reflect accurately the events of a natural infection, particularly those early events associated with parasite establishment. Moreover, in humans, protozoan infection is often silent or less aggressive than mouse models, while also having a longer timeframe. An additional caveat in studies with human patients is the lack of analysis in deep tissues during the chronic phase, as often only peripheral blood is available. All this demands the introduction of alternative models that mimic the natural routes and more closely resemble human infection. An illustrative example comes from rats infected with *T. gondii* that develop a long-lasting chronic infection similar to human infection [105,106]. However, this model remains poorly documented in the literature. Also, non-human primates (NHP) have proven to be faithful models of several human infectious diseases, particularly in AIDS research [107,108]. Unfortunately, the use of NHP models in parasitic infections has been limited to pre-clinical drug or vaccine evaluation or clinical description of the infection [109–111], though recent studies have started to employ these models for more in-depth immunological descriptions [112,113], (VR, ACS, ML, AO, RS, and JE, submitted manuscript).

The mechanisms of impaired T cell function that we reviewed here should be considered as complementary in effecting the immune escape responsible for parasite persistence and disease. In this context, the design of novel immunotherapies, such as therapeutic vaccines, may gain advantage in incorporating strategies that converge to restore immune competence.

Acknowledgments

The authors would like to thank Anne-Marie Anderson, Bernard Krust, and Rui Appelberg for critical reading and contribution to the manuscript.

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DRAM Triggers Lysosomal Membrane Permeabilization and Cell Death in CD4⁺ T Cells Infected with HIV

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Abstract

Productive HIV infection of CD4⁺ T cells leads to a caspase-independent cell death pathway associated with lysosomal membrane permeabilization (LMP) and cathepsin release, resulting in mitochondrial outer membrane permeabilization (MOMP). Herein, we demonstrate that HIV infection induces damage-regulated autophagy modulator (DRAM) expression in a p53-dependent manner. Knocking down the expression of DRAM and p53 genes with specific siRNAs inhibited autophagy and LMP. However, inhibition of Atg5 and Beclin genes that prevents autophagy had a minor effect on LMP and cell death. The knock down of DRAM gene inhibited cytochrome C release, MOMP and cell death. However, knocking down DRAM, we increased viral infection and production. Our study shows for the first time the involvement of DRAM in host-pathogen interactions, which may represent a mechanism of defense via the elimination of infected cells.

Citation: Laforge M, Limou S, Harper F, Casartelli N, Rodrigues V, et al. (2013) DRAM Triggers Lysosomal Membrane Permeabilization and Cell Death in CD4⁺ T Cells Infected with HIV. *PLoS Pathog* 9(5): e1003328. doi:10.1371/journal.ppat.1003328

Editor: Christopher Aiken, Vanderbilt University School of Medicine, United States of America

Received: August 20, 2012; **Accepted:** March 12, 2013; **Published:** May 2, 2013

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Funding: ML was supported by a fellowship from ANRS. RS was supported by Programa Ciência – financed by Programa Operacional Potencial Humano POPH – QREN – Tipologia 4.2 – Promoção do Emprego Científico, co-funded by Fundo Social Europeu and National funding from Ministry of Science, Technology and Higher Education (MCTES). “Fondation pour la Recherche Médicale,” to JE and from CNRS and ARC to FH. VR is supported by fellowship from FCT code SFRH/BD/64064/2009. JE thanks the Canada Research Chair program for financial assistance. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Several proteolytic processes are involved in programmed cell death. Lysosomal membrane permeabilization (LMP) and mitochondrial outer membrane permeabilization (MOMP) have been identified as major events triggering programmed cell death. Thus, in a number of models, lysosomal destabilization plays an early and important role in cell death [1,2]. Lysosomes are acidic organelles that contain numerous acid hydrolases capable of digesting macromolecules of the cell. Upon LMP, the cathepsins are released to the cytosol, where they can initiate the intrinsic apoptotic pathway. This process is mediated in part by the proteolytic activation of the pro-apoptotic molecules, Bid and Bax, resulting in MOMP and cytochrome C release [3]. Thereafter, the release of cytochrome C causes the activation of effector caspases and triggers a caspase-dependent apoptotic pathway. However, lysosome leakage can also induce a caspase-independent non-apoptotic cell death pathway [4]. Thus, lysosomal hydrolases and proteases are acting as initiators and effectors of programmed cell death. CD4⁺ T cells productively infected with HIV-1 die through a caspase-independent death pathway [5,6,7,8,9]. Treatment of productively infected CD4⁺ T cells with the reverse transcriptase inhibitor DDI prevents programmed cell death [5,7,10]. The death of HIV-infected CD4⁺ T cells is associated with the limited permeabilization of lysosomes and lysosomal efflux of cathepsins to

the cytosol [10]. Cathepsin D induces conformational change of Bax and its insertion into the OMM promotes the release of cytochrome C [10]. LMP is induced by both the X4 and R5 laboratory strains and by HIV-1 isolates from infected patients. Thus, the permeabilization of lysosomes precedes that of mitochondria and represents an early commitment to cell death in HIV-infected CD4⁺ T cells [10].

Activation of the tumor suppressor p53 can trigger a primary lysosomal destabilization [11,12]. Induction of its proapoptotic target genes in virally infected cells has been considered as an altruistic suicide mechanism that limits viral infection. Thus, many viruses, including simian virus 40 (SV40), human papilloma virus (HPV) and adenoviruses (Ad), have evolved mechanisms to prevent p53 responses [13], whereas active p53 was detected with several other types of viruses, such as vesicular stomatitis virus (VSV), Newcastle disease virus (NDV) [14] and human immunodeficiency virus (HIV) [15]. However, the mechanism by which p53 mediates LMP is so far unknown.

Lysosomes are also important by their ability to regulate the terminal steps of autophagy [16,17,18]. Autophagy is an evolutionarily conserved process first defined genetically in yeast [19,20]. The primary function of autophagy in most cell types is thought to be an adaptive response to starvation, and is essential for cell survival by degrading proteins and organelles damaged during oxidative stress. In some cellular settings, it can serve as a

Author Summary

Lysosomes are acidic organelles capable of digesting macromolecules and regulating autophagy. In the context of host-pathogen interactions, productive viral infections are associated with lysosome membrane permeabilization (LMP) and programmed cell death (PCD). At a molecular level, the tumor suppressor protein 53 (p53), which is a key player in the detection of DNA damage, acts also as a sensor of pathogen replication. Activation of p53 has been considered to be an altruistic cell suicide mechanism that limits viral infection. Here, we provide new evidence that damage-regulated autophagy modulator (DRAM), a p53 target gene, regulates both LMP and PCD of HIV-infected CD4⁺ T cells. Whereas the inhibition of DRAM or p53 prevents autophagy in infected cells, the inhibition of the autophagy machinery has a minor role in this context. As a consequence, the silencing of DRAM leads to increased HIV viral infection. This is the first report describing the role of DRAM in the context of host-pathogen interaction. Whereas it is to the advantage of the pathogens to preserve their hosts and thus facilitate their multiplication and dissemination, hosts have developed altruistic cellular processes to defend themselves and limit the spread of the infectious agent in multicellular organisms. We propose that the ancestral DRAM protein represents a mechanism of self-defense, inducing elimination of infected cells through LMP.

cell death pathway by itself [21] or in collaboration with apoptosis [22], although its role in this regard is still debated. The cross-talk between apoptosis and autophagy is therefore quite complex, and sometimes contradictory. It has been suggested that autophagy is involved in HIV viral replication [23], in the death of uninfected CD4⁺ T cells following the interaction of the HIV envelope glycoprotein and its co-receptor CXCR4 [24]. On the opposite, HIV infection inhibits autophagy in macrophages, dendritic cells [25,26,27] or even in CD4⁺ T cell lines [28].

The damage-regulated autophagy modulator (DRAM), a lysosomal protein, has been reported to link p53 to autophagy [29]. It shows a high degree of conservation throughout evolution. Interestingly, its expression was reported to be lower in certain tumors, suggesting it plays a role in human pathology. However, whether DRAM links p53 and LMP in the context of host-pathogens relationships has never been addressed, to the best of our knowledge.

Our results highlight the major role played by DRAM in the regulation of LMP and autophagy in HIV-infected CD4⁺ T cells downstream from p53 activation. A specific siRNA blocking DRAM protein expression inhibited LMP and prevented the death of CD4⁺ T cells, which leads to a higher number of infected cells. We propose that the ancestral DRAM protein represents a mechanism of self-defense involved in the elimination of microbe-infected cells and constitutes a critical aspect of antiviral immunity.

Results

a) HIV-1 infection increases DRAM expression

Based on our previous results [7,10], we infected purified primary CD4⁺ T cells with HIV-1_{LAI} for 12 h at a MOI of 0.01, then activated the cells with ConA and IL-2. The number of infected cells (intracellularly stained with anti-HIV-Gag antibody) peaked five days after infection (**Figure S1A, B**), and a concomitant increase in lysosomal destabilization, as demonstrated

by the release of cathepsin D (B and L, not shown), was observed (**Figure S1C**). Thus, lysosomes are rapidly permeabilized in primary CD4⁺ T lymphocytes infected with HIV-1, resulting in the release of cathepsins into the cytosol [10].

We then assessed the expression of DRAM in primary CD4⁺ T cells infected with HIV-1. We detected more DRAM protein at 31 kDa by western blots at day five in HIV-infected CD4⁺ T cells than in non-infected cells (**Figure 1A, B**). Consistently with previous results [15,30], we found by western blot that p53 in HIV-1-infected primary CD4⁺ T cells is phosphorylated on serine 15 (P-p53) and increases concomitantly with the increase of viral replication (**Figure S1D**). By cell fractionation, we found an accumulation of p53 within the nucleus (**Figure S1E**). Fluorescent microscopic analysis of P-p53 showed that the percentages of CD4⁺ T cells expressing P-p53 increase (**Figure S1F**) and P-p53 occurred in HIV-infected primary CD4⁺ T cells (Gag⁺) but not in non-infected cells (Gag⁻) (**Figure S1G**). More than 80% of the CD4⁺ T cells expressing P-p53 were positive for Gag antigen. We then assessed mRNA levels of DRAM in HIV-infected primary CD4⁺ T cells versus non-infected cells. Quantitative gene expression analysis with real-time RT-PCR showed that DRAM expression was increased on days 4 and 5 post-infection in HIV-infected CD4⁺ T cells (**Figure 1C**). We also found an up-regulation of the mRNA levels of p53-inducible genes such as Bax, p21, and HDM2 (data not shown). Altogether, these results indicate that HIV infection in primary CD4⁺ T cells causes p53 transcriptional activation associated with increased expression of DRAM.

In order to confirm the link between p53 activation and DRAM expression, we used specific siRNAs targeting DRAM and p53 mRNA. As expected, the inhibition of DRAM protein production had no effect on p53 expression, whereas the siRNA targeting p53 resulted in lower levels of both the p53 and DRAM proteins in HIV-infected primary CD4⁺ T cells (**Figure 1D, E and F**). Interestingly, DRAM (in red) forms aggregates in Gag⁺ cells (in green) (**Figure 1F**). Confocal microscopy revealed the colocalization of DRAM and the lysosome-associated membrane glycoprotein 2 (LAMP2) that confirms its lysosomal localization (**Figure S2**). Quantification of DRAM and LAMP2 expression using ImageJ software indicated that the level of DRAM is increased in HIV-infected CD4⁺ T cells but not of LAMP2. Altogether, our results demonstrated for the first time that HIV-1 induces DRAM expression, which is linked to p53.

b) DRAM triggers autophagy in HIV-1-infected CD4⁺ T cells

Next, we examined autophagy-related ultrastructures in CD4⁺ T infected by HIV. Electron microscopic analyses of HIV-infected primary CD4⁺ T cells with budding HIV viruses (arrow heads) showed that cells contained large numbers of vacuoles with double-membrane structures (arrows) (**Figure S3A, B**). These vacuoles were not observed in non-infected cells (**Figure S3, panel Aa**). The presence of cytoplasmic material within these double-membrane structures represents autophagosomes (large arrows), the first autophagic-related structures to be produced (**Figure S3, panel Ca, b**). We also observed lysosomes located near autophagosomes (**Figure S3, panel Cb**). Microphotographs also show autophagolysosomes (dashed arrows) resulting from the fusion of lysosomes with autophagosomes associated with degradation of the sequestered content, in HIV-infected CD4⁺ T cells (**Figure S3, panel Cc**). In the latter, autophagolysosomes were more frequently detectable than autophagosome structures (**Figure S3D**). Moreover, in CD4⁺ T cells, in contrary to the situation reported for differentiated macrophages [25] or monocytes

DRAM and HIV-1 Infection

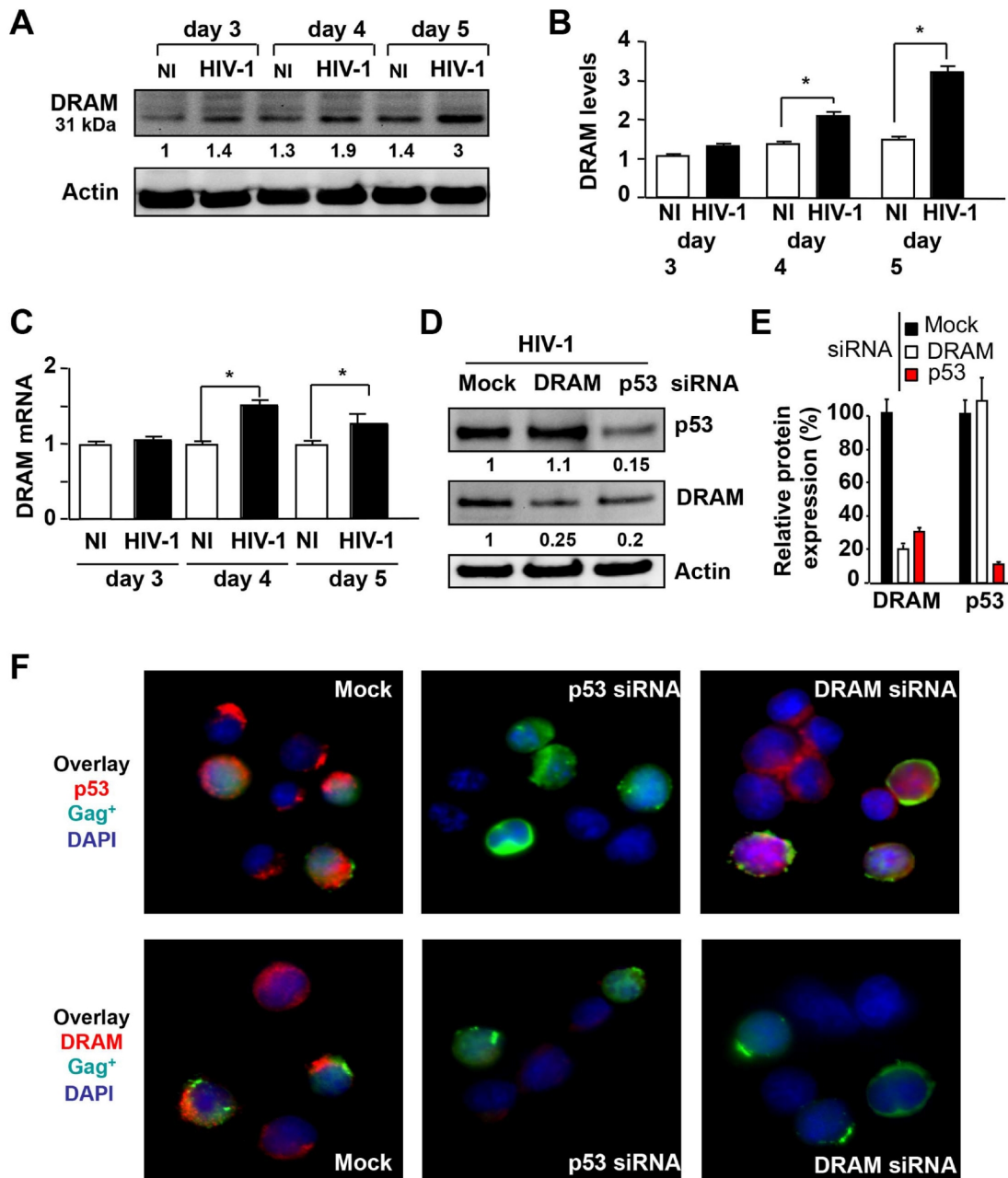


Figure 1. HIV-1 infection induces DRAM expression in CD4⁺ T cells. Primary CD4⁺ T cells in the absence (NI) or presence of HIV-1_{LAI} (HIV-1) have been analyzed. **(A)** Lysates of CD4⁺ T cells were prepared on days 3, 4 and 5 post-infection and separated by western blots, and then immunoblotted with specific antibody against DRAM. Actin was used as a control for protein loading. **(B)** DRAM protein levels were quantified in three independent experiments. Non-infected cells on day 3 were considered arbitrarily equivalent to 1 for normalization. Bars show the mean $\pm$ SD. The asterisk (*) indicates statistically significant difference, $p < 0.05$. **(C)** DRAM mRNA level was quantified by RT-PCR on days 3, 4 and 5 post-infection. Non-infected cells were considered arbitrarily equivalent to 1. Bars show the mean $\pm$ SD of three independent experiments. The asterisk (*) indicates statistically significant difference, $p < 0.05$. **(D)** HIV-infected CD4⁺ T lymphocytes were transfected with either control siRNA (mock) or siRNAs specific for p53 or DRAM. Cells extracts at day 5 post-transfection were analyzed and immunoblotted with specific p53 and DRAM antibodies. Actin was used as a control of loading. **(E)** Bars show the mean $\pm$ SD of four independent experiments. **(F)** Cells were stained with antibodies against p53 or DRAM (in red) or with an antibody against Gag (in green). Nuclei were counterstained with DAPI (blue). doi:10.1371/journal.ppat.1003328.g001

infected with HIV at day 5 (**Figure S3E**), we detected no viral particles in autophagosome structures.

The identification of several genes encoding proteins responsible for the execution of autophagy has facilitated the detection and manipulation of the autophagy pathway (see reviews [16,18]. LC3 is a protein marker reliably associated with autophagosomes during autophagy [31] although its localization in phagosomes after TLR stimulation has also been reported [32]. We investigated LC3 status, to identify the machinery involved in autophagy at the biochemical level. Detection of both LC3-I and LC3-II revealed that the ratio of LC3-II/LC3-I was higher in HIV-infected CD4⁺ T cells than in non-infected CD4⁺ T cells (**Figure 2A**). In order to examine the relationship between Gag⁺ target cells and LC3 proteins, we analyzed the formation of LC3 puncta by confocal microscopy. Consistent with immunoblotting results, larger numbers of CD4⁺ T cells with punctate LC3 were found in HIV-infected cultures (Gag⁺)

than in non-infected ones (**Figure 2B, C**). Moreover, we compared LC3 staining in uninfected bystander cells (Gag⁻) and HIV-1 infected CD4⁺ T cells (Gag⁺, green) found within infected samples five days after infection and found that the formation of LC3 puncta was mostly confined to Gag⁺ target cells (**Figure 2D**).

We next analyzed the early events associated with the autophagy pathway. The initiation of autophagy involves a complex of Beclin 1 and PIK3C3, whereas Atg5 (30 kDa) is required for autophagosome precursor synthesis. Atg5 forms a complex with Atg12 (the Atg5-Atg12 complex), which has a molecular weight of 64 kDa and participates in the autophagosome membrane elongation. Consistent with the accumulation of LC3-II in HIV-infected CD4⁺ T cells, on day 5 post-infection, Beclin 1 protein level was higher in HIV-infected CD4⁺ T cells than in uninfected CD4⁺ T cells (**Figure 3A, B**). We also found that the expression of Beclin mRNA is significantly increased on

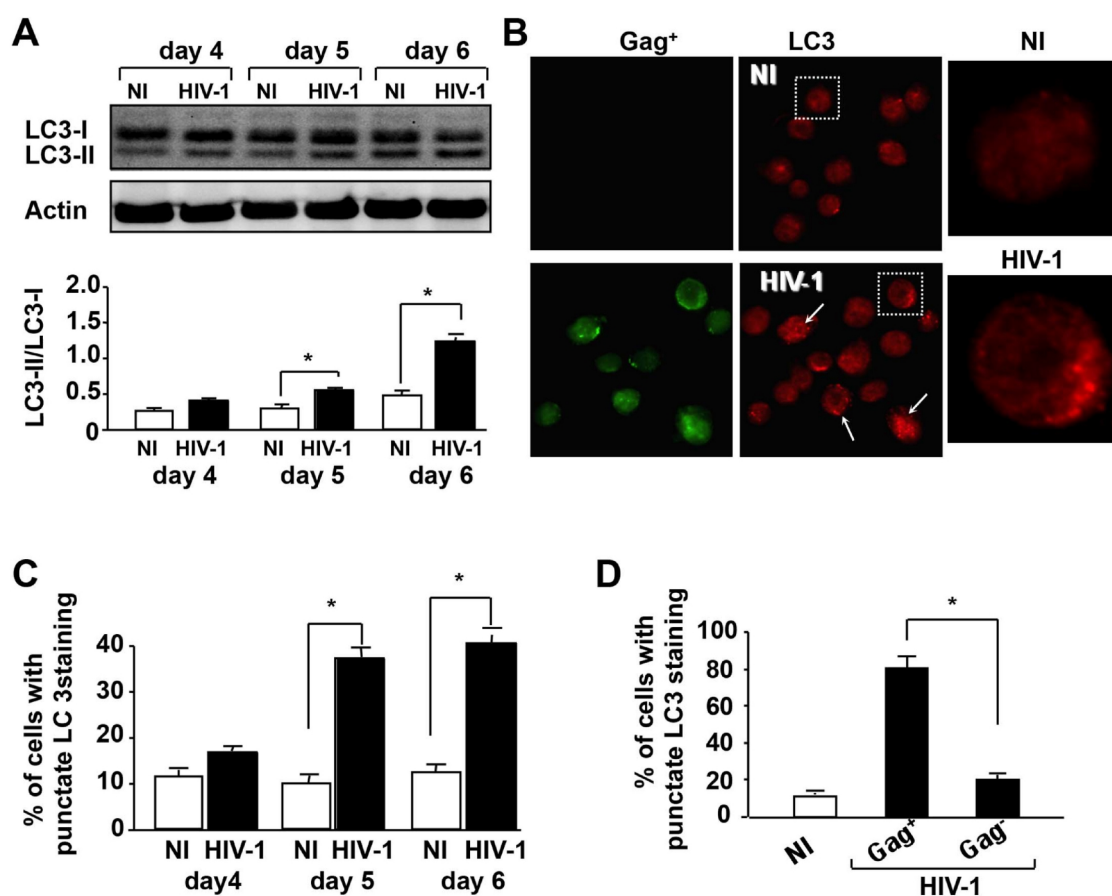


Figure 2. HIV-1 infection induces autophagic machinery in CD4⁺ T cells. (A) Western-blot analysis of LC3-I and LC3-II proteins in CD4⁺ T cells in the absence (NI) or presence of HIV-1_{1AI} (HIV-1) on days 4, 5 and 6 post-infection. Actin was used as a control for protein loading. Ratio of LC3-II/LC3-I was calculated and bars show the mean $\pm$ SD of four independent experiments; *, $p < 0.05$. (B) Cells were stained on day 5 with mAbs recognizing the LC3 antigen (red) and the Gag antigen (green), and examined by confocal microscopy. The insets show magnified images of LC3-II puncta in infected cells. (C) Percentages of CD4⁺ T cells displaying punctate LC3-II staining on days 4, 5 and 6 post-infection. Histograms show the means $\pm$ SD of five individual experiments; *, $p < 0.05$. More than 200 cells were counted for each condition. (D) Within infected samples, quantification of Gag⁺ and Gag⁻ cells with punctate LC3-II accumulation. More than 200 cells were counted for each staining, 5 days post-infection, and the results presented are means $\pm$ SD of five independent experiments; *, $p < 0.05$. doi:10.1371/journal.ppat.1003328.g002

day 5 post-infection (**Figure 3C**), whereas the expression of DRAM mRNA increases at day 4 (**Figure 1C**). Moreover, the Atg5 protein level was higher in HIV-infected CD4⁺ T cells than in non-infected ones, and a band at 64 kDa band corresponding to the Atg5/Atg12 complex was detected (**Figure 3A, B**). Using small interfering RNAs (siRNAs) directed against the mRNAs for BECLIN1 and ATG5, LC3-II protein levels were lower, as expected, in HIV-1-infected CD4⁺ T cells than in control siRNA (mock)-transfected cells, as shown by western blotting and fluorescence microscopy (**Figure S4A, B**). Altogether, these results support the hypothesis that HIV infection of primary CD4⁺ T cells induces autophagy.

It has been reported that depletion of LAMP2 by siRNA leads to the accumulation of autophagic vacuoles [33,34]. Thus, the accumulation of autophagic vacuoles in HIV-infected CD4⁺ T cells could be the consequence of LAMP depletion. However, confocal microscopy of LAMP2 in HIV-infected cells revealed no major difference with uninfected CD4⁺ T cells (**Figure S2**). Now, the appearance of autophagosomes can result from an increase in their induction or, since virtually all cells have a basal autophagic rate, from a block of their turnover. We used Pepstatin A (PA) to inhibit lysosomal proteases in order to influence the lysosome-autophagy pathway and block the flux of autophagy. We detected increased protein levels of LC3-II in HIV-infected primary CD4⁺ T cells treated with PA (**Figure 3D**). One of the best-characterized substrates of selective autophagy is p62 (sequestosome 1/SQSTM1). The p62 protein binds to the autophagy regulator Atg8/LC3 and is incorporated into the autophagosome. Lysosomal degradation of autophagosomes leads to a decrease in p62 levels during autophagy [35,36]; conversely, impairment of autophagy is accompanied by the accumulation of p62 [37]. Thus, we measured the expression of p62 as a marker of the terminal step in the autophagy pathway. We found that the amount of p62 decreased in HIV-infected CD4⁺ T cells (**Figure 3E**). These results show that autophagy results more from induction of autophagosomes rather than from reduced autophagy flux in HIV-infected CD4⁺ T cells.

Finally, we investigated whether knocking down DRAM and p53 expressions inhibits autophagy in HIV-infected CD4⁺ T cells. Immunoblotting showed that the inhibition of DRAM and p53 protein production decreased the formation of LC3-II (**Figure 3F**). Moreover, staining for LC3 remained punctuate in Gag⁺ CD4⁺ T cells treated with control siRNA (mock), whereas lower levels of puncta were displayed after treatment with siRNA targeting DRAM or p53 (**Figure 3G**). Altogether these results demonstrated that HIV infection of primary CD4⁺ T cells mediates autophagy in a DRAM/p53 dependent pathway.

c) DRAM triggers lysosomal destabilization in HIV-1-infected CD4⁺ T cells

Because we have previously showed that HIV mediates LMP, we investigated whether the knockdown of DRAM and p53 affected LMP. First, to monitor lysosomal destabilisation, CD4⁺ T cells isolated from HIV-1-infected cultures at day 5 were loaded with FITC-conjugated dextran of 40-kDa, and after a 2-h chase, the cells were visualized by laser scanning confocal microscopy (**Figure 4A, B**). The redistribution of the FITC-dextran molecules was observed in Gag⁺ cells (diffuse staining), but not in Gag⁻ cells (**Figure 4A**). Thus, a diffuse staining pattern for the 40-kDa FITC-dextran molecules was observed in 60% of the Gag⁺ cells (≥ 200 cells were examined) (**Figure 4B**), which is indicative of lysosomal efflux. By inhibiting the expression of DRAM and p53 using siRNAs, the 40-kDa molecules were almost totally confined to punctate structures, consistent with exclusive lysosomal

localization (**Figure 4A, B**). We then assessed by confocal microscopy whether the inhibition of p53 and DRAM prevented cathepsin D release in the cytosol. By modifying the levels of p53 and DRAM proteins with specific siRNAs, we showed a significant decrease of cathepsin D release in cytosol in Gag⁺ target cells (**Figure 5A, B**). Digitonin-based subcellular fractionation and immunoblot analysis was used to visualize cathepsin D release. Our results showed higher amount of cathepsin D in the cytosol of HIV-1 CD4⁺ T cells in comparison to uninfected cells. In presence of specific siRNAs against p53 and DRAM, cathepsin D release is reduced (**Figure 5C**). Anti-Lamp-1 was used to verify the absence of lysosomal contamination in the cytosolic fraction.

We previously reported that cathepsin D release from lysosomes is an early event resulting in mitochondrial destabilization and cytochrome C release in HIV-1-infected CD4⁺ T cells [10]. This led us to investigate whether the knockdown of DRAM and p53 affected MOMP. Our results clearly show that siRNAs specific for DRAM and p53 inhibited cytochrome C release in Gag⁺ as assessed by fluorescence microscopy (**Figure 5A, B**) and by immunoblot (**Figure 5C**). Moreover, MOMP ($\Delta\Psi_m$) and cell death (PI^+) assessed by flow cytometry using DiOC₆ probe (**Figure 6A, B**) and using propidium iodide (**Figure 6C, D**), respectively, confirms a preventive effect of specific siRNA for DRAM and p53. We also evaluated whether the inhibition the Beclin 1 and Atg5 gene products impacts on LMP. In fact, as demonstrated by the diffuse staining of cathepsin D in Gag⁺ cells, inhibition of BECLIN1 and ATG5 expression by specific siRNAs had no major effect on LMP (**Figure S5A, B**) and cell death (**Figure S5C**). Because it has been proposed that HIV mediates death of bystander CD4⁺ T cells through autophagy [24], we incubated quiescent CD4⁺ T cells in the presence of HIV and dIL. At days 4 and 5, we analyzed mitochondrial depolarization ($\Delta\Psi_m$), cell death (PI^+) and viral replication (Gag detection) by flow cytometry (**Figure S6**). In the absence of viral replication (less than 2% of the CD4⁺ T cells are Gag⁺) the cells do not undergo death [7,10]. We also demonstrated by western blot the absence of DRAM augmentation and the absence of LC3-II in bystander cells in contrast to what happen in productively infected CD4⁺ T cells (**Figure S6**).

HIV-1 accessory proteins such as the viral protein R (Vpr) or nef have been associated with T cell death. Thus, Vpr causes G2 arrest and apoptosis in cycling cells [38,39]. Therefore, we assessed whether Vpr modulated LMP. We infected purified CD4⁺ T cells with either wild type or a Vpr-defective NL4-3 virus. Our results showed that the release of cathepsin D and cytochrome C is similar in the presence or absence of Vpr, and that inhibition of DRAM using specific siRNA prevents LMP (cathepsin D release, **Figure S7B, C**) and MOMP (cytochrome c release, **Figure S7B**). Thus, Vpr encoded by HIV is dispensable to trigger LMP in primary CD4⁺ T cells. Another viral protein, Nef, induces in CD4⁺ T lymphocytes the accumulation of lysosomes [40] and a limited lysosomal permeabilization [10]. Therefore, we assessed whether the inhibition of DRAM and p53 prevents Nef-mediated LMP and MOMP [10]. Whereas overexpression of Nef induced cathepsin D and cytochrome C releases, in contrast to Nef antisense (**Figure S8A, B**), siRNA against DRAM or p53 did not impact LMP (**Figure S8A, B**) and MOMP (**Figure S8C**) mediated by Nef. However, we cannot exclude that residual DRAM is enough to induce LMP.

Because LMP is upstream from MOMP and cell death, we evaluated the consequence of DRAM inhibition on viral infection. Thus, we evaluated the numbers of infected cells (Gag⁺) in the culture as previously described [10]. The siRNAs targeting p53 and DRAM clearly resulted in the presence of greater number of

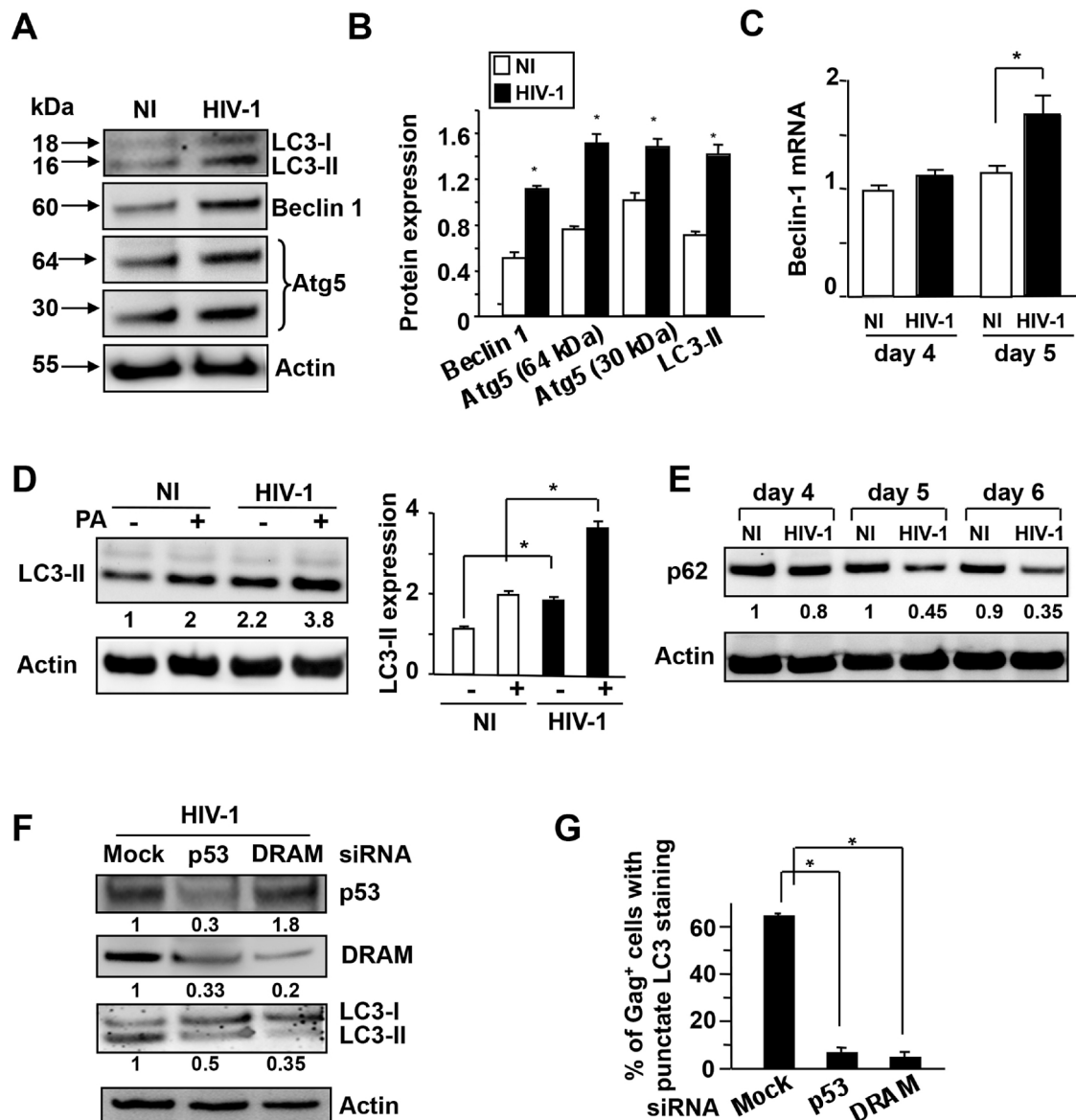


Figure 3. DRAM regulates autophagy in CD4⁺ T cells infected with HIV-1. (A) Immunoblots of lysates from CD4⁺ T cells in the absence (NI) or presence of HIV-1_{LAI} (HIV-1) at day 5 after infection. Membranes were probed for LC3, beclin 1, Atg5, and actin. (B) The amount of each protein was measured and normalized with respect to the loading control (Actin) (relative expression). Bars show the mean $\pm$ SD of five independent experiments. *, $p < 0.05$. (C) Beclin 1 mRNA level was quantified by RT-PCR on days 4 and 5 post-infection. Non-infected cells (NI) on day 4 were considered arbitrarily equivalent to 1 for normalization. Bars show the mean $\pm$ SD of three independent experiments (*, $p < 0.05$). (D) Samples from uninfected (NI) and HIV-infected CD4⁺ T cells incubated in the absence or presence of Pepstatin A (PA). At day 5 the cells were dissolved in SDS. Membranes were probed for LC3-II and actin. Bars show the mean $\pm$ SD of three independent experiments; *, $p < 0.05$. (E) Western-blot analysis of p62 protein in CD4⁺ T cells in the absence (NI) or presence of HIV-1_{LAI} (HIV-1) on days 4, 5 and 6 post-infection. Actin was used as a control for protein loading. The level of p62 was calculated, and the levels in non-infected cells (NI) on day 4 were considered arbitrarily equivalent to 1. (F) CD4⁺ T cells were transfected with siRNA specific for p53 and DRAM or the control siRNA (mock) and then infected with HIV-1. On day 5 post-infection, cells were analyzed by immunoblotting using LC-3 antibody. Actin was a control of loading. Values represent LC3-II/LC3-I ratio. (G) LC3-II staining (number of puncta per cell ≥ 6) was examined by fluorescence microscopy in Gag⁺ target cells. The values shown are means $\pm$ SD of three independent experiments (≥ 200 cells were examined per experiment).

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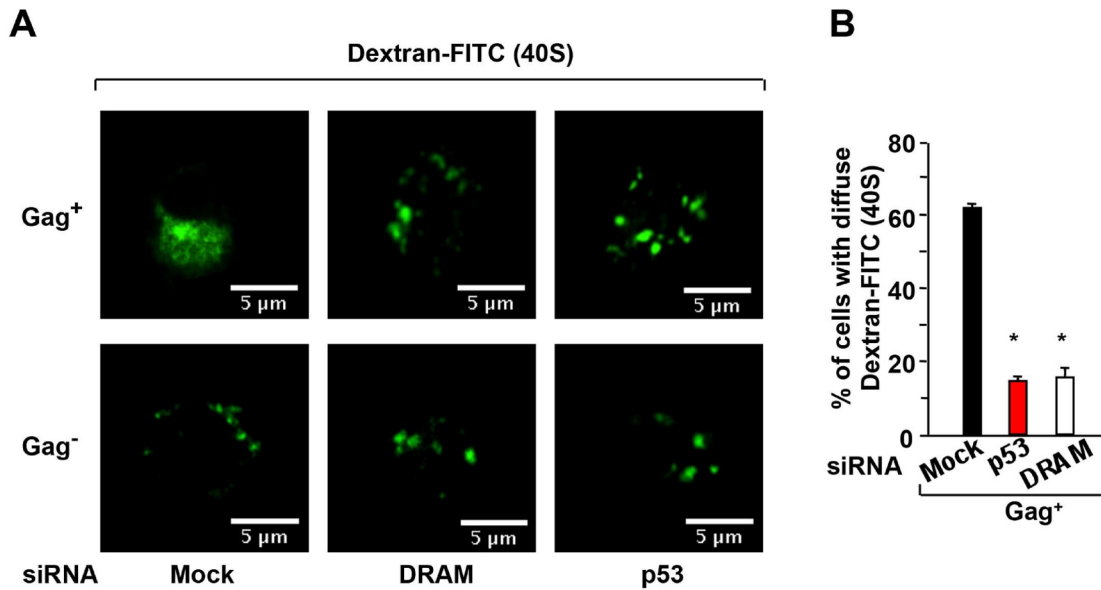


Figure 4. DRAM induces lysosomal membrane permeabilization (LMP) in HIV-infected CD4 T cells. CD4⁺ T cells were transfected with siRNA specific for p53 and DRAM or the control siRNA (mock). (A) Cells were loaded with fluorescent dextran molecules of 40 kDa and examined by laser scanning confocal microscopy. Pictures show gag⁺ and gag⁻ cells from infected samples. Diffuse staining revealed lysosomal permeabilization. (B) The values shown are means $\pm$ SD of two independent experiments (≥ 200 cells were examined). doi:10.1371/journal.ppat.1003328.g004

HIV-infected CD4⁺ T cells than control siRNA (mock) (Figure 6E). This is consistent with the levels of HIV DNA measured (data not shown). Thus, the knock-down of DRAM and p53 protein levels by specific siRNAs resulted in numbers of infected cells three to four times greater than the control. We also measured by ELISA in the supernatants the p24 production in the presence or absence of DRAM. Our results revealed that viral production is increased following DRAM knock-down as compared to the control (Figure 6F).

Taken together our results demonstrate the major role for DRAM in regulating LMP and cell death in HIV-infected cells.

Discussion

Many different viruses induce lysosomal damage and kill the cells in which they replicate [41,42,43,44]. Our results provide new insight in virus-cell interaction, showing a clear relationship between p53 activation, DRAM and LMP in a model of viral replication-mediated cell death. The data presented here demonstrate for the first time that DRAM controls LMP; the depletion of p53 and DRAM indeed prevents LMP and cell death in HIV-infected CD4⁺ T cells. Thus, this provides a rationale for the previous observation that the tumor suppressor p53 can trigger a lysosomal destabilization that contributes to cell death [11,12].

These results reinforce the concept that productive HIV infection is associated with a caspase-independent cell death pathway associated with early lysosomal destabilization. The discovery of the role of DRAM during HIV infection identifies this molecule as a new regulator of host cell-pathogen interactions, contributing to the control of viral infection. This may represent a process of altruistic cell suicide developed by multicellular organisms to defend themselves against microbial infections.

Lysosomes are permeabilized in CD4⁺ T lymphocytes productively infected with HIV-1, resulting in the early release of cathepsins into the cytosol. The released cathepsin D acts upstream from the conformational change in Bax and MOMP [10]. On the contrary, bystander cells exposed to HIV do not express higher levels of DRAM and LC3, in agreement with idea that viral replication induces a death signal [5,6,7,10]. Accumulating evidence indicates that lysosomes function as death signal integrators in response to a wide variety of death stimuli [1]. We demonstrated that the inhibition of DRAM by specific siRNA prevents cathepsin D release, demonstrating for the first time that DRAM is critical for LMP in the context of host-pathogen interaction. We found higher levels of both DRAM mRNA and protein in HIV-infected CD4⁺ T cells than in non-infected cells. However, it was previously shown that the overexpression of DRAM was not enough for inducing autophagy and cell death [29]. This initial observation suggests that additional partners and/or translational modifications are probably required for DRAM-mediated LMP in HIV-infected CD4⁺ T cells. We found higher amounts of DRAM that form puncta in Gag⁺ cells, as visualized by fluorescence microscopy. Bax and Bak, the gatekeepers that induce MOMP, also form aggregates. Unfortunately, attempts at immunoprecipitating DRAM in primary CD4⁺ T cells infected by HIV using commercial antibodies to identify partners have been unsuccessful so far. Therefore, further analysis is necessary to specify the mechanism by which DRAM destabilizes lysosome membranes in primary CD4⁺ T cells.

Our results demonstrated that inhibition of p53 by specific siRNA prevent DRAM expression and autophagy. The infection of T-cell lines and primary CD4⁺ T cells with HIV was initially reported to be associated with stronger expression of pro-apoptotic genes, such as those encoding Bax, p21 and MDM2 [45,46]. CD4⁺ T cells productively infected with HIV display phosphor-

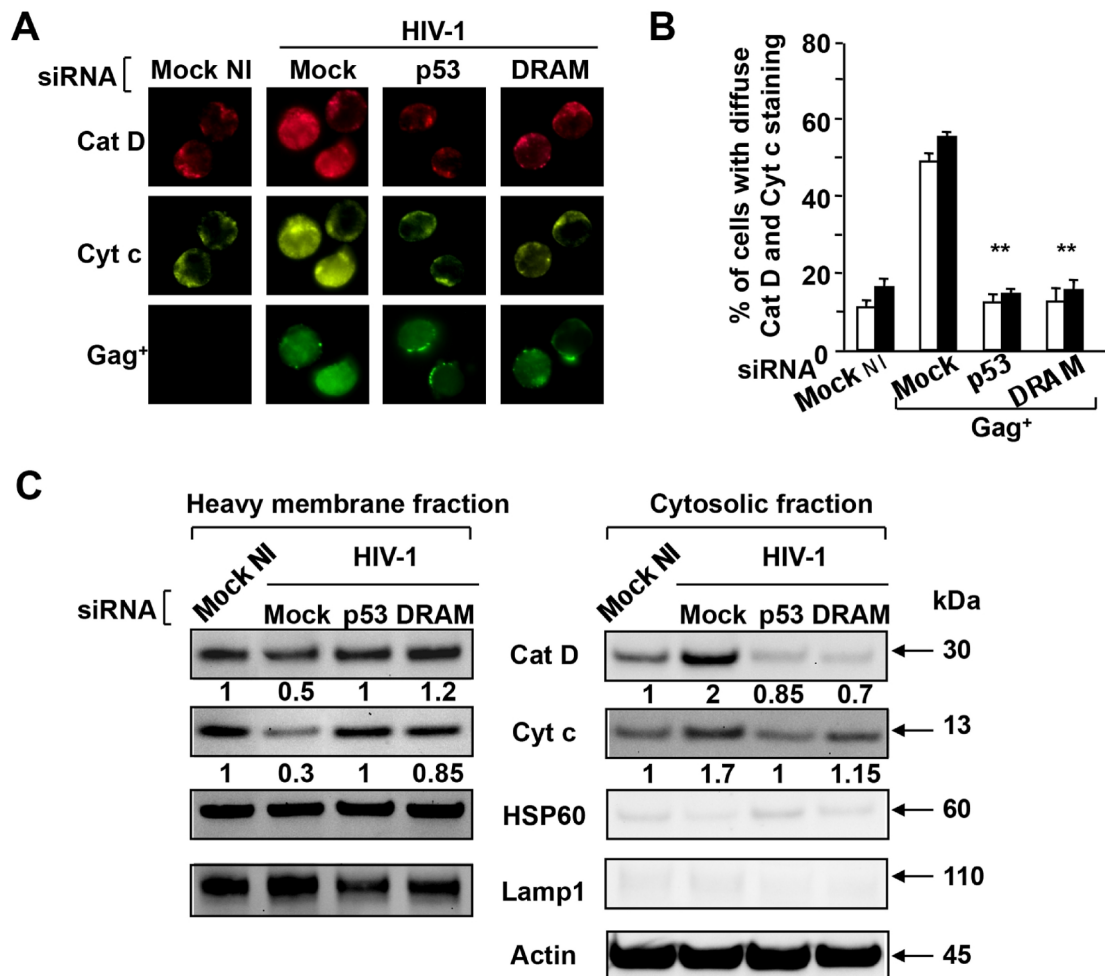


Figure 5. DRAM induces cathepsin D and cytochrome C release in HIV-infected CD4⁺ T cells. HIV-infected CD4⁺ T cells were transfected with siRNA specific for p53 and DRAM or the control siRNA (mock) and then infected with HIV-1. Non-infected cells were used as a control. **(A)** At day 5 post-infection, cells were stained with specific antibodies against cathepsin D (Cat D), cytochrome C (Cyt c) and Gag antigen. **(B)** The subcellular distribution of Cat D and Cyt c in the Gag⁺ cells was analyzed. More than 200 cells were counted for each staining and the results shown are the means $\pm$ SD of three independent experiments; * $p < 0.05$. **(C)** Heavy membrane and cytosolic fractions derived from CD4⁺ T cells, five days after infection, were analyzed by western blotting for the presence of Cat D and Cyt c. Anti-HSP60 and anti-Lamp-1 were used to verify the absence of mitochondrial and lysosomal contamination, respectively. Actin was used as a control of loading. One representative experiment out of three performed giving similar results is shown.
doi:10.1371/journal.ppat.1003328.g005

ylation of p53 on serine 15 leading to the accumulation of p53 within the nucleus in agreement with other observations [15,30]. However, in these models the role of p53 was not directly addressed. Here, we demonstrated using siRNAs that inhibition of p53 prevents LMP and programmed cell death in productively infected CD4⁺ T cells. Viral accessory protein Vpr has been proposed to induce p53 phosphorylation [38], and it causes G2 arrest and apoptosis via ATR [39]. However, resting CD4⁺ T cells infected by HIV and stimulated with mitogens accumulate predominantly in the G₀/G1 phase of the cell cycle at day 5 [7,10,47]. Consistently with the observation in cell cycle in primary CD4⁺ T cells, our data indicate that Vpr is dispensable for LMP. Other candidates encoded by HIV such as the gp120

envelope glycoprotein [48] have been proposed to induce p53 phosphorylation. However, these proteins in primary CD4⁺ T cells are dispensable for HIV infection-mediated cell death [9,47] and no syncytia are observed in the culture [7,10,47]. Moreover, in bystander CD4⁺ T cells, DRAM and LC3-II are not increased.

Nef is in itself an important pathogenic factor in HIV-1 infection. In severe combined immunodeficient (SCID)-hu (thymus/liver) mice, Nef is pathogenic, as evidenced by thymocyte depletion [49]. It has also been reported that Nef can induce in CD4⁺ T lymphocytes the accumulation of lysosomes [40] and a limited lysosomal permeabilization [10]. Here, we found that DRAM or p53 siRNA has no preventive effect on LMP-mediated by Nef. In agreement with the absence of a p53 effect,

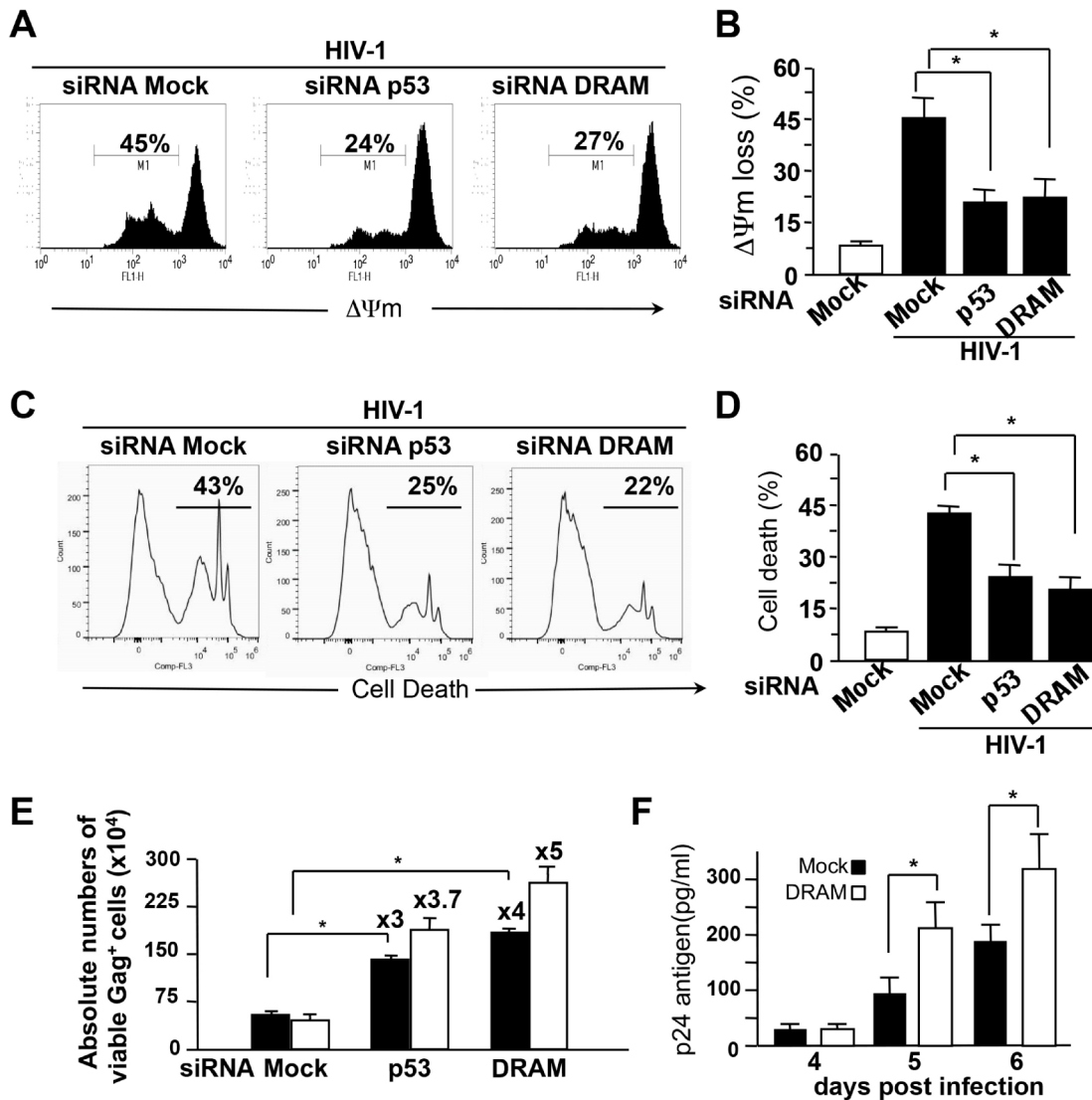


Figure 6. DRAM induces cell death in HIV-infected CD4⁺ T cells. CD4⁺ T cells were transfected with siRNAs specific for p53 or DRAM or the control siRNA (mock), and then infected with HIV. (A) $\Delta\Psi_m$ loss was assessed using a DioC₆ probe at day 5 post-infection. A representative experiment out five independent experiments is shown. (B) The values shown are means $\pm$ SD; *, $p < 0.05$. (C) Cell death was assessed using a propidium iodide (PI) probe at day 5 post-infection. A representative experiment is shown. (D) The values shown are means $\pm$ SD of three independent experiments; *, $p < 0.05$. (E) Absolute numbers of infected cells were calculated on days 5 (black square) and 6 (white square) post-infection. Histograms show means $\pm$ SD of three independent experiments; * $p < 0.05$. (F) Viral production was followed by measuring p24 release in the supernatants of CD4⁺ T cells. Results are the mean $\pm$ SD of three independent experiments; * $p < 0.05$. doi:10.1371/journal.ppat.1003328.g006

overexpression of Nef is not associated with p53 activation (data not shown). Previous observations have indicated that Nef, instead of activating p53, inhibits it [50,51]. Nef is a critical factor that enhances virus replication *in vitro* in primary CD4⁺ T cells and is clearly associated with AIDS [52,53,54,55,56]. Thus, it is conceivable that Nef indirectly acts as a cofactor in the onset of the permeabilization of lysosomes by favoring viral replication [10]. Activation of p53 – the guardian of the genome – has been reported as a sensor detecting pathogen replication in order to

eliminate infected cells [14]. Therefore, prompt induction of LMP and apoptosis of virally-infected cells via p53/DRAM activation will be beneficial for the host as an altruistic cell suicide to limit virus dissemination.

We demonstrated that DRAM regulated autophagy in virally-infected CD4⁺ T cells downstream from active p53. Thus, whereas DRAM was initially described as regulating autophagy in cancer [29], we extended here its role in the context of host-pathogen interactions. Within HIV-infected CD4⁺ T cells, we have observed

(i) ultrastructural autophagy-associated structures, such as autophagosomes and autophagolysosomes; (ii) higher levels of Beclin 1, which is essential for the induction of autophagy; (iii) Atg5-Atg12 complexes, which are considered essential for membrane elongation; (iv) LC3-II aggregates in p24⁺ cells, involved in the formation of the autophagosome membrane; and (v) the degradation of p62. These results suggest that HIV mediates autophagy flux in primary CD4⁺ T cells. It has been suggested that HIV infection inhibits starvation-induced or rapamycin-induced autophagy in CD4⁺ T cell lines [28]. Thus, using primary CD4⁺ T cells and in the absence of additional stimuli to trigger autophagy, we demonstrated that HIV infection increased autophagy instead of inhibiting it. In macrophages infected with HIV, the virus inhibits autophagy flux through Nef, leading to the accumulation of autophagic vacuoles [25]. Thus, although autophagy is observed in both cellular models, the mechanisms are distinct. Indeed, macrophages are resistant to apoptosis, contrary to infected CD4⁺ T cells. Autophagy is suspected of participating in the elimination of damaged organelles and preserving cell integrity from the accumulation of abnormal proteins [21]. Thus, autophagy could be induced to limit damage induced by disrupted lysosomes. The clearance of ruptured lysosomes by autophagy has been previously shown [57]. Our results demonstrate in primary CD4⁺ T cells that siRNAs blocking the expression of the Atg5 and Beclin 1 proteins have no effect on LMP and cathepsin D release. Therefore, the induction of autophagy is not directly responsible for the lethality of HIV-infected CD4⁺ T cells. Viral protein clearance requires intact lysosomes to remove protein aggregates and limit cell death [58]. Herein, by preserving LMP by knocking down DRAM, we prevented cell death mediated by HIV infection. Interestingly, we found greater viral infection and production. Altogether, these data demonstrate that although DRAM is controlling both autophagy and LMP, only this latter process is essential for survival of the infected cells. Viral replication is thought to directly induce CD4⁺ T-cell death during the acute phase of HIV infection, particularly in the intestine [59,60,61,62]. This suggests that an absence of DRAM could be favorable for viral dissemination and persistence of virally-infected CD4⁺ T-cells in HIV-infected individuals. Pharmacological intervention to modulate DRAM in HIV-infected CD4⁺ T cells may thus help to eliminate viral reservoirs and delay development of clinical AIDS.

Our results demonstrate for the first time that the destabilization of lysosomes is induced by DRAM and is an early event in the commitment to cell death contributing in the control of viral infection.

Methods

Isolation of lymphocytes and viral infection

PBMC were isolated from the peripheral blood of anonymous healthy volunteers. All blood donors were informed and agreed to a written consent prior to blood donation in accordance with the guidelines of the Etablissement Français du Sang. CD4⁺ T cells were obtained by negative selection with a CD4 T-cell Isolation kit (Miltenyi Biotec). The CD4⁺ T-cell preparation was at least 98% pure. Monocytes (5%) were added to the purified cells, to ensure full T-cell activation. The cells were incubated with HIV-1_{LAI} for 12 h at a multiplicity of infection (MOI) of 0.01, and activated with 1 µg/ml ConA (Sigma-Aldrich) and 100 units/ml recombinant human IL-2 (Roussel-Uclaf, France), as previously described [7,10]. The virus was produced from CEM-LTRgfp that allows to monitoring viral replication [63]. HIV-1_{LAI} was recovered from supernatant at the peak of replication. Virus stocks were prepared by transfection of 293T cells with plasmids of Wt and Vpr-

defective NL4-3 (kindly provided by S. Benichou). HIV replication was assessed by monitoring the Gag⁺ cells by flow cytometry using anti-HIV-Gag (KC57-PE, Beckman-Coulter) and by measuring Gag-p24 release in supernatants by an Innostest HIV antigen enzyme-linked immunosorbent assay (ELISA) kit (Ingen). The number of live infected CD4⁺ T cells was assessed by flow cytometry as previously described [7,10,64,65]. Dead cells were detected using 1 µM of propidium iodide (PI) from Molecular Probes and Annexin V-FITC from Beckman Coulter. To evaluate changes in the inner mitochondrial transmembrane potential $\Delta\Psi_m$, cells were stained for 15 min at 37°C with 40 nM of the potential-sensitive fluorescent dye DiOC₆ (3,3'-diethyloxycarbocyanine) from Molecular Probes.

Immunofluorescence analyses

The reagents used for immunofluorescence studies were: rabbit polyclonal antibodies recognizing anti-MAP LC3 (H-50) purchased from Santa Cruz, anti phospho-p53 (Ser 15) antibodies purchased from Cell Signaling Technology, anti-DRAM antibodies purchased from ϕ ProSci, anti-cathepsin D antibodies from Zymed Laboratories, anti-p53 mAb (DO-1) purchased from Santa Cruz, anti-Lamp-2 mAb from Calbiochem, and a sheep anti-cytochrome c antiserum from Sigma. Intracellular Gag antigen was assessed by flow cytometry after fixation and permeabilization of the cells (Intraprep permeabilization reagent, Coulter), which were then stained with FITC- or RD1-labeled mAb against p24^{gag} antigen (KC-57, Beckman coulter). Otherwise, the cells were fixed by incubation with 1% paraformaldehyde, span on glass slides, washed with PBS, and permeabilized by incubation with 0.05% Triton X-100. The cells were washed and incubated with the antibodies indicated in PBS supplemented with 0.5% BSA and 2% FCS. The cells were stained with an Alexa-conjugated secondary antibody (Molecular Probes). Nuclei were counterstained for 5 minutes with 5 µM DAPI (Molecular Probes). The cells were examined by conventional or confocal fluorescence microscopy (Zeiss Microsystems). For the formation of LC3-II aggregates, cells with more than 6±2 vesicles were considered positive as previously described [31]. To monitor lysosomal destabilization, CD4⁺ T cells were incubated for 2 h with 5 mg/ml FITC-dextran of 40-kDa (Sigma) as previously described [10]. After a 2-h chase period, the cells were stained with anti-p24 mAb (Gag⁺) and examined by laser scanning confocal microscopy.

Immunoblotting

Pellets of 1×10⁶ CD4⁺ T cells were either directly resuspended in Laemmli buffer containing 2% SDS and 10% 2-ME and boiled for 5 minutes, or lysed in Nonidet P-40 buffer (1% NP-40, 50 mM Tris-HCl (pH 7.4), 150 mM NaCl) supplemented with protease inhibitors. Pepstatin was purchased from Sigma. Cytosolic and nuclear fractions were obtained by extraction with the NE-PER kit (Nuclear and Cytoplasmic Extraction Reagents from PIERCE). Lysates were then subjected to electrophoresis in NUPAGE 4–20% polyacrylamide gels (Invitrogen). The proteins were transferred to polyvinylidene difluoride membranes (Amersham Bioscience) and then incubated with primary antibodies and with horseradish peroxidase-coupled secondary reagents (Amersham Biosciences). The primary antibodies used for western blotting were: rabbit antisera against Beclin 1 (H-300, Santa Cruz), phospho-p53 (Ser 15) (Cell Signaling Technology), DRAM (Stressgen), Atg5 (Novus) and tubulin (Santa-Cruz); mouse mAbs against p53 (DO-1, Santa Cruz), Cathepsin D (BD Transduction Laboratories), Cytochrome c (Pharmingen), p62 (Cell Signaling Technology), Lamp-1 (BD Transduction Laboratories), lamin B (Ab-1, Oncogene Research Products Calbiochem) and actin

(Millipore). Rabbit antisera against MAP LC3 was purchased from MBL. Productive HIV infection was visualized by western blotting that allows detection of the presence of viral antigens in cell extracts. The immunoblots were incubated with sera obtained from a pool of HIV-infected patients, and then revealed with horseradish peroxidase-linked goat anti-human secondary antibodies (Amersham Biosciences). The blots were then developed by enhanced chemiluminescence methods (ECL⁺ from GE Healthcare), photographed with a CCD camera (GBOX, SYNGENE), and the optical densities measured and normalized with respect to the loading control (tubulin or Hsp60 for mitochondrial fractions). It must be noted that we have tested all commercial antibodies directed against DRAM for the immunoprecipitation assay; none of them are functional.

Subcellular fractionation

Cytosolic and heavy membrane fractions were generated from 10^7 cells, using a selective digitonin-based permeabilization and subcellular-fractionation technique as previously described [7,10]. In brief, CD4⁺ T lymphocytes were washed with cold PBS and then suspended in a lysis buffer (250 mM sucrose, 20 mM Hepes, 5 mM MgCl₂, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, pH 7.4, supplemented with protease inhibitors purchased from Roche). The digitonin concentration was 35 µg/ml. After 5 min, the cells were centrifuged and the supernatant was removed (cytosolic fraction). The remaining pellet was resuspended in lysis buffer (150 mM NaCl, 1.0% NP-40, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl, pH 8.0) and incubated 30 min. The supernatant comprising the membrane fraction was retained after centrifugation (30 min at 15000 g).

Transfection experiments

Pre-designed small interfering RNA (siRNA) molecules targeting p53, DRAM, BECLIN 1 and ATG5 were synthesized by Dharmacon. Scrambled controls were also used. Gene expression was silenced by the small interfering RNA (siRNA) technique [10], using duplexes of 21-nucleotide siRNAs with two 3'-overhanging TT residues (Prologo). The sense strand of the siRNA used to silence the BECLIN 1 gene was CAGTTTGGCACAATCAATCAATATT, that of ATG5 gene seq 1 was GCAACTCTGGATGGGATTGTT and that of seq 2 was CATCTGAGCTACCCGGAATATT, whereas that of the DRAM gene was CCACAGAAATCAATGGTGATT. For p53 we used a smart pool. Purified resting CD4⁺ T cells were transfected, by electroporation with siRNAs (0.75 µM/4 × 10⁶ cells), mediated by the Nucleofection system (Amaxa). Cells were allowed to rest for 16 hours, exposed to HIV-1 cultured for an additional 12 h and then stimulated with either Nef-WT or Nef-AS. We also used the autophagy inhibitor 3-methyladenine in some experiments, but the results obtained were inconclusive, due to a strong toxicity of this drug in primary cultures of human cells.

Transfection of CD4⁺ T lymphocytes with Nef expression vectors

Plasmids carrying the Nef gene (from the LAI isolate) in a sense (Nef-WT) and antisense (Nef-AS), under the control of the cytomegalovirus promoter, were kindly provided by O. Schwartz (55). CD4⁺ T cells were first transfected with siRNA directed against DRAM and p53, and after overnight culture were restimulated with ConA and IL-2 for 4 days. Thereafter, the cells were transfected with either Nef-WT or Nef-AS using the Nucleofection system (Amaxa). After 24 h, the cells were washed and fixed for flow cytometry and confocal microscopy.

Real-time RT-PCR analysis

RNAs were isolated from uninfected and HIV-1 infected CD4⁺ T-cells at days 3, 4 and 5 post-infection. cDNA synthesis was performed using the AffinityScript QPCR cDNA Synthesis Kit (Stratagene, Agilent Technologies) with 1 µg of total RNA and random primers. The resulting RT product was expanded using the Brilliant II SYBR Green QPCR Master Mix (Stratagene, Agilent Technologies) and specific primers for DRAM (forward: 5'-AGACTCCATCTTTTCACCCAAA-3', reverse: 5'-GCTC-TTCACCTTTCAAGCCTAA-3'), Beclin-1 (forward: 5'-AAGACAGAGCGATGGTAG-3', reverse: 5'-CTGGGCTGTGGTAA-GTAA-3') and the housekeeping gene ribosomal RNA S14 subunit (forward: 5'-GGCAGACCGAGATGAATCCTCA-3', reverse: 5'-CAGGTCCAGGGGTCTTGGTCC-3'). Detection was performed with the Mx3000P QPCR System (Stratagene, Agilent Technologies). Threshold cycle (Ct) values were obtained for each gene at the different time points using the instrument software. Differences in the levels of gene expression over time were determined for each condition by relative quantification using the Delta Ct method.

Electron microscopy

Pellets of uninfected or infected CD4⁺ T cells were fixed by incubation for 1 h in phosphate buffer pH 7.2 supplemented with 1.6% glutaraldehyde and were then post-fixed by incubation for 2 h in 0.1 M phosphate buffer supplemented with 1% osmium tetroxide. Pieces of cell pellet were washed for five minutes in water and then dehydrated in a series of increasing concentrations of ethanol before embedding in Epon 812 [66]. Ultrathin sections were cut and stained with 4% uranyl acetate and lead citrate. They were then examined under a ZEISS 902 electron microscope, at 80 KV, or under a FEI Technai 12 microscope at 80 KV.

Statistical analysis

Data are reported as means ± SEM. The significance of differences was assessed by Student's t test (Prism software) with p < 0.05 considered significant.

Supporting Information

Figure S1 HIV-1 infection induces p53 activation in CD4⁺ T cells. CD4⁺ T cells in the absence (NI) or presence of HIV-1_{LAI} (HIV-1) have been analyzed. **(A)** Flow cytometric analyses of HIV-1 Gag antigen expression (Gag-PE) at day 5 after infection. **(B)** Percentage of Gag⁺ cells on days 3, 4 and 5 after infection. Histograms are means ± SD of 10 individual experiments. **(C)** Percentages of cells releasing cathepsin D (Cat D) in CD4⁺ T-cells. Cells (Gag⁺ and Gag⁻) were analyzed by confocal microscopy, after staining for cathepsin D. Results expressed as the mean ± SD of 10 individual experiments. In each condition 100 cells were counted. **(D)** Nuclear fractions from CD4⁺ T cells were analyzed for P-p53 on days 3, 4 and 5 post-infection. Antibody against lamin B was used as a control for protein loading. A typical experiment out of four is shown on the left, and the means ± SD are shown in histograms on the right. *, p < 0.05. **(E)** Nuclear and cytoplasmic fractions from CD4⁺ T cells were analyzed for p53 on day 5 post-infection. **(F)** Cells on day 5 were stained with mAbs against P-p53 (red) and p24 antigen (green) and analyzed by fluorescent microscopy. Nuclei were counterstained with DAPI (blue). Representative cells are shown and, in **(G)**, a histogram shows the percentages of cells that are P-p53⁺ on days 3, 4, and 5. Results expressed as the mean ± SD of 4

individual experiments. In each condition 100 cells were analyzed. *, $p < 0.05$. (TIF)

Figure S2 Colocalization of Lamp-2 and DRAM in infected CD4⁺ T cells. CD4⁺ T cells are infected with HIV-1 and stained on day 5 post-infection for LAMP2 (green) and DRAM (red). (A) Gag⁺ and Gag⁻ (NI) cells are shown. (B, C) Quantification of DRAM and LAMP2 expressions was assessed using ImageJ software. For each cell, area and pixel value statistics were calculated accordingly and mean fluorescence intensity per cell is shown. Results expressed as the mean $\pm$ SD of 2 individual experiments. In each condition 100 cells were analyzed. *, $p < 0.05$. (TIF)

Figure S3 Autophagy-related ultrastructures in CD4⁺ T infected by HIV. (A) a, b Electron microscopy analyses of autophagy-related ultrastructures in CD4⁺ T cells in the absence (NI) or presence of HIV-1_{LAI} (HIV-1); (c) higher magnification of the inset in (b); arrows indicate autophagosomes with double-membrane-structures in cells with HIV-1 particles budding at the surface. (B) Quantitation of CD4⁺ T cells displaying autophagic vacuoles. Results expressed as the mean $\pm$ SD of 3 individual experiments. In each condition 150 cells were analyzed; *, $p < 0.05$. (C) Representative electron micrographs of the cytoplasmic regions of CD4⁺ T cells with productive HIV-1 infection; (a, b) autophagosomes (arrows) and budding HIV-1 particles (arrowhead); (c) dashed arrows indicate autophagolysosomes with electron-dense structures in HIV-infected CD4⁺ T cells. (D) Frequency of autophagosome (a) and autophagolysosome (c) in HIV-infected CD4⁺ T cells. Budding virus on cell surface was used to monitor infected cells. A total of 150 cells were analyzed. *, $p < 0.05$. (TIF)

Figure S4 Inhibition of Beclin 1 and Atg5 reduces autophagy in infected cells. (A) CD4⁺ T cells transfected with either control siRNA (Mock) or siRNAs specifically targeting BECLIN1 and ATG5 were infected with HIV-1. Two sequences for Atg5 were used: sequence 1 (ATG5₁) and sequence 2 (ATG5₂). Immunoblots of lysates at day 5 after infection are shown. Membranes were probed for Beclin 1, Atg5 and LC3. Actin was used as a control for protein loading. One representative experiment out to three performed is shown. (B) The distribution of LC3-II (number of puncta per cell ≥ 6) was determined by fluorescence microscopy in Gag⁺ cells. The values shown are means $\pm$ SD of three independent experiments (≥ 200 cells were examined); *, $p < 0.05$. (TIF)

Figure S5 HIV-1 infection induces LMP in the absence of Beclin 1 and Atg5. HIV-infected CD4⁺ T cells were transfected with siRNA specific for BECLIN1 and ATG5 or the control siRNA (mock) and then infected in the absence (NI) or in the presence of HIV-1 (HIV-1). (A) At day 5 post-infection, cell extracts were analyzed for Beclin and Atg5. (B) Cells were stained with specific antibodies against Cathepsin D (Cat D) and Gag

antigen. The subcellular distribution of Cat D in the Gag⁺ cells was analyzed. More than 200 cells were counted for each staining and the results shown are the means $\pm$ SD of three independent experiments. No statistical difference was observed. (C) Percentage of cell death assessed by flow cytometry using propidium iodide (PI). Results are the means $\pm$ SD of three independent experiments. No statistical difference was observed in the absence or presence of specific siRNAs. (TIF)

Figure S6 Productive infection induces DRAM. CD4⁺ T cells were transfected with siRNA specific for p53, DRAM or the control siRNA (mock) and then infected with HIV-1. Cells were then cultured in the absence (bystander) or presence of ConA+IL-2 (activation). (A) $\Delta\Psi_m$ loss and cell death were assessed using DioC₆ and propidium iodide (PI), respectively. Flow cytometric analysis shown is performed at day 5 post-infection. A representative experiment is shown and in (B), histograms show the means $\pm$ SD of three individual experiments. Cells were analyzed on days 4 and 5 post-infection (C) Percentage of HIV infection was determined by intracellular staining with specific Gag antibody Gag antigen. (D) Western blots of DRAM and LC3 expression in CD4⁺ T cells infected by HIV-1 in the absence or presence of cell activation on day 5 post-infection. Actin was used to a loading control. (E) Histograms show the means $\pm$ SD of three individual experiments; *, $p < 0.05$. (TIF)

Figure S7 Vpr is dispensable for DRAM-mediated LMP and MOMP in HIV-1 infected CD4⁺ T cells. CD4⁺ T cells were transfected with siRNA specific for DRAM or the control siRNA (mock) and then infected with either Wt or Vpr-defective NL4-3 virus (Vpr⁻). (A) Immunoblots of lysates from CD4⁺ T cells. Membranes were probed for DRAM and Actin. (B) Cells were stained with specific antibodies against cathepsin D (Cat D) (Red), cytochrome C (Cyt c) (yellow) and Gag antigen (green). (C) More than 200 cells were counted for each staining shown in B, and the results shown are the means $\pm$ SD of three independent experiments. (TIF)

Figure S8 Nef-mediated LMP is DRAM-independent. CD4⁺ T cells were transfected with siRNA specific for p53, DRAM or the control siRNA (mock) and then transfected overnight with (A) Nef-WT or (B) Nef-AS). Cells were stained with specific antibodies against cathepsin D (Cat D) (blue) and cytochrome C (Cyt c) (red). (C) $\Delta\Psi_m$ loss was assessed using DioC₆ probe. Histograms show the means $\pm$ SD of three individual experiments. (TIF)

Author Contributions

Conceived and designed the experiments: ML AS JE. Performed the experiments: ML SL FH NC VR RS HH. Analyzed the data: ML SL FH NC VR RS JFZ AS JE. Contributed reagents/materials/analysis tools: ML SL FH NC VR RS JFZ AS JE. Wrote the paper: ML AS JE.

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