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UNIVERSIDADE DO PORTO

# CD38 and immune function: role in infections by intracellular bacteria and systemic autoimmunity

Marta Susana de Almeida Viegas da Silva

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# CD38 and immune function: role in infections by intracellular bacteria and systemic autoimmunity

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Orientador – Doutora Teresa Martins

Categoria – Investigadora Auxiliar

Afiliação – Instituto Português de Oncologia de Coimbra Francisco Gentil, Coimbra

Co-orientador Professor Doutor Artur Águas

Categoria – Professor Catedrático

Afiliação – Instituto de Ciências Biomédicas Abel Salazar da Universidade do Porto.

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## Contents

## Abreviations

ADP – adenosine diphosphate

ADPR – adenosine diphosphate ribose

ANA – anti-nuclear autoantibodies

ANCA – antineutrophil cytoplasmic autoantibodies

ART – ADP-ribosyl transferase

ATP – adenosine triphosphate

BCR – B cell receptor

cADPR – cyclic adenosine diphosphate ribose

CCL14 – C-C chemokine ligand 14

CCL2 – C-C chemokine ligand 2

CCL21 – C-C chemokine ligand 21

CCR2 – C-C chemokine receptor 2

CCR7 – C-C chemokine receptor 7

CXCL12 – CXC chemokine ligand 12

CXCR4 – CXC chemokine receptor 4

CXCR5 – CXC chemokine receptor 5

DC – dendritic cells

DC-SIGN – dendritic cell-specific intercellular adhesion molecule-grabbing non-integrin

DN T cells – double negative T cells

DP T cells – double positive T cells

dsDNA – double-stranded

ERK – extracellular signal-regulated kinase

ESAT-6 – early secreted antigenic target of 6kDa

FbpA – mycolyl-transferase complex of enzymes

FcγR – Fc gamma receptor

FcRIIIγ – Fc gamma receptor type III

fMLP –formyl-Met-Leu-Phe

GM-CSF – granulocyte macrophage-colony stimulating factor

GN – glomerulonephritis

HIV – human immunodeficiency virus

IC – immune complexes

ICAM – intercellular adhesion molecule

IFNγ – interferon gamma

Ig – immunoglobulin

IL-1 – interleukin 1

IL-10 – interleukin 10

IL-12 – interleukin 12

IL-17 – interleukin 17

IL-18 – interleukin 18

IL-23 – interleukin 23

IL-4 – interleukin 4

IL-6 – interleukin 6

IL-8 – interleukin 8

iNOS – inducible nitric oxide synthase

IRAK – interleukin-1 receptor-associated kinase

IRF-1 – interferon regulatory factor 1

JNK – c-Jun N-terminal kinase

LAM – lipoarabinomannan

Lck – leukocyte-specific protein tyrosine kinase

LN – lymph nodes

MAC – *Mycobacterium avium* complex

MAPK – mitogen-activated protein kinase

MCP-1/3 – monocyte chemoattractant protein 1/3

MHC I – major histocompatibility complex I

MHC II – major histocompatibility complex II

MIP1$\alpha$/$\beta$ – macrophage inflammatory protein 1alpha/beta

Mtb – *Mycobacterium tuberculosis*

NAADP – nicotinic acid adenine dinucleotide phosphate

NAD – nicotinamide adenine dinucleotide

NAD(P) – nicotinamide adenine dinucleotide phosphate

NF-IL6 – nuclear factor-interleukin 6

NF-$\kappa$B – nuclear factor kappa B

NGD – nicotinamide guanine dinucleotide

NK cells – natural killer cells

NO – nitric oxide

NOD – nucleotide-binding and oligomerisation domain

pDC – plasmacytoid dendritic cells

PI3K – phosphatidylinositol kinase 3

PLC$\gamma$1 – phospholipase C-gamma 1

RANTES – regulated upon activation normal T cell express sequence

RF – rheumatoid factor

RNI – reactive nitrogen intermediates

ROI – reactive oxygen intermediates

SLE – systemic lupus erythematosus

SP T cells – single positive T cells

TB – tuberculosis

TCF-1$\alpha$ – T cell transcription factor 1alpha

TCR – T cell receptor

TEC – thymic epithelial cells

TGF$\beta$ – tumour growth factor beta

Th1 cells – type 1 T helper cells

Th17 – type 17 T helper cells

Th2 – type 2 T helper cells

Th3 cells – type 3 T helper cells

TLR – toll-like receptors

TNF$\alpha$ – tumour necrosis factor alpha

Tr1 cells – type 1 regulatory T cells

Treg cells – regulatory T cells

Type I IFN – type I interferon

UV light – ultra-violet light

ZAP-70 – zeta-chain associated protein kinase 70

# Chapter One

# 1. Introduction

## 1.1. CD38

CD38 belongs to a family of multifunctional ectoenzymes, which possess ADP-ribosyl cyclase activity. All members of this family share biological functions, as well as structural characteristics. CD38 has been identified in a number of different species. CD38 sequence is homologous in over 80% in these species, suggesting a highly conserved structure throughout evolution (Lund *et al.*, 1998). It is believed that CD38 may have arisen several million years ago. Its primary function would have been that of an enzyme and only later, in the course of evolution, it would have acquired receptor functions. Nevertheless, these two functions seem to have remained independent of each other.

Human *CD38*, which is located on chromosome 4, was identified in 1980 by Reinherz and co-workers (Reinherz *et al.*, 1980). The murine homologue, located on chromosome 5, was identified in 1993 (Harada *et al*., 1993). Human *CD38* spans over 62kb and is composed by 8 exons and 7 introns. It possesses a large intron, intron 1, with more than 20kb, which contains several regulatory elements, such as a retinoic acid responsive element; transcription factor responsive elements, such as TCF-1$\alpha$, NF-IL-6, IRF-1; and a CpG island. Transcription of *CD38* is not restricted to one site, as no TATA or CAAT boxes are present (Ferrero *et al*., 1999; Ferrero *et al*., 2000).

CD38 is a type II glycoprotein with a molecular weight of 45kDa. It has a short cytoplasmic tail (20 aminoacids), a transmembranar region (23 aminoacids) and an extracellular domain (257 aminoacids) (Deaglio *et al*., 2001). CD38 may assume different forms and sizes. When the extracellular portion is hydrolysed, a p39 isoform is originated, which possesses full or partial enzyme activity, suggesting that CD38 may act as a circulating enzyme or a local hormone (Lund *et al*., 1998). CD38 may also assume a dimeric form of 78kDa or form tetramers with a molecular weight of 190kDa. This last form is usually associated with the cellular membrane. CD38 can be internalized and is able to recirculate from the membrane to the cytoplasm, and possibly to the nucleus, suggesting that CD38 can be a quite promiscuous molecule (Deaglio *et al*., 2001).

So far, three CD38 ligands have been identified: hyaluronic acid (Nishina *et al*., 1994), NAD (Funaro *et al.*, 1999), and CD31 (Deaglio *et al*., 2000). Nevertheless, the interaction of these ligands with CD38 does not fully explain all the cellular effects observed upon CD38 ligation by monoclonal antibodies, suggesting the existence of other ligands (Malavasi *et al*., 2008).

CD38 has intracellular and extracellular enzymatic activity. The extracellular domain of CD38 was found to be highly homologous to a formerly identified ADP-ribosyl

cyclase isolated from the cytosol of the invertebrate *Aplysia californica.* In humans, CD38 was found to have several distinct enzymatic activities: i) transformation of $NAD^+$ into ADPR ($NAD^+$ glycohydrolase activity) or cADPR (ADP-ribosyl cyclase activity); ii) production of pyridinium analogues of $NAD(P)^+$, such as $NAADP^+$ (transglycosylation reaction); and iii) hydrolysis of cADPR into ADPR (cADPR hydrolase activity) (Howard *et al*., 1993). The most widely accepted model for the enzymatic activity of CD38 is the "partition" mechanism. This model postulates the existence of a reactive intermediate which would be metabolized to yield either ADPR or cADPR. The metabolism of $NAD^+$ would generate a common reactive intermediate, E·ADP-rybosyl, which would be stabilized by CD38. This intermediate would then evolve to the reaction products through two competing pathways: i) an irreversible intermolecular reaction with water, generating ADPR (hydrolysis pathway); or ii) a reversible intramolecular reaction, yielding cADPR (cyclase pathway). This intermediate is able to react with numerous nucleophylic acceptors, such as water, $CH_3OH$ and pyridines, giving rise to a number of different products (Schubber *et al*., 2004; Kim *et al*., 1993; Kuhn *et al*., 2006). cADPR is a second messenger endowed with the capacity to mediate intracellular calcium release from the ryanodine receptor-regulated stores (Lee *et al*., 1991; Lee *et al*., 1997; Galione *et al*., 1991). Thus, CD38 is in the middle of a "topological paradox", as it utilizes NAD as a substrate to yield extracellular ADPR that, in turn, regulates intracellular calcium mobilization. To resolve this paradox, it has been proposed that CD38 could either be directly responsible for the transport of cADPR into the cells or act as an accessory molecule, helping cADPR transport through specific nucleoside transporters (Schubber *et al*., 2004). Although $NAD^+$ is the most common substrate, CD38 is also able to catalyze other substrates, such as NAADP and NGD (Lee *et al*., 2006; Schubber *et al*., 2004).

CD38 is expressed by several cell populations within and outside the haematopoietic system (Table 1). Outside the haematopoietic system, CD38 can be found in the brain (Mizuguchi *et al*., 1995), heart, kidneys (Koguma *et al*., 1994), intestine and pancreas (Fernandez *et al*., 1998; Takasawa *et al*., 1993). With regard to the haematopoietic system, CD38 is expressed by B cells (Lund *et al*., 1996) and T lymphocytes (Morra *et al*., 1998), Natural Killer (NK) cells (Deaglio *et al*., 2002; DiRosa *et al*., 1997), granulocytes (Partida-Sànchez *et al*., 2001), myeloid cells (Inove *et al*., 1997), monocytes/macrophages and dendritic cells (DC) (Fedele *et al*., 2004; Zilber *et al*., 2000).

**Table 1:** Expression of CD38 within and outside the haematopoietic system.

| **Haematopoietic system** | **References** |
|---|---|
| B lymphocytes | Lund *et al*., 1996 |
| T lymphocytes | Morra *et al*., 1998 |
| NK cells | Deaglio *et al*., 2002; DiRosa *et al*., 1997 |
| Granulocytes | Partida-Sànchez *et al*., 2001 |
| Myeloid cells | Inove *et al*., 1997 |
| Monocytes/Macrophages | Zilber *et al*., 2000 |
| Dendritic cells | Fedele *et al*., 2004 |
| **Non-haematopoietic system** | |
| Brain | Mizuguchi *et al*., 1995 |
| Heart | Koguma *et al*., 1994 |
| Kidneys | Koguma *et al*., 1994 |
| Intestine | Fernandez *et al*., 1998 |
| Pancreas | Takasawa *et al*., 1993 |

The role of CD38 outside the haematopoietic system is still unclear, except for pancreatic β-cells. Okamoto and co-workers (2002) proposed that glucose metabolism results in ATP synthesis which, in turn, would inhibit CD38-mediated degradation of cADPR. This would result in an increase in cADPR availability for mobilization of calcium from intracellular stores and increased release of insulin (Kato *et al*., 1995; Kato *et al*., 1999). It has been suggested that CD38 may also have an anti-apoptotic effect in

pancreatic cells (Johnson *et al*., 2006). More recently, CD38 was also proposed to be involved in regulation of oxytocin release. Although the exact mechanism is still unclear, it seems to involve synthesis of cADPR (Jin *et al*., 2007; Young, 2007).

The wide expression of CD38 in immune cells suggests an important function for this ectoenzyme in the immune system. CD38 expression is tightly controlled during hematopoiesis, suggesting that CD38 may play a relevant physiological role in this process (Alessio *et al*., 1990; Kumagai *et al*., 1995). In general, CD38 expression is low in stem cells, increases during maturation, being downregulated after lineage commitment (Fig. 1). It is then upregulated upon activation (Malavasi *et al*., 1994). However, there are some differences between humans and mice (Dónis-Hernandez *et al*., 2001). While in murine B cells expression of CD38 increases throughout the maturation process; in humans, the opposite is observed (Alessio *et al*., 1990). In both species, CD38 expression has been reported in thymic $\alpha\beta TCR^{+}CD4^{-}CD8^{-}$ and in peripheral $CD45RB^{lo}CD4^{+}$ T cells (Bean *et al*., 1995; Sandoval-Montes *et al*., 2005; Alessio *et al*., 1990). High levels of CD38 were found on activated T cells whereas resting T cells express low levels of this ectoenzyme (Fig. 1). The distinctive expression pattern of CD38 in various cell populations has allowed its use as a cell marker in numerous pathological situations (Antonelli *et al*., 2001; Antonelli *et al*., 2001a; Pupilli *et al*., 1999; Durig *et al*., 2002; Kramer *et al*., 1995; Marinov *et al*., 1993; Echaniz *et al*., 1993; Giorgi *et al*., 1993).

*In vitro* data has long suggested that CD38 may have important immunological functions (Table 2). CD38 behaves as a receptor, this way modulating the outcome of cellular activation. However, due to its short cytoplasmatic tail, CD38 needs to be physically associated with other cell receptors to initiate signaling cascades: B-cell receptor (BCR), on B cells (Lund *et al*., 1996; Lund *et al*., 1998); T-cell receptor (TCR), on T cells (Morra *et al*., 1998); CD16, CD26 and CD39, on NK cells (Deaglio *et al*., 2002; DiRosa *et al*., 1997); CD32, on myeloid cells (Inove *et al*., 1997); and major histocompatibility complex (MHC) class II, on macrophages (Zilber *et al*., 2000).

**Table 2:** Immunological functions of CD38 in different cell populations.

| Cell type | Associated molecule | Functional outcome |
|---|---|---|
| B Lymphocytes | BCR | Lowering BCR signaling[a] |
| | | Rescue from apoptosis[b] |
| | | Ig class switching[a] |
| T Lymphocytes | TCR | Increased production of cytokines[c, d] |
| NK cells | CD16, CD26, CD39 | Increased cytokine production[e] |
| | | Enhancement of cytotoxic responses[f] |
| Myeloid cells | CD32 | Increased superoxide production[g, h] |
| Monocytes | MHC class II | Increased response to respiratory burst activators[i, j] |
| DC | Unknown | Chemotaxis[k, l, m] |
| Neutrophils | Unknown | Chemotaxis[n] |

[a], Lund *et al*., 1996; [b], Boffil *et al*., 2000; [c], Cesano *et al*., 1998; [d], Morra *et al*., 1998; [e], Deaglio *et al*., 2002; [f], DiRosa *et al*., 1997; [g], Tsujimoto *et al*., 1997; [h], Inove *et al*., 1997; [i], Zilber *et al*., 2005; [j], Zilber *et al*., 2000; [k], Fedele *et al*., 2004; [l], Partida-Sànchez *et al*., 2004; [m], Frasca *et al*., 2006; [n], Partida-Sànchez *et al*., 2001.

Two models for CD38 signaling on immune cells have been proposed (Lund *et al*., 1996). In the first model, CD38 is either constitutively or transiently associated with an antigen receptor associated protein. For instance, in B cells, antigen receptor associated protein would associate with CD38 and Ig$\alpha$/Ig$\beta$ at the same time, through their transmembrane or extracellular domains. Upon CD38 ligation, CD38/antigen receptor associated protein conformation would change, allowing antigen receptor associated protein dissociation from CD38 and subsequent association with the BCR. This would initiate the BCR signaling cascade. Alternatively, CD38 could directly co-crosslink Ig$\alpha$/Ig$\beta$ and, in this way, bypass the requirement for antigen receptor associated protein. In this case, after activation, CD38 would associate with Ig$\alpha$/Ig$\beta$. This association could occur through the transmembrane or the extracellular domain. The synergy observed between CD38 and the BCR can be explained by either model. Initiation of CD38-mediated

signaling cascade in B cells is dependent on the ligand present, being triggered by CD38/CD31, but not CD38/NAD interactions.

As stated earlier, CD38 expression is tightly controlled in the lymphoid lineage. Lymphoid progenitor cells are capable of differentiating into B and T lymphocytes and NK cells. On B cells, CD38 is upregulated on pro- and pre-B cells, during BCR rearrangements, and is downregulated after BCR assembly, suggesting a role for CD38 in this process (Malavasi *et al*., 1994). As immature B cells leave the bone marrow and migrate to the spleen, where they complete their maturation process, the expression of CD38 is downregulated (Hardy *et al*., 2001). Upon activation, germinal center B cells upregulate CD38 expression (Banchereau *et al*., 1992) (Fig. 1). On mature B cells, CD38 associates with the BCR and, acting as an accessory molecule, can either augment or dampen B cell activation (Lund *et al*., 1995; Lund *et al*., 1996; Lund *et al*., 1998). B cell activation via CD38 induces tyrosine phosphorylation, calcium mobilization, and increased expression of Btk, Lyn and Fyn and an overall increase of phosphatidylinositol kinase (PI3K) activity (Kitanaka *et al*., 1997; Boffil *et al*., 2000). In addition, it initiates the mitogen-activated protein kinase (MAPK) and phospholipase C-$\gamma$1 (PLC-$\gamma$1) signaling cascades (Boffil *et al*., 2000), leading to cytokine production (Lund *et al*., 1996). CD38 is also involved in immunoglobulin class switching (Kikuchi *et al*., 1995; Yasue *et al*., 1999; Mizoguchi *et al*., 1999). CD38 activation on B cells leads to expression of the germline $\gamma$1 transcripts and increased expression of the IL-5 receptor leading to immunoglobulin class switching, a process that was associated with CD38-dependent activation of c-Rel, p65 and p50 (Kaku *et al*., 2002). The end result of CD38-mediated activation differs between mice and humans: while in mice, activation of B cells through CD38 leads to cell survival (Boffil *et al*., 2000); in humans, it induces cell cycle arrest or apoptosis (Alessio *et al*., 1990; Kumagai *et al*., 1995; Boffil *et al*., 2000). CD38 was recently suggested to play a role in the generation of murine mature splenic B cells. Depending on the maturation state of B cells, different effects can be observed upon CD38 ligation: immature B cells die by apoptosis, whereas more mature B cells proliferate and further progress in the maturation process, suggesting a role for CD38 in splenic B cell selection (Rodriguez-Alba *et al*., 2008).

Expression of CD38 in the thymus is also tightly controlled, suggesting a role for this ectoenzyme in thymic selection (Bean *et al*., 1995). CD38 expression is low at DN1 stage, upregulated during DN2 and DN3 stages and $\beta$-selection, decreasing thereafter (Malavasi *et al*., 1994; Spits, 2002; Dik *et al*., 2005; Staal *et al*., 2001) (Fig. 1). CD38 may have a role in TCR rearrangements and during $\beta$-selection, most likely being involved in

the definition of the TCR reactivity. In the periphery, CD38 expression is low on resting T lymphocytes and is upregulated upon activation. On human T cells, CD38 was found to be constitutively associated with a subset of lipid rafts that possess high levels of Lck and CD3ζ, and plays an essential role in the initiation of CD38-mediated signaling cascade (Zubiaur *et al*., 2002; Munoz *et al*., 2003). Activation through CD38 is followed by phosphorylation of CD3ζ/ZAP-70 and Lck, mobilization of intracellular calcium, activation of the Raf1/MAPK signaling cascade and NF-κB translocation to the nucleus leading to transcription of *IL-2* (Zubiaur *et al*., 1997; Cho *et al*., 2000). CD38-activated T cells produce increased amounts of IFNγ, TNFα, TGFβ and GM-CSF (Cesano *et al*., 1998).

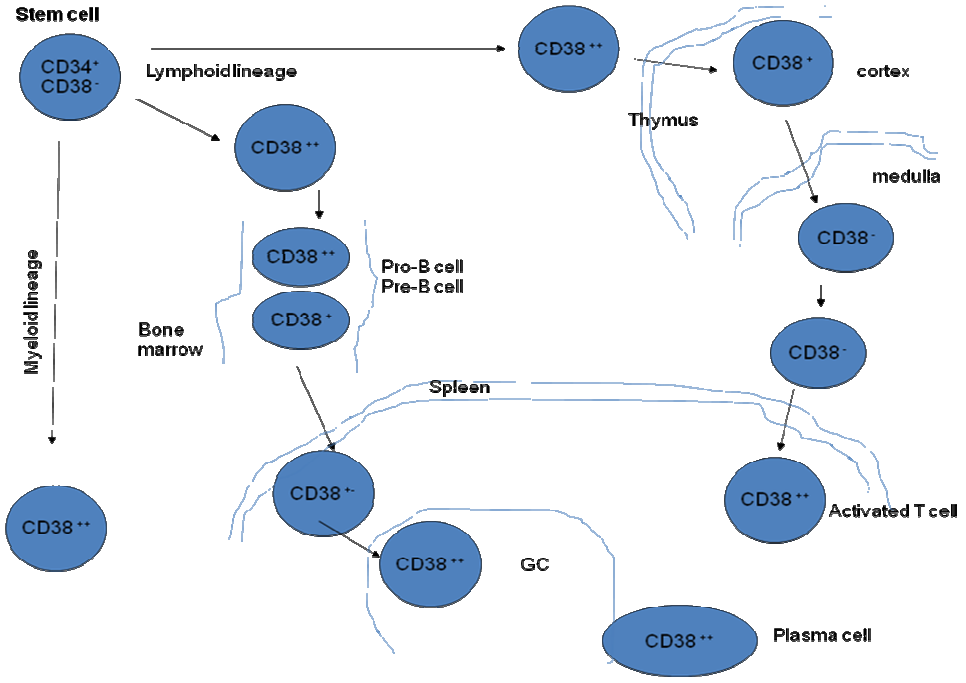


**Figure 1.** CD38 expression throughout lymphocyte development. Expression of CD38 is low on stem cells and is upregulated when cell lineage is decided. Its expression is high during T and B cell differentiation in the thymus and bone marrow, respectively. The expression of CD38 is downregulated when lymphocytes enter circulation. Upon activation, T and B lymphocytes reexpress CD38.

NK cells also express CD38. Activation of human NK cells via CD38 leads to calcium mobilization, CD3ζ, FcRIII-γ, ZAP-70 and c-Cbl phosphorylation, and IFNγ production (Deaglio *et al*., 2002). CD38 was found to be a cytotoxic trigger on IL-2-activated NK cells, being able to initiate signaling events that culminate in granzyme release by these cells (Scnocchia *et al*., 1999).

Expression of CD38 on cells of the myeloid lineage has also been reported (Tsujmoto *et al*., 1997; Inove *et al*., 1997). It was suggested that CD38 may have

functionally important functions on these cells, namely on monocytes/macrophages, neutrophils and DC. On myeloid cells, CD38 physically associates with CD32 (Inove *et al*., 1997). CD38 stimulation induces calcium mobilization and activates tyrosine kinases, contributing for the tyrosine phosphorylation of c-Cbl, which then binds to several targets, including PI3K (Matsuo *et al*., 1996), Fyn, Lck and Btk, GTPase-activating protein, PLC$\gamma$1, Nck, Lyn and Grb-2 (Kontani *et al*., 1996; Ausiello *et al*., 1995). CD38 ligation initiates superoxide production by formyl-Met-Leu-Phe (fMLP)-stimulated myeloid cells, through triggering of the chemotactic receptor/G1-mediated pathway (Tsujimoto *et al*., 1997). On human monocytes, CD38 was found to be physically associated with MHC II molecules (Zilber *et al*., 2000). Activation of monocytes via CD38 induces intracellular calcium mobilization and phosphorylation of many intracellular substrates that belong to the MHC II pathway, such as src-type phosphotyrosine kinases hck and fgr (Zilber *et al*., 2000). In addition, CD38 controls the response to respiratory burst activators (Zilber *et al*., 2005). CD38 was proposed to act as a co-receptor for MHC II molecules augmenting macrophages' responses to superantigens (Zilber *et al*., 2000). On human DC, the cascade elicited by CD38 ligation leads to intracellular calcium mobilization, upregulation of CD83 and production of IL-12, this way modulating DC maturation (Fedele *et al*., 2004). In humans and mice, CD38 was shown to be required for DC migration towards CCL2, CCL19, CCL21 and CXCL12, as it controls the CCR2-, CXCR4- and CCR7-mediated signaling cascades (Frasca *et al*., 2006; Partida-Sànchez *et al*., 2004). On neutrophils, CD38 was shown to control chemotaxis towards fMLP peptides, a consequence of reduced production of cADPR (Partida-Sànchez *et al*., 2001).

CD38 may also behave as a selectin, through interaction with CD31 (Malavasi *et al*., 1994; Deaglio *et al*., 1998; Deaglio *et al*., 2000; Dianzani *et al*., 1994). CD38/CD31 interactions initiate a signaling cascade characterized by fast and complex effects, including modulation of expression of cell surface molecules and alterations in intracellular calcium, which acts as a second mensager inside the cell (Deaglio *et al*., 1998). The changes induced by CD38/CD31 interactions include CD38 internalization, up-regulation of CD29 ($\beta$ chain of VLA$\beta$1 integrin subunit platelet GPII$\alpha$, expressed on leukocytes) and CD54 (also known as ICAM-1, expressed on endothelial cells, T and B cells and monocytes), and cytokine secretion, namely IL-6, IL-10, IFN$\gamma$ and GM-CSF (Deaglio *et al*., 2000). Moreover, CD38/CD31 ligation also leads to NF-$\kappa$B translocation to the nucleus and upregulation of other ectoenzymes, such as PC-1 and CD73 (Deaglio *et al*., 1998). CD38/CD31 interactions paralleled some, but not all *in vitro* observations made after ligation of CD38 by monoclonal antibodies in several cell lines, suggesting the existence of other yet unidentified ligands (Deaglio *et al*., 2000; Malavasi *et al*., 2008).

CD38 is the predominant ADP-ribosyl cyclase expressed by haematopoietic cells (Partida-Sànchez *et al*., 2001). CD38 is known to control mono (ADP-ribosyl) transferase (ART)-mediated NAD metabolism by competing for the substrate, thereby limiting ART access to NAD (Krebs *et al*., 2005). Cumulating evidence supports a role for purinoceptors in immune modulation; their ligands, including NAD, are currently recognized as danger signals (Haag *et al*., 2007; Di Virgilio *et al*., 2001; la Sala *et al*., 2003; Hanley *et al*., 2004; Di Virgilio, 2005). NAD activates P2X7 receptors through ART. This interaction is very stable, requires micromolar concentrations of NAD, and induces CD62L shedding, exposure of phosphotidylserine and cell death (Koch-Nolte *et al*., 1996; Di Virgilio *et al*., 2001; Seman *et al*., 2003). The role of NAD-induced killing *in vivo* is far from being understood. Purine nucleotides are normally released during inflammatory or cellular stress and may alert the immune system to tissue damage. The released NAD accumulates in the extracellular environment and may induce selective killing of regulatory and naive T cells, thus limiting the chance of activation of undesired T cells while allowing primed T cells to proliferate. When tissue damage is controlled, NAD levels decrease, allowing regulatory T-cell activation and proliferation and, thus, termination of the ongoing immune response. So, CD38, by competing with ART for NAD, is able to control ART-mediated NAD metabolism. As such, CD38 may be an active player in tailoring of T cell responses to damage/challenge.

The use of CD38KO mice provided a further insight to the functions of this ectoenzyme. B6.129P2-Cd38$^{tm1/Lnd}$ mice (CD38KO mice) were generated by substitution of exons two and three of *CD38* with the neomycin gene. CD38KO mice share the C57Bl.6 genetic background, are viable and fertile. They have a normal distribution and ratio of T and B lymphocytes and the numbers of granulocytes, macrophages and neutrophils are within normal ranges (Cockayne *et al*., 1998). CD38KO mice present a deficit in intracellular calcium mobilization (Takasawa *et al*., 1993; Partida-Sànchez *et al*., 2001). These mice produce decreased amounts of IgM, $IgG_1$ and IgE and have diminished humoral responses against T-dependent antigens, suggesting that CD38 is functionally important in B cells. In contrast, humoral responses against T-independent antigens are increased (Cockayne *et al*., 1998).

In conclusion, there is cumulating evidence that suggest that CD38 has important immunological functions, being able to modulate the outcome of cellular activation. However, the mechanisms by which CD38 is able to achieve this modulation are far from being understood. As pathological situations are usually very informative on the physiological significance of different molecules, it is relevant to study the role of CD38 in a disease context.

## 1.2. CD38 IN DISEASE

CD38 appears to play a role on distinct human pathological situations, such as HIV infection (Echaniz *et al*., 1993; Giorgi *et al*., 1993), myeloid cell malignancies (Marinov *et al*., 1993), prostate cancer and lymphocytic leukemias (Kramer *et al*., 1995; Durig *et al*., 2002), diabetes (Pupilli *et al*., 1999) and chronic autoimmune thyroiditis (Antonelli *et al*., 2001). It is currently used as a marker with clinical significance for some of these conditions. In mice, CD38 appears to play a role in immunity against *Steptococcus pneumoniae* (Partida-Sànchez *et al*., 2001). Furthermore, CD38 was suggested to be required for resistance to development of type I diabetes (Chen *et al*., 2006; Krebs *et al*., 2005).

### 1.2.1 CD38 and infection

As CD38 is functionally important in several immune cells, it was hypothesized that it could play a role in development of immune responses against invading pathogens, such as viruses and bacteria (Lee *et al*., 1997). This hypothesis has been confirmed in several studies, namely with regard to human HIV infection and murine infection by extracellular bacteria.

CD38 has been used as a prognosis marker in HIV infection. The increased expression of CD38 correlates with a bad prognosis (Giorgi *et al*., 1993). CD38 specifically inhibits gp120/CD4 binding and, in this way, prevents HIV access to target cells (Savarino *et al*., 1999; Savarino *et al*., 2003). An increase in the number of CD38 expressing $CD8^+$ and $CD4^+$ T cells was observed in patients with active HIV infection. However, a high percentage of these cells was also found to have a reduced effector activity and to be more susceptible to Fas-mediated apoptosis. These features could explain the inability of patients to control HIV infection and the higher plasma viremia observed (Chun *et al*., 2004; Zaunders *et al*., 2005). However, these studies failed to elucidate the mechanisms in which CD38 may be involved.

As for bacteria, CD38 was proposed to play a role in immunity against *S. pneumoniae*, as CD38KO mice were shown to be more susceptible to infection by these extracellular pathogens. This increased susceptibility was associated with absence of CD38 in the haematopoietic system and to a reduced chemotactic response to some but not all chemoattractants. For instance, CD38KO neutrophils are unable to migrate towards fMLP but not to IL-8. This effect was associated with CD38-mediated production of

cADPR, which is required for intracellular calcium mobilization and migration of neutrophils towards peptides of bacterial origin (Partida-Sànchez *et al*., 2001).

Being important in viral infections and having a key role in the modulation of immune responses, namely at the level of Th1 cytokine secretion, it is possible that CD38 may also be involved in immunity against intracellular bacteria, namely mycobacteria.

#### 1.2.1.1 Mycobacteria

The genus *Mycobacterium* is composed of nonmotile, non-spore forming, rod-shaped, slightly curved or straight, aerobic bacilli ranging from 0.2x1mm to 0.6x10mm in size. The members of this family share over 99% DNA similarity being distinguished by the presence of single nucleotide polymorphisms (Smith, 2003). The mycobacterial genome has $4.4x10^6$ base pairs and is composed of approximately 4000 genes. Another striking characteristic of mycobacteria is their complex lipid-rich envelope, constituted by a peptidoglycan skeleton and a cord factor. This envelope is responsible for many of the characteristic properties of mycobacteria, such as acid-fastness, slow growth, resistance to detergents, resistance to common antibacterial antibiotics and antigenicity (Smith, 2003).

Mycobacteria strains are able to grow over a wide temperature range, to rapidly adapt to new conditions and to grow well between pH 4.0 - 7.5 (Chapman *et al*., 1962; Portaels *et al*., 1982). Mycobacteria are primarily found in the soil. It is thought that domestication of cattle allowed mycobacteria to evolve, cross the species barrier and parasite humans (Kirschner *et al*., 1992; Kirschner *et al*., 1999; Covert *et al*., 1999; Thorel *et al*., 1997). Taxonomically, mycobacteria fall into two main groups: slow- and fast-growers, the most pathogenic members being found in the former. Mycobacteria have the ability to infect a number of species including rodents, birds, fish and humans. Despite the high number of mycobacterial species, more than 95% of all human infections are caused by only seven species, *M. tuberculosis*, *M. leprae*, *M. avium-intracellulare* complex, *M. kansaii*, *M. fortuitum*, *M. chelonae* and *M. abscessus*.

Among the human most life threatening intracellular bacteria are *Mycobacterium tuberculosis* (Mtb) and *Mycobacterium leprae*. *Mycobacterium avium* complex (MAC), which is usually not life threatening, is becoming an increasing problem especially in immunocompromised and immunodeficient individuals (Orme *et al*., 1992) especially in the context of HIV infections. Mtb is the causative agent of tuberculosis (TB) (Koch, 1882). Mtb infection is spread by close person-to-person contact through the inhalation of

infectious aerosols, which then travel to the terminal airways and establish infection. Even though TB can virtually involve any organ, it is usually restricted to the lungs. In contrast to Mtb, the MAC route of infection is yet to be determined, but the gastrointestinal and the respiratory tract are thought to be the primary routes (Orme *et al*., 1992; Chin *et al*., 1994; von Reyn *et al*., 1994).

#### 1.2.1.2 Immunity to Mycobacteria

Mycobacteria are intracellular pathogens that mainly infect macrophages, in which they can survive by circumventing macrophages' microbicidal mechanisms.

The majority of studies regarding the immune response to mycobacteria have concerned Mtb infection. Wallgren (1948) described the progression and resolution of TB in four stages. The first stage begins with inhalation of Mtb containing aerosols. This stage lasts from three to eight weeks and is characterized by dissemination of mycobacteria through the lymphatic circulation to the lymph nodes (LN) of the lungs. The second stage is characterized by haematogenous circulation of mycobacteria. This stage lasts about three months. At this point, some individuals can suffer from an acute fatal disease, also known as disseminated tuberculosis. The next three to seven months (3$^{rd}$ stage) are marked by pleural surface inflammation due to either haematogenous mycobacterial dissemination or release of mycobacteria into the pleural space. This stage can be delayed for up to two years. The fourth stage corresponds to resolution of infection. The disease does not progress, but becomes persistent. At this point, the mycobacteria are still viable, but are growing and being killed at similar rates. Even though some mycobacteria may be released into the surrounding microenvironment, they are unlikely to survive as the bacterial foci are surrounded by a fibrous wall that impedes delivery of nutrient and oxygen supply to the mycobacteria. In addition, free mycobacteria are ingested by fully activated macrophages and are readily killed. The disease may be reactivated when Mtb-infected individuals become immunosupressed.

Protective immunity against mycobacteria requires development of cell-mediated responses, but is also dependent on innate defence mechanisms. Resident macrophages are among the first cells that encounter this pathogen and, as such, the initial containment of mycobacterial growth and proliferation is dependent on the intrinsic microbicidal capacities of macrophages.

Mycobacteria have several secreted immunodominant proteins, such as early secreted antigenic target of 6kDa (ESAT6) and 19kDa protein, which are common to all

pathogenic mycobacteria, and may have a role in virulence. Mycobacteria also have a number of immunodominant cell surface components, such as lipoarabinomannan (LAM) and the mycolyl-transferase complex of enzymes (FbpA). LAM is a complex glycolipid of repeated arabinose-mannose disaccharide subunits, which seems to be responsible for downmodulation of host defences, thus, favouring mycobacterial survival. The FbpA protein complex (also known as antigens 85A, B and C) is a component of the mycobacterial cell wall and envelope that is unique to the pathogenic members of this family (Smith, 2003).

Host cells recognize mycobacterial pathogen-associated molecular patterns (PAMPs) through Toll-like receptors (TLR) and cytosolic nucleotide-binding and oligomerisation domain-like receptors, such as NOD1 and NOD2 (Visintin *et al*., 2001; Yang *et al*., 2007; Ferwerda *et al*., 2005; van Crevel *et al*., 2002; Quesniaux *et al*., 2004; Zahringer *et al*., 2008). TLRs are phylogenetically conserved and are expressed by macrophages and DC (Visintin *et al*., 2001). There are nine different TLRs, recognizing different mycobacterial proteins. For instance, TLR2 recognizes LAM, the heterodimer TLR2/TLR6 binds the 19kDa mycobacterial lipoprotein, and TLR9 recognizes CpG dinucleotides of mycobacterial origin (van Crevel *et al*., 2002). TLRs signal through IRAK and MyD88, leading to translocation of NF-$\kappa$B to the nucleus and production of type I IFNs and TNF$\alpha$, by macrophages and DC (O' Neill *et al*., 2007; O' Neill, 2008; Trinchieri *et al*., 2007). Activated macrophages and DC then induce IFN$\gamma$ production by NK, Th1 and $CD8^+$ T cells (Quesniaux *et al*., 2004; Ryffel *et al*., 2005; Reiling *et al*., 2008). As for NOD receptors, of the twenty different receptors described so far, only NOD1 and NOD2 have been implicated in recognition of intracellular bacteria. These receptors recognize *meso*-diaminopimelic acid and muramyl dipeptide (Quesniaux *et al*., 2004; Zahringer *et al*., 2008). NOD2 receptors act in synergy with TLR2, activating the NF-$\kappa$B signalling cascade and the inflammasome/caspase1 pathway to generate proinflammatory cytokines and chemokines (Yang *et al*., 2007; Ferwerda *et al*., 2005), this way contributing to recruitment of neutrophils to the site of infection (Fritz *et al*., 2006).

Phagocytosis of mycobacteria can be triggered through a number of different receptors (Aderen *et al*., 1999; Hirsch *et al*., 1994; Schlesinger *et al*., 1993; Tailleux *et al*., 2003; Yadav *et al*., 2006; Bermudez *et al*., 1991; Bermudez *et al*., 1991a; Bohlson *et al*., 2001). For instance, mycobacteria are able to use complement receptors (CR1, CR3 or CR4), facilitating their uptake in opsonin-poor microenvironments, such as the lung. Mycobacterial terminal mannose residues can be recognized by macrophages. Furthermore, mycobacteria may also be ingested through type A scavenger receptors, Fc$\gamma$

receptors, surfactant proteins and C1q. The choice for one or another entrance mechanism appears to relate to the fate of the intracellular pathogen. For instance, the Fcγ receptor is linked to an inflammatory response; entrance through this receptor may be deleterious to the mycobacterium. In contrast, the use of complement receptors does not elicit an inflammatory response favouring mycobacterial survival. The use of surfactant proteins and mannose receptors as means of mycobacterial entrance inhibits the production of oxygen and nitrogen intermediates and is mainly used by virulent mycobacteria, suggesting that this route is also advantageous for the mycobacterium (van Crevel *et al*., 2002).

After phagocytosis, mycobacteria are directed to specialized phagosomes. These vesicles fuse with lysosomes forming the phagolysosome. One of the mechanisms employed by macrophages to control mycobacterial growth is the acidification of the phagolysosome to pH values of 4-5, to allow for optimal functioning of the lysosomal enzymes. However, mycobacteria can prevent or delay fusion of phagosomes with lysosomes and are able to modulate the phagosomal compartment by preventing the incorporation of the vesicular proton ATPase and, consequently, phagolysosome acidification (Sturgill-Koszycki *et al*., 1994; Gomes *et al*., 1999; Kelley *et al*., 2003; Mueller *et al*., 2006). Another microbicidal mechanism is the respiratory burst leading to the production of reactive oxygen intermediates (ROI), such as superoxide and hydrogen peroxide. Reactive nitrogen intermediates (RNI) are also produced. While it is clear that the respiratory burst is an important microbicidal mechanism in Mtb infection (Cooper *et al*., 2002a), conflicting evidence has been published in MAC infection (Appelberg *et al*., 1993; Ohga *et al*., 1997; Tian *et al*., 1995; Doherty *et al*., 1997; Gomes *et al*., 1999a). Mycobacteria have also evolved ways to circumvent this microbicidal mechanism and survive. Mycobacteria have oxygen radical scavengers, such as sulfatides and LAM, and are able to modulate the host acquired immune response, by dampening the production of IFNγ and, consequently, the expression of IFNγ-induced genes, such as *iNOS* (Martino, 2008; van Crevel *et al*., 2002; Kusner, 2005; Sturgill-Koszycki *et al*., 1994; Xu *et al*., 1994; Stugill-Koszycki *et al*., 1996; Clemens *et al*., 1990).

Also in response to infection, cells resident at the site of infection produce chemokines and cytokines, initiating an inflammatory response. It is a physiologic response that aims at containment and restriction of the damage to the site. Damaged cells secrete cytokines and chemokines, such as macrophage inflammatory protein-1 alpha and beta (MIP-1α and MIP-1β that attract leukocytes. Once at the site, these leukocytes are activated and help in clearance of the invading pathogen. The first

responders to damage are the neutrophils which peak at 6 hours and decline thereafter. A wave of monocytes and T cells follows. Until the damage is contained or eliminated the inflammatory response is continuous. The inflammatory response is a complex process and depends on the tight control of leukocyte migration. This is a well orchestrated and sequential process that starts with casual collisions of leukocytes with the endothelial layer surrounding vessels of every type. Migration of blood cells can be divided into three stages: i) exit of blood cells from the circulation by adhering loosely to the vessel wall, a process known as rolling; ii) termination of rolling followed by a tight adhesion to the endothelium; and iii) transendothelial migration (Muller *et al*., 1993; Albelda *et al*., 1991). Four distinct families of transmembrane glycoproteins have been implicated in this process: integrins (largely distributed group of receptors that mediate leukocyte-leukocyte, leukocyte-endothelial cell adhesions, and cellular interactions with extracellular matrix components); cadherins (glycoproteins that mediate calcium-dependent cellular adhesion in a hemophilic and tissue-specific manner); selectins (Lec-CAM; bind leukocytes to endothelial cells) and cell adhesion molecules (CAM; belong to the immunoglobulin superfamily and are believed to participate in a variety of homophilic and heterophilic cellular interactions) (Albelda *et al*., 1991).

Macrophages are not the only innate immune cells involved in the response against mycobacteria. Neutrophils, NK cells, NK T cells and $\gamma\delta$ T cells also play a role. In Mtb infection, the role of neutrophils is still controversial (Pedrosa *et al*., 2000; Sugawara *et al*., 2004; Seiler *et al*., 2000; Eruslanov *et al*., 2005; Aleman *et al*., 2002; Aleman *et al*., 2005; Aleman *et al*., 2007; Tan *et al*., 2006). In MAC infection, decreased recruitment of these cells to the site of infection leads to increased susceptibility, a feature proposed to be due to the neutrophils' ability to enhance macrophages' bacteriostatic activity (Flórido *et al*., 1997; Appelberg, 1992; Appelberg *et al*., 1995; Ogata *et al*., 1992). Despite the reduced evidence on the role of NK and NK T cells in mycobacterial infection, they were proposed to have protective effects (Bermudez *et al*., 1990; Junqueira-Kipnis *et al*., 2003; Feng *et al*., 2006). Upon activation, NK and NK T cells produce IFN$\gamma$ this way being able to activate the macrophages' microbicidal mechanisms before the development of an acquired immune response. These cells are also able to induce Th1 differentiation (Scharton-Kersten *et al*., 1997; Scharton *et al*., 1993; Sieling *et al*., 1995). $\gamma\delta$ T cells also play an important role in protection against Mtb (Boom, 1996; Chein *et al*., 1996; Tsukaguchi *et al*., 1995). However, their role appears to be limited to the production of cytokines and chemokines that allow the recruitment of inflammatory cells into the infected areas (D'Souza *et al*., 1997; Roberts *et al*., 1996; Thoma-Uszynski *et al*., 1997). Regarding MAC infection, conflicting evidence on the role of these cells has been

published: while *in vitro* studies support a role for γδ T cells in protection against MAC infection (Bermudez *et al*., 1995), *in vivo* data failed to confirm these observations (Saunders *et al*., 1998; Petrofsky *et al*., 2005).

$CD8^+$ T cells have also been implicated in anti-mycobacterial responses (Flynn *et al*., 1992). These cells recognize and directly lyse infected macrophages. It was proposed that, in humans, the protective role of $CD8^+$ T cells against Mtb was mediated through the secretion of granulysin (Shen *et al*., 2002).

DC uptake mycobacterial antigens and, once activated, travel to peripheral lymphoid organs where they engage in antigen presentation. Mycobacterial antigens are processed in phagolysosomal compartments and presented, in the context of MHC II, to antigen-specific $CD4^+$ T cells. In the presence of co-stimulatory signals, acquired immune responses are initiated. Mycobacterial antigens can, for some reason, escape from the phagolysosomal compartment to the cytosol. In this case, MHC I molecules are responsible for the presentation of mycobacterial antigens to $CD8^+$ T cells (van Crevel *et al*., 2002).

In response to microbial stimuli, macrophages and DC produce IL-12 (D'Andrea *et al*., 1992; Ma *et al*., 2001; Langrish *et al*., 2004) and TNFα (Valone *et al*., 1988; Henderson *et al*., 1997). IL-12 is an important regulator of innate and acquired immune responses; it leads to the production of IFNγ by NK and NK T cells, at early stages of infection, and drives development of Th1-mediated immune responses, which are protective against mycobacterial infections (Trinchieri, 2003; Manetti *et al.*, 1994; Cooper *et al.*, 1997; Cooper *et al*., 2002). Upon activation, DC upregulate the expression of costimulatory molecules, such as CD40. CD40 interacts with its ligand on T cells, CD40L, leading to their activation (Kennedy *et al*., 1996; Grewal *et al*., 1998). CD40/CD40L interactions have been shown to play a role in immunity against Mtb and MAC (Flórido *et al*., 2004; Samten *et al*., 2000; Samten *et al*., 2003). Ligation of CD40 to CD40L is associated with production of nitric oxide by macrophages (Tian *et al*., 1995). Disruption of CD40/CD40L interactions lead to delayed IL-12 production which may compromise timed development of Th1-mediated immune responses, suggesting an accessory role for CD40/CD40L interactions in immunity against this pathogen (Flórido *et al*., 2004; Hayashi *et al*., 1999; Kennedy *et al*., 1996).

Immunity to mycobacteria requires development of Th1 immune responses (Winslow *et al*., 2008; Sohal *et al*., 2008; Flynn *et al*., 2001; Raupach *et al*., 2001) and granulomatous responses (Hansch *et al*., 1996; Saunders *et al*., 2007; Orme *et al*., 1994;

Cooper *et al*., 2002). Granulomata are organized host immune structures, composed of tightly interposed macrophages and other cells, such as T cells, that are essential for host containment of mycobacteria (Ehlers *et al*., 2000; Volkman *et al*., 2004). Granulomatous lesions appear to be initiated by non-specific inflammatory signals arising from the interaction of tissue macrophages with microbial products. Macrophages are attracted to the site of microbial invasion through a process mediated by chemokines and cytokines which cause up-regulation of adhesion molecules on both leukocyte and endothelial cells (Volkman *et al*., 2004, Roach *et al*., 2002). Following the accumulation and activation of macrophages, the inflammatory lesion begins to take the granulomatous form. With arrival of antigen-specific $CD4^+$ T cells, the lesion develops into a mature granuloma where activation of macrophages by IFN$\gamma$ and TNF$\alpha$ results in inhibition of microbial growth. IFN$\gamma$ is essential for full activation of macrophages' microbicidal mechanisms (Cooper *et al*., 1993; Pearl *et al*., 2001) whereas TNF$\alpha$ plays a central role in the formation of granulomata (Kindler *et al*., 1989; Orme *et al*., 1999; Tsenova *et al*., 1999). Absence of either cytokine results in disseminated mycobacterial infection that is usually fatal for the host. Eventually, the granuloma becomes encapsulated by a fibrous wall and the centre becomes necrotic (Sneller, 2002; Flórido *et al*., 2004a). The fully matured granuloma protects the host not only by promoting mycobacterial containment, but also by reducing nutrient supply to the pathogens (Sneller, 2002).

Newly described cytokines, such as IL-23 and IL-17, have also been proposed to play a protective role in mycobacterial infections, although the mechanisms by which this protection is achieved are still unclear (Zelante *et al*., 2007; Khader *et al*., 2007; Umemura *et al*., 2007; Wozniak *et al*., 2006; Khader *et al*., 2005; Sergajava *et al*., 2005). IL-23, produced by DC, was shown to induce production of IFN$\gamma$ by activated T cells and is suspected to have a more prominent role during priming and expansion of memory responses against mycobacteria than during effector responses (Khader *et al*., 2005). IL-17, produced by activated Th17 cells, was proposed to be responsible for recruitment of mononuclear and polymorphonuclear cells into the lungs of mycobacteria-infected mice and to play a role in granulomata maintenance (Umemura *et al*., 2007; Sergajava *et al*., 2005; Dragon *et al*., 2008; Zelante *et al*., 2007; Cruz *et al*., 2006). Similarly to IL-23, IL-17 is suspected to participate in the late phases of mycobacterial infection (Cooper *et al*., 2008).

### 1.2.2 CD38 and autoimmunity

CD38 expression is under tight control during lymphopoiesis being expressed by discrete T and B cell subsets. These observations suggest that CD38 may be involved in differentiation of these cells, namely at the level of receptor rearrangements. As deregulated selection of the mature T and B cell repertoire may lead to altered tolerance mechanisms, it was hypothesized that CD38 could to play a role in development of autoimmunity. This hypothesis has been further supported by studies on organ-specific autoimmune diseases, namely diabetes. Autoantibodies against CD38 were detected in Caucasians patients with type 1 diabetes (Pupilli *et al*., 1999) and in chronic autoimmune thyroiditis patients (Antonelli *et al*., 2001). Anti-CD38 autoantibodies were shown to correlate with a more aggressive form of chronic autoimmune thyroiditis and were found to be independent of classical markers for thyroid autoimmunity (Antonelli *et al*., 2001a). However, how CD38 is involved in pathogenesis is still unclear. In mice, absence of CD38 was associated with accelerated development of diabetes in NOD mice. Earlier development of disease was linked to a peripheral effector: regulatory T-cell imbalance, which was proposed to be due to increased NAD-induced killing of the latter. It is believed that CD38 competes with ART for NAD, this way, preventing the selective death of T regulatory cells (Chen *et al*., 2006; Krebs *et al*., 2005).

However, while cumulating evidence suggest a role of CD38 in the control of organ-specific autoimmune diseases, only indirect evidence, suggestive of the involvement of CD38 in systemic autoimmune diseases, was published, namely in the context of systemic lupus erythematosus (SLE). Due to its localization and functional role within the immune system, *CD38* was proposed as a candidate gene for lupus-susceptibility *loci* in humans (Harley *et al*., 2002; Olson *et al*., 2002; Kelly *et al*., 2002) and mice (Vidal *et al*., 1998). Moreover, in humans, the CC genotype of *CD38,* at position 182 of intron 1, was found to be associated with resistance to discoid rash development in Spanish SLE patients (Ferrero *et al*., 1999a; González-Escribano *et al*., 2004). Furthermore, SLE patients presented an increase in CD38 expressing B and T lymphocytes (Al-Janedi *et al*., 1993; Spronk *et al*., 1996; Lindquist *et al*., 1999; Pavón *et al*., 2006). In mice, the murine *Lmb2 locus,* located on chromosome 5, was associated with susceptibility to develop a lupus-like disease. This region is syntenic to the human 4p15 region and has been linked to the production of anti-double stranded DNA (dsDNA) autoantibodies (Vidal *et al*., 1998).

### 1.2.2.1 Systemic Lupus Erythematosus

SLE and discoid lupus were first described on the second half of the 19th century by Cazenave, Kaposi and Osler (Benedek, 1997). It is a systemic autoimmune disease afflicting various organs. Disease onset varies from person to person. It can have an acute, occasionally catastrophic, onset with fever, fatigue and weight loss, or a relatively mild onset with few symptoms. A number of systems can be affected, including muscle, joints, central and peripheral nervous system, lungs, heart, skin, serous membranes, blood vessels and kidneys (Lahita, 2004).

SLE is a multifactorial disease with an unknown ethiology that is characterized by a number of cellular abnormalities. SLE development involves a combination of genetic, hormonal and environmental factors. A number of chromosomal abnormalities have been linked to the development of SLE. Genes that play a role in development of SLE are, most likely, involved in immune pathways, such as immune-complex (IC) clearance, B-cell signalling, regulation of apoptosis, antigen processing and presentation, TCR conformation and Ig structure. Genes that encode natural autoantibodies or regulate hormone structure or hormonal receptors may also play a role in lupus development. One of the first genetic factors to be identified and linked to the initiation of SLE was the *MHC* (Reveille, 1999). However, it became clear that other genes had to be involved. In fact, known SLE susceptibility genes include various *loci* on chromosomes 1, 4, 5, 7 and 17. Non-MHC genes have different degrees of influence and low penetrance and, as such, no individual gene is, by itself, sufficient for lupus development (Criswell, 2008).

Hormonal factors that contribute to SLE development include estrogens (Zandman-Goddard *et al*., 2007), prolactin (Orbach *et al*., 2007), estradiol (Lahita *et al*., 1981) and vitamin D (Arnson *et al*., 2007).

Environmental factors, such as drugs and UV light (Yung *et al*., 1996), viruses (Barzilai *et al*., 2007) and vaccines (Schoenfel *et al*., 2000; Cohen *et al*., 1996) may also predispose for SLE development. A number of autoantigens produced in lupus share homology and immunological crossreactivity with viral proteins (Barzilai *et al*., 2007a). Microbial CpG DNA may induce a breach in peripheral tolerance, through the induction of cytokine production by leukocytes, inhibition of apoptosis or prevention of autoreactive B cell deletion. Furthermore, it may act as an antigen being preferentially uptaken by anti-DNA specific B cells (Krieg, 1995).

Due to the impossibility of using human subjects to study the immunopathological mechanisms of SLE, as a result of ethical considerations and the inability to control an

immense number of variables, researchers have turned to murine models. In fact, much of what is currently known on this subject comes from studies using murine models that share many of the characteristics of human SLE. These models can be divided into two groups: spontaneous (New Zealand Black (NZB), NZB/W, MRL/*lpr*, C57Bl.6-*lpr/lpr* and BXSB) (Kono *et al*., 2004), and induced (heavy metal-induced lupus, drug-induced lupus, pristine-induced lupus) models (Satoh *et al*., 1994; Satoh *et al*., 1995; Monestier *et al*., 1992). Despite different timings of disease onset (Andrews *et al*., 1978; Mellors, 1965), all spontaneous models share histopathologic and serologic features as well as immunological abnormalities. All these models develop glomerulonephritis (GN) caused by the increased production autoantibodies, due to B cell hyperactivity. This hyperactivity seems to be a consequence of loss of regulatory function of T cells and abnormal T cell help (Rudofsky *et al*., 1999; Powrie *et al*., 2003; Shevach *et al*., 2000; Rajagopalan *et al*., 1990; Desai-Mehta *et al*., 1994; Takeno *et al*., 1997; Holyst *et al*., 1997; Fields *et al*., 2005).

### 1.2.2.2 Immunopathogenesis of lupus

Two models have been proposed for lupus development and both place DC as central players. The first model proposes viral infections as triggers of autoimmunity development, whereas the second supports a role for apoptotic material as the cause of disease initiation (Baccala *et al*., 2007). Apoptotic material may become available to plasmacytoid DC (pDC) following excessive tissue damage or defective clearance of apoptotic material (Marshak-Rothstein *et al*., 2007). pDC uptaken apoptotic material bind to intracellular TLR7 (RNA-containing IC) or TLR9 (DNA-containing IC) that are specialized in recognition of nucleic acids. Activation of these TLRs initiate a signalling cascade that culminates in the production of proinflammatory cytokines, such as type I IFNs, IL-1, IL-6, IL-8, IL-18 and TNF$\alpha$ (Means *et al*., 2005; Wada *et al*., 1999; Ronnblom *et al*., 2006), and chemokines, including MIP-1$\alpha$ and MIP-1$\beta$, *regulated upon activation normal T cell express sequence* (RANTES) and monocyte chemoattractant protein 1 (MCP-1) (Means *et al*., 2005a; Wada *et al*., 1999), and in the upregulation of MHC II molecules (Pisetsky, 1996; Janeway *et al*., 2002; Leadbetter *et al*., 2002; Viglianti *et al*., 2003; Hoffman *et al*., 2004; Scheinecker *et al*., 2001). Type I IFNs induce maturation of lymphoid and myeloid DC. These will then present autoantigens to and activate autoreactive T cells. Activated autoreactive T cells provide help to autoreactive B cells leading to their differentiation, isotype switching and production of autoantibodies (Sigurdsson *et al*., 2005; Gregersen *et al*., 2006; Hron *et al*., 2004; Ronnblom *et al*., 2006;

Banchereau *et al*., 2006). The increased production of autoantibodies leads to the formation of IC that will be deposited virtually anywhere in the body, including the kidneys, and initiate local inflammatory events. Those IC that do not accumulate in the kidneys remain in circulation, and will be recognized by circulating pDC, this way perpetuating the autoimmune loop.

Kidneys, due to their filtering function, are the most affected organs in SLE. GN is caused by autoantibodies, a consequence of hyperactivation of autoreactive B cells. Autoantibodies can mediate damage either directly, through binding to glomerular antigens (Ohnishik *et al*., 1994; Tsao *et al*., 1992; Ehrenstein *et al*., 1995; Madaio *et al.*, 1996; Vlahakos *et al*., 1992; Gilkeson *et al*., 1995; Tsao *et al*., 1992a), or indirectly, through the formation of IC (Sasaki *et al*., 1991; Van Bruggen *et al*., 1996; Lefkowith *et al*., 1996; Termaat *et al*., 1990). These mechanisms impair the kidneys' normal physiological function, trigger local inflammation, due to their ability to initiate the complement cascade, and consequently disrupt the kidney's structure and function (Kishore *et al*., 2000). Damaged kidneys become a continuous source of autoantigens that contribute to the perpetuation of the undesired immune response.

Production of autoantibodies is a consequence of autoreactive B cell hyperactivation. Autoreactive B cells contribute to maintenance of tolerance and development of immune responses against pathogens (Arnold *et al*., 1993; Kim *et al*., 2002; Ehrenstein *et al*., 2000; Werwitzke *et al*., 2005; Davidson *et al*., 2004; Roosnek *et al*., 1991). These cells are normally found in healthy individuals but are under the control of peripheral regulatory mechanisms (Wardemann *et al*., 2003; Yurasov *et al*., 2006; Arnold *et al*., 1993). In lupus, a number of defects in B cells have been identified (Chan *et al*., 1999; Mamula *et al*., 1994; Chan *et al*., 1998; Chan *et al*., 2000; González *et al*., 1999; Lipsky, 2001). These defects can occur at three different levels: i) B cell activation thresholds (Kumar *et al*., 2006; Nimmerjahn *et al*., 2008; Xiang *et al*., 2007; Su *et al*., 2007; Bolland *et al*., 2000; Fukuyama *et al*., 2005; McGaha *et al*., 2005); ii) B cell longevity (Mackay *et al*., 1999; Gross *et al*., 2000; Lesley *et al*., 2004; Thien *et al*., 2004); and iii) apoptotic cell and/or antigen processing (Leadbetter *et al*., 2002; Viglianti *et al*., 2003; Boule *et al*., 2004; Bave *et al*., 2000; Bave *et al*., 2003; Means *et al*., 2005). Under conditions still to be defined, autoreactive B cells become hyperactivated and produce high amounts of autoantibodies against a variety of autoantigens, driving development of lupus (Martin *et al*., 2004; Harris *et al*., 2000; Lund, 2008; Fillatreau *et al*., 2008). Several mechanisms seem to be responsible for the activation of these cells, including excessive T cell help (Rajagopalan *et al*., 1990; Desai-Mehta *et al*., 1994; Takeno *et al*., 1997; Holyst *et al*., 1997; Fields *et al*., 2005), alterations in the availability of self-antigens (Walport *et*

*al*., 2002; Herrmann *et al*., 1998; Schlomchik, 2008; Lanzavecchia, 1995; Sercarz *et al*., 1993; Dighiero *et al*., 1999; Doyle *et al.*, 2002) and reduced function of regulatory T cells (Powrie *et al*., 2003; Shevach *et al*., 2000).

It is important to note that contribution of autoreactive B cells to lupus pathogenesis is not limited to their antibody-dependent functions (Chan *et al*., 1999a; Schlomchik, 2008). B cell antibody-independent functions, such as antigen presentation and recruitment of $CXCR5^+$ follicular T helper cells into germinal centers, may also play a role in lupus development (Mamula *et al*., 1994; Chan *et al*., 1998; Chan *et al*., 1999a; Chan *et al*., 2000; Schlomchik, 2008; Ebert *et al*., 2004; Vinuesa *et al*., 2005). However, B cell defects are *per se* insufficient to drive lupus development. A close interplay with autoreactive T cells is required for the initiation of the autoimmune process.

Autoreactive T cells that recognize lupus autoantigen-MHC complexes, such as anti-DNA, -histones, -SmB, -SmD, -U1-70kD, -U1-A and -hnRNPA2, have been identified in lupus patients and murine models of lupus (Hoffman *et al*., 1993; Fenning *et al*., 1995; Wolff-Vorbeck *et al*., 1994; Gallegos *et al*., 2006; Rajagopalan *et al*., 1990; Desai-Mehta *et al*., 1994; Lu *et al*., 1999; Desai-Mehta *et al*., 1996; O'Brien *et al*., 1990; Okubo *et al*., 1993; Holyst *et al*., 1997; Talken *et al*., 1999; Talken *et al*., 1999a). Similarly to autoreactive B cells, in healthy individuals, autoreactive T cells are usually under the control of peripheral regulatory mechanisms (Scalzo *et al*., 2006). They have decreased activation thresholds, altered signalling cascades, abnormal effector functions (Kammer *et al*., 2002; Elliot *et al*., 2003; Desai-Mehta *et al*., 1996; Yi *et al*., 2000; Prokunina *et al*., 2002; Kaliyaperumal *et al*., 1999; Vratsanos *et al*., 2001; Bouzahzah *et al*., 2003; Kammer *et al*., 1990; Horwitz *et al*., 1997; Dayal *et al*., 1996) and are refractory to the suppressive action of regulatory T cells (Budagyan *et al*., 1998; Gershon, 1975; Shevach, 2000; Powrie *et al*., 2003). Under unknown conditions, autoreactive T cells may be activated, providing excessive co-stimulatory signals to B cells, in the form of increased expression of CD40L, over-expression of LFA-1 and cytokine production (Desai-Mehta *et al*., 1994; Bijl *et al*., 2001; Richardson, 2003; Desai-Mehta *et al*., 1996; Yi *et al*., 2000; Yellin *et al*., 2000; Crow *et al*., 2001; Hondowicz *et al*., 2008). The role of different cytokines in lupus is still controversial. Classically, disease was thought to be dependent on T cell help mediated through Th2 cytokines. Hyperactivated $CD4^+$ lupus T cells overproduce IL-4 that, due to its B-cell stimulatory activity, was proposed to have a deleterious role (Erb *et al*., 1997; Snapper *et al*., 1992; Wong *et al*., 2000). IL-10, whose production is associated with accelerated proteinuria onset, GN and increased production of anti-dsDNA autoantibodies (Prud' home *et al*., 1995; Ishida *et al*., 1994) was also considered to be detrimental in a lupus context.

Antigen processing, presentation and defective clearance of apoptotic bodies may also be altered in lupus. It was proposed that, during apoptosis, the available autoantigens could be modified by the action of caspases, granzyme B or oxidative cleavage. These altered autoantigens could be perceived by the immune system as foreign eliciting an immune response (Walport, 2002; Herrmann *et al*., 1998; Yan *et al*., 2002; Kuchtey *et al*., 2003; Scheinecker *et al*., 2001; Casciola-Rosen *et al*., 1994; Andrade *et al*., 2000; Gridinger *et al*., 2001; Greidinger *et al*., 2002).

CD38 has been shown to have a non-ending number of immunological functions. So far, the evidence published has only clearly supported a role for CD38 in immunity against extracellular parasites and organ-specific autoimmunity. Nevertheless, a role for CD38 in immunity against intracellular bacteria or in systemic autoimmunity can be envisaged. We have thus decided to investigate the role of CD38 in these contexts. The compiled data will allow us to have a clearer picture and extend our understanding on the role of CD38 in immune homeostasis.

## 1.3. AIMS

CD38 belongs to a family of multifunctional ectoenzymes, the ADP-ribosyl cyclases, possessing signaling and cell adhesion properties, and extracellular/intracellular enzymatic activity. CD38 is expressed by a number of cell populations within and outside the immune system and was shown to have immunologically relevant functions in these cells. Within the haematopoietic system, CD38 is expressed by T and B cells, monocytes/macrophages, granulocytes and DC, and, by acting as a co-receptor, it is able to modulate the end result of cell activation. In mice, CD38 appears to play a role on distinct pathological situations, including infection by extracellular parasites, such as *S. pneumoniae* and autoimmune diseases, such as type I diabetes.

### 1.3.1 Role of CD38 in infection by intracellular pathogens.

In mice, CD38 was shown to be required for immunity against *S. pneumoniae*, as its absence rendered mice more susceptible to infection by this pathogen. This increased susceptibility was associated with a diminished neutrophil chemotaxis in response to peptides of bacterial origin. In humans, CD38 was proposed to have a protective role in HIV infection. However, the mechanisms by which this protection is achieved are, at this point, unclear. As CD38 appears to be important in viral infections and is able to modulate immune responses, namely at the level of Th1 cytokine secretion, it is conceivable that it could also play a role in immunity against intracellular pathogens, such as mycobacteria. One of the aims of the herein presented work was to investigate whether CD38 was required or participated in immunity against intracellular pathogens. The microorganism of choice was *M. avium*, as it was well established and studied model in our laboratory. To investigate the role of CD38 in development of immune responses against *M. avium*, we have used CD38KO and compared their immune response to this pathogen to that of C57Bl.6 mice, which share the same genetic background.

### 1.3.2 Role of CD38 in systemic autoimmune diseases.

In mice, absence of CD38 was shown to accelerate development of type I autoimmune diabetes in NOD mice. Early development of disease was associated with a peripheral effector: regulatory T-cell imbalance, due to increased NAD-induced killing of the latter cells. However, only indirect evidence pointing to a possible role for CD38 in systemic autoimmunity has been published. Human patients with active SLE have higher

numbers of CD38 expressing $CD8^+$ T cells and Ig-secreting B cells. CD38, due to its localization and functional role in the immune system, is a candidate gene for lupus-susceptibility *loci* in humans and mice. The other objective of this work was to investigate whether CD38 played a role in development of lupus-like disease. For that we have developed a new murine strain, CD38KO-*lpr/lpr* mice. These mice resulted from the cross of CD38KO with C57Bl.6-*lpr/lpr* mice. The latter strain was chosen because it is a well-established murine lupus model, shares the same genetic background of CD38KO mice and had been extensively studied in our laboratory. Development of a lupus-like disease in CD38KO-*lpr/lpr* was monitored and compared to that of C57Bl.6-*lpr/lpr* mice. The immunophenotype of these mice was also analysed.

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# Chapter Two

## 2. Role of CD38 in mycobacterial infections

2.1 **Viegas MS, Carmo A, Silva T, Seco F, Serra V, Lacerda M, Martins TC** (2007) CD38 plays a role in effective containment of mycobacteria within granulomata and polarization of Th1 immune responses against *Mycobacterium avium*. *Microbes Infect* 9: 847-854.

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Original article

# CD38 plays a role in effective containment of mycobacteria within granulomata and polarization of Th1 immune responses against *Mycobacterium avium*

Marta S. Viegas [a,*], Anália do Carmo [c,d], Teresa Silva [c], Francisco Seco [c], Vasco Serra [b], Manuela Lacerda [b], Teresa C. Martins [a,d]

[a] *Molecular Pathology Laboratory, Portuguese Institute for Oncology of Coimbra (IPOCFG, EPE), Av. Bissaya Barreto, 98, Apartado 2005, 3000-075 Coimbra, Portugal*

[b] *Histopathology Laboratory, Portuguese Institute for Oncology of Coimbra (IPOCFG, EPE), Av. Bissaya Barreto, 98, Apartado 2005, 3000-075 Coimbra, Portugal*

[c] *Vasco da Gama University School, Quinta de S. Jorge, Estrada da Conraria, 3040-714 Castelo Viegas, Portugal*

[d] *Center for Neuroscience and Cell Biology (CNC), University of Coimbra, 3004-517 Coimbra, Portugal*

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**Abstract**

CD38 is a multifunctional ectoenzyme that behaves either as an enzyme, a cell adhesion molecule or as a cell surface receptor involved in cell signalling. It is expressed in cells of several lineages, including B and T lymphocytes, and macrophages. CD38 was shown to be important for the development of T-cell dependent humoral immune responses against extracellular pathogens. It also appears to be functionally important in macrophages, which are the host cells of *Mycobacterium avium*, an intracellular parasite that survives within these cells by avoiding a number of their microbicidal strategies. The present work aimed at investigating whether CD38 had any role on the immune response against mycobacterial infection. After intraperitoneal *M. avium* infection, the immune response of CD38KO mice was compared to that of their parental strain, C57Bl.6 mice. Absence of CD38 rendered mice more susceptible to mycobacterial infection. This susceptibility seems to be due to ineffective Th1 differentiation and polarization, which is essential for the control of *M. avium* infection. In addition, absence of CD38 seems to compromise the maintenance of the granulomatous barrier, leading to dissemination and unrestrained growth of mycobacteria.
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*Keywords:* CD38; *M. Avium*; Granulomatous response; Th1 polarization

## 1. Introduction

CD38 belongs to a family of multifunctional ectoenzymes possessing signalling and cell adhesion properties, and extracellular/intracellular enzymatic activity [1]. CD38 catalyzes hydrolysis of $NAD^+$ to nicotinamide and ADP, and synthesis and hydrolysis of cADPR. CD38 was shown to have several immunologically relevant functions. CD38 is expressed in many cell types, including B and T lymphocytes [2,3]. CD38 physically associates with cell surface receptors that mediate signaling cascades [4–8]. CD38 ligation induces activation of transcription factors involved in development of inflammatory responses, such as NF-κB [9]. Activation of cells via CD38 leads to increased production of cytokines, namely IFN-γ. It may behave as an adhesion molecule, induce cytokine secretion, and enhance macrophages' antigen presenting function [10]. Partida-Sànchez and co-workers [11] showed that CD38KO mice were more susceptible to infection by *Streptococcus pneumoniae*, a feature that was proposed to be due to diminished neutrophil chemotaxis in response to

* Corresponding author. Tel.: +351 239 400 329; fax: +351 239 484 317.
*E-mail address:* marta.s.viegas@gmail.com (M.S. Viegas).

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peptides of bacterial origin. At present, it is not known whether CD38 plays a role in the immune response against intracellular bacteria.

*Mycobacterium avium* is a facultative intracellular bacterium that mainly infects macrophages guaranteeing its survival by circumventing macrophages's microbicidal strategies [12]. Effective immune responses to mycobacteria involve Th1 cells and production of high amounts of IFN-γ [12], along with the development of granulomatous responses. Granulomata are highly organized structures, composed of macrophages and T lymphocytes that have a central role in protection against *M. avium* [12,13].

To investigate the role of CD38 in development of immune responses against *M. avium*, CD38KO and C57Bl.6 mice were infected with this pathogen and their immune responses compared. We have found that CD38 plays a role in the immune response against mycobacteria, as CD38KO mice are more susceptible to *M. avium* infection than C57Bl.6 mice. In addition, we have observed that although CD38 does not seem to be essential for the formation of granulomata, its absence compromises the maintenance of the granulomatous barrier and the control of mycobacterial growth.

## 2. Material and methods

### 2.1. Mice

C57Bl.6 mice breeding pairs were purchased from Harlan Iberica (Barcelona, Spain). B6.129P2-*Cd38*$^{tm1Lnd}$ (CD38KO) mice breeding pair was purchased from The Jackson Laboratories (Maine, USA). Both mice share the same genetic background. Mice were bred and kept in animal housing facilities of the Institute for Molecular and Cell Biology (IBMC, Porto, Portugal) under SPF conditions. This research was approved by the Animal Welfare Division of the Portuguese Veterinary Council.

### 2.2. Mycobacteria

*M. avium* strain ATCC 25291, serotype 2, was grown in liquid culture at 37 °C in Middlebrook 7H9 broth (Difco Labs, Detroit, MI) containing 0.04% Tween-80 (Sigma Chemical CO, St Louis, MO). The mycobacteria were harvested from liquid culture by centrifugation (6000 × *g*), washed three times in PBS, as described before [14], and kept frozen at −80 °C.

#### 2.2.1. Mycobacterial infection

Bacteria were suspended in saline containing 0.04% Tween-80 (Sigma Chemical CO, St Louis, MO) and diluted to a concentration of $2 \times 10^6$ viable bacilli of *M. avium* per millilitre. Two-month old C57Bl.6 and CD38KO mice were intraperitonealy (i.p.) infected with 0.5 ml of *M. avium* suspension. Aged-matched animals were inoculated with 0.04% Tween-80 in saline.

### 2.3. Colony forming units (CFU)

One, 30 and 45 days after inoculation with $10^6$ viable bacilli of *M. avium*, mice were sacrificed and the spleen and liver collected and homogenized in 3 ml of deionised water containing 0.05% Tween-80 (Sigma Chemical CO, St Louis, MO). Serial dilutions were performed and 10 µl of each cell suspension were plated onto Middlebrook 7H10 solid medium (Difco Labs, Detroit, MI). Mycobacteria were allowed to grow at 37 °C. After a period of 15 days, CFU were counted.

### 2.4. Mycobacterial antigens

Cell suspensions of *M. avium* were disrupted at 0 °C by ultrasonication for six periods of 30 s in a Branson sonifier set at 100 W. Protein concentration was determined by a modified Lowry procedure [15].

#### 2.4.1. In vitro stimulation

Thirty and 45 days after infection with $10^6$ viable *M. avium* bacilli, the mice were sacrificed and the spleen collected. Spleen cell suspensions were obtained as described elsewhere [15]. Viable cells were counted by a trypan blue exclusion test. A total of $10^6$ cells were stimulated with *M. avium* antigens (4 µg/ml) in complete RPMI 1640 containing 10% FCS. Control cells were cultured in complete RPMI 1640 containing 10% FCS. After a 24-h stimulation (37 °C, 5% $CO_2$), the supernatants were collected for cytokine quantification and frozen at −20 °C.

### 2.5. Cytokine quantification by ELISA

After *in vitro* stimulation, cytokine production was quantified by ELISA. The antibodies used were purified and biotynilated anti-mouse IL-4 and anti-mouse IFN-γ (all from BD Pharmingen, San Jose, CA). Standard dilution curves were obtained using the relevant recombinant cytokines (sensitivity limit for IFN-γ: 156,25 pg/ml and for IL-4: 39,0625 pg/ml). Avidin-HRP and OPD (both from Sigma Chemical CO, St Louis, MO) were used for colour development. The plate was read in an ELISA plate reader at 450 nm.

### 2.6. Histological analysis of granulomata

Two-month old female CD38KO and C57Bl.6 mice were i.p. infected with $10^6$ viable *M. avium* bacilli. Thirty and 45 days after infection, mice were sacrificed and the livers recovered, fixed in 10% paraformaldehyde and embedded in paraffin. Histological slides were prepared, stained with haematoxylin-eosin (H-E) and by reticulin stain for morphologic evaluation of granulomata. For quantification of mycobacteria present in the granulomata, histological slides were stained by Zhiel-Neelsen. An Axiophot microscope (Zeiss, Germany) with a digital still camera (DSC-S85, Sony, Japan) interfaced with a PC was used for image acquisition.

### 2.7. Statistical analysis

The numerical data were compared using Student's *t*-test. Numerical populations considered to be significantly different are indicated in the figures.

## 3. Results

### 3.1. Bacterial burden in M. avium target organs

To assess the susceptibility of CD38KO mice to mycobacterial infection, we have quantified the bacterial burden present in target organs (spleen and liver) of CD38KO and C57Bl.6 mice. We have found that, at 45 days post-infection, mycobacterial growth in the spleen and liver of CD38KO mice was increased with regards to the one observed in C57Bl.6 mice (Fig. 1). We have found splenic and liver CFU in CD38KO and C57Bl.6 mice as soon as one day after i.p. inoculation with *M. avium*. At this time point, CFU counts were lower in CD38KO mice than in C57Bl.6 mice (Fig. 1).

### 3.2. Cytokine secretion

Resistance to mycobacterial infection is associated with Th1 immune responses whereas susceptibility involves activation of Th2 lymphocytes. We have, thus, quantified two of the cytokines that distinguish Th1 and Th2-mediated immune responses: IFN-γ and IL-4, respectively. We have observed that, at day 30 post-infection, splenocytes from C57Bl.6 and CD38KO mice released vestigial amounts of IFN-γ in the absence of antigenic stimulation. Upon antigenic stimulation, cells from infected CD38KO and C57Bl.6 mice produced increased amounts of IFN-γ with regards to cells from control mice (Fig. 2A, left). Nevertheless, splenocytes from infected CD38KO produced significantly lower amounts of this cytokine than cells from infected C57Bl.6 mice (Fig. 2A, left). By day 45 post-infection, we have found that cells from C57Bl.6 and CD38KO mice released only trace amounts of this cytokine, whether or not antigenic stimulation was performed (Fig. 2A, right).

Regarding the production of IL-4, we were unable to detect IL-4 production in the absence of antigenic stimulation by day 30 post-infection. Upon antigenic stimulation we have observed that splenocytes from infected CD38KO mice secreted increased amounts of this cytokine when compared to cells of infected C57Bl.6 mice (Fig. 2B, left). By day 45 post-infection we have found that, in the absence of antigenic stimulation, splenocytes from infected and control CD38KO mice produced similar amounts of IL-4, whereas cells from infected C57Bl.6 mice produced higher amounts of this cytokine than cells from control C57Bl.6 mice (Fig. 2B, right). Upon antigenic stimulation, splenocytes from infected CD38KO mice secreted higher amounts of IL-4 than cells from control CD38KO mice, while no differences were seen when cells from control and infected C57Bl.6 mice were compared (Fig. 2D).

### 3.3. Granuloma formation

*M. avium* infection induces the formation of granulomata. Granulomata can be classified according to their organization, type and number of cells present, and nature of cell death occurring inside the granuloma. We have analysed liver granulomata as this organ is one of the major targets of mycobacteria after peritoneal inoculation. Also, granulomata and the surrounding parenchyma can be easily distinguished in the liver, in contrast to the spleen. We have found that, by day 45 post-infection, CD38KO and C57Bl.6 mice presented well-organized, non-necrotizing granulomata with epithelioid cells, macrophages and lymphocytes (Fig. 3A,B). However, we have

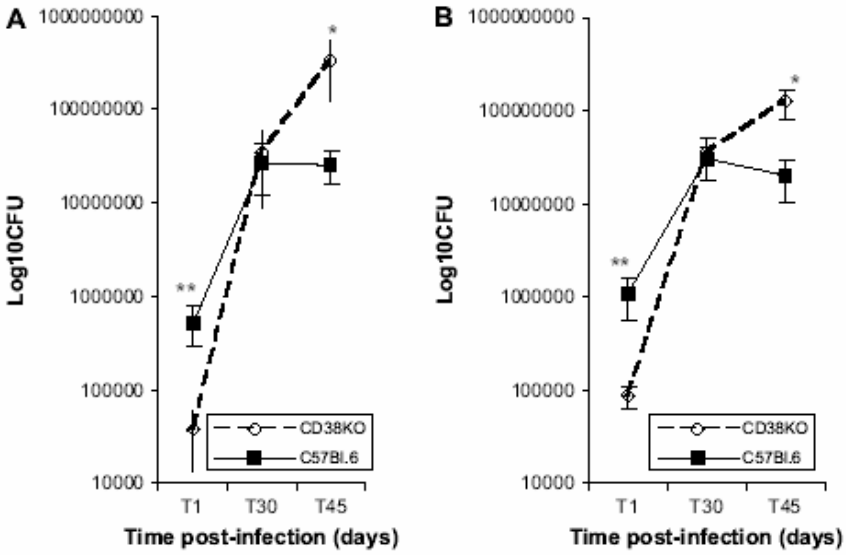


Fig. 1. CD38KO mice have an increased mycobacterial burden in target organs of *M. avium*. The animals were i.p. infected with $10^6$ viable *M. avium* bacilli. Spleen (A) and liver (B) were collected at several time points and CFU counted. Data represent the mean value of groups of 6 mice and the vertical line the standard deviation of the mean (SD). *, $p < 0.05$; **, $p < 0.01$.

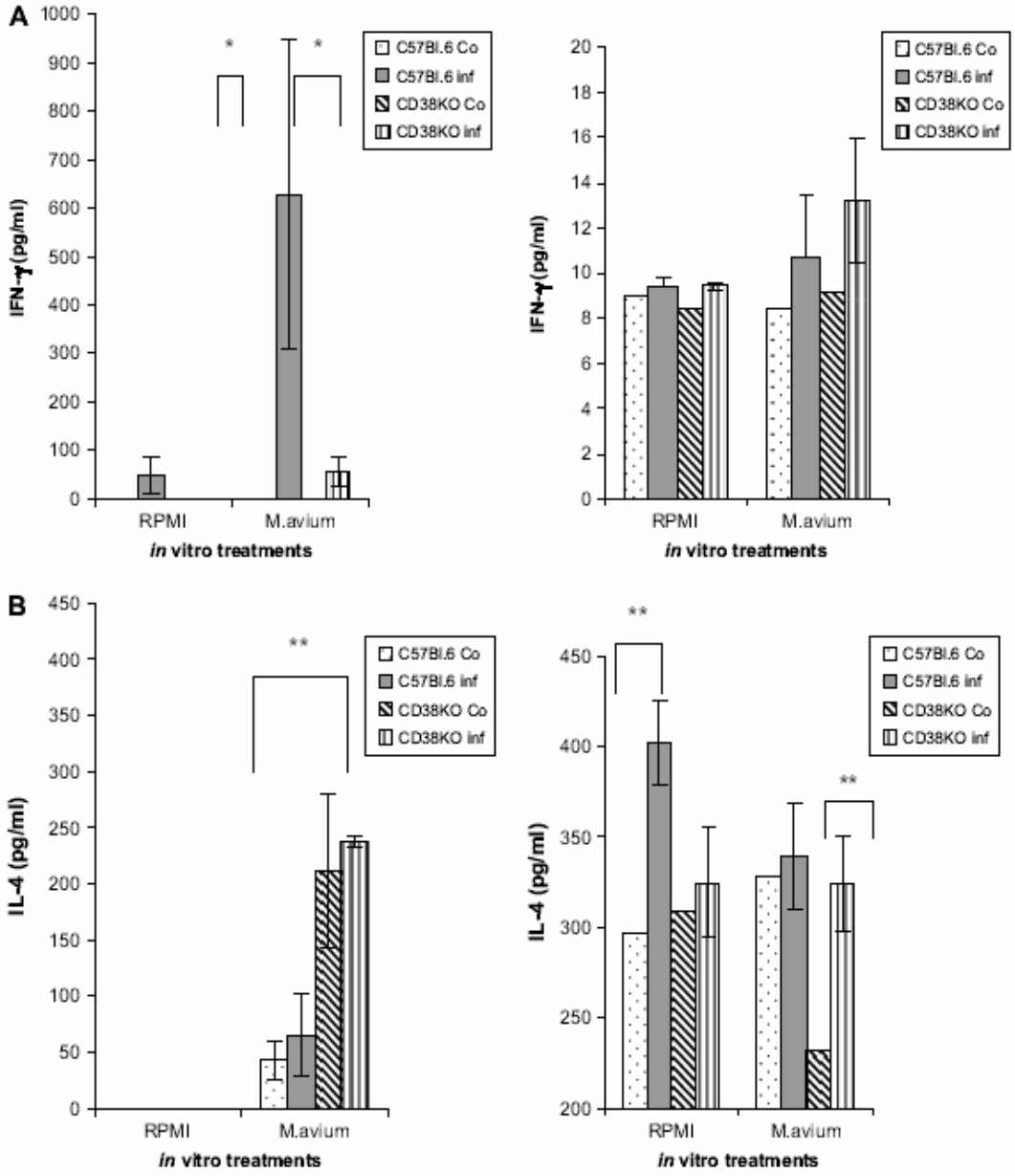


Fig. 2. CD38KO mice secrete reduced amounts of IFN-γ and increased amounts of IL-4 in response to *M. avium* infection. (A) IFN-γ secretion. (B) IL-4 production (left, 30 days post-infection; right, 45 days post-infection). Mice were i.p infected with $10^6$ viable *M. avium* bacilli. Control mice were injected with 0.04% Tween-80 in saline. After 30 days of infection, spleens were collected and cells were stimulated overnight with *M. avium* sonicate. Each bar represents the mean value of groups of 8 animals and the vertical line the SD. Regarding IFN-γ statistically significant differences were found when CD38KO and C57Bl.6 mice and when control and infected C57Bl.6 mice were compared (*$p < 0.01$; **$p < 0.001$). With regards to IL-4 production, no significant differences were found when infected CD38KO and C57Bl.6 mice were compared. Statistically significant differences were found when control and infected mice were compared (**$p < 0.001$).

observed that CD38KO mice, in contrast to C57Bl.6 mice, were unable to form a closed fibrotic rim around the granulomata (Fig. 3C,D). With respect to the number and size of granulomata, we have found that, by 30 days of infection, they were similar in both strains (Fig. 4A,B). However, by day 45 post-infection, we have seen that the number of granulomata was increased in CD38KO when compared to C57Bl.6 mice (Fig. 4A). We have observed that granulomata of CD38KO mice were smaller than those of the parental strain (Fig. 4B). With regards to bacterial load, we have found that, by day 30 post-infection, comparable numbers of mycobacteria were found within granulomata of CD38KO and C57Bl.6 mice (Fig. 5A,B). However, by day 45 post-infection, we have observed that granulomata of CD38KO mice contained higher amounts of mycobacteria than those of the parental strain (Fig. 5C,D).

## 4. Discussion

In mice, immunity to *M. avium* requires augmentation of macrophage's bactericidal activity by IFN-γ, which may be produced by Th1 cells, NK cells and even macrophages. Resistance to *M. avium* infection evolves through two stages: one of innate immunity and a second of acquired $CD4^+$T cell mediated resistance. The former involves protective effects mediated by both IFN-γ and TNF-α, and the latter, which

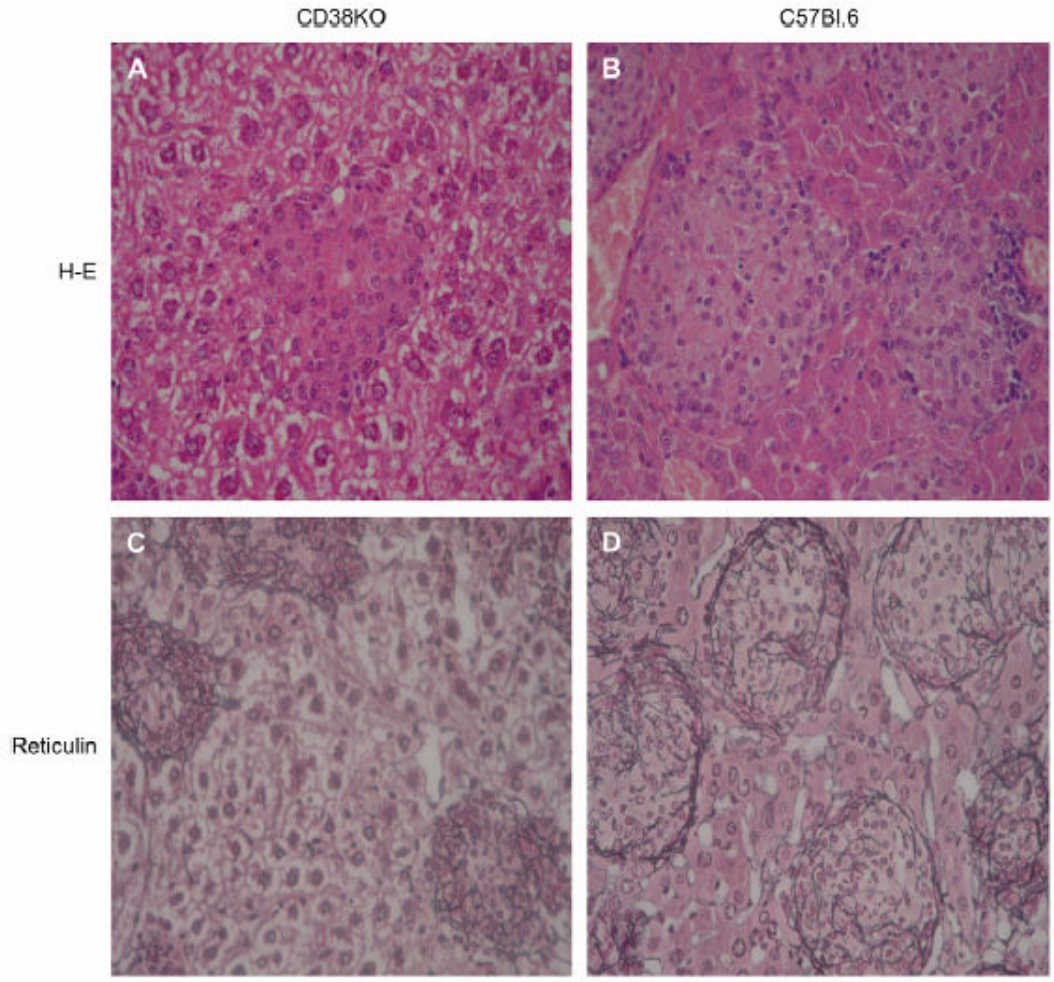


Fig. 3. CD38KO mice develop well defined granulomata but are unable to form a closed fibrotic rim. Photomicrographs of granulomata in livers of CD38KO (A, C) and of C57Bl.6 mice (B, D). The mice were i.p. infected with $10^6$ viable *M. avium* bacilli. At 45 days, the livers were collected and H-E (A, B) and reticulin (C, D) stained for histological analysis (×400).

takes place around 2–3 weeks after infection, requires IFN-γ [12,16]. Another characteristic feature of the response against *M. avium* is the development of granulomata. Granulomatous lesions may be initiated by non-specific inflammatory signals arising from the interaction of tissue macrophages with microbial products [17]. These structures start as confluent masses of cells organized around a core of macrophages and epithelioid cells and surrounded by lymphocytes (occasionally also polymorphonuclear cells), but later increase in size, become encapsulated by a fibrotic rim and may evolve to show areas of central necrosis, which later progress into large caseous areas [17,18]. Granulomata provide a highly ordered juxtaposition of macrophages and T cells in such a way that antimycobacterial mechanisms may be effectively coordinated and regulated [19]. With the arrival of antigen-specific T cells, the lesion develops into a mature granuloma where activation of macrophages by IFN-γ results in inhibition of microbial growth [17].

Lack of CD38 may impact the immune response against *M. avium* at several levels. For instance, it may affect the arrival of immune cells, namely macrophages, to the infection site, not only due to its function as an adhesion molecule [1], but also because of its ability to modulate the response of

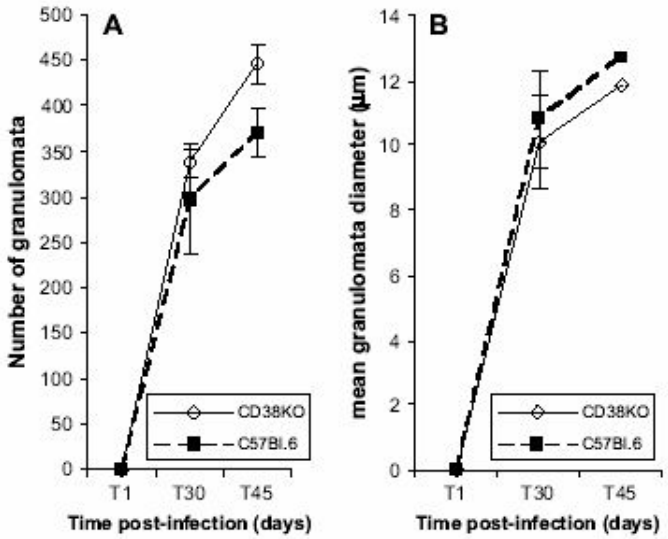


Fig. 4. CD38KO mice have a higher number of granulomata than C57Bl.6 mice. (A) Granulomata were counted in 12 random fields per liver (×100). Five livers were examined per group at each time point. Error bars represent SD. (B) Granulomata were aligned within a grid (×100). The diameter represents the mean of two perpendicular measurements within the grid. Twenty granulomata were measured per liver and 5 livers were examined per group at each time point. Error bars represent SD.

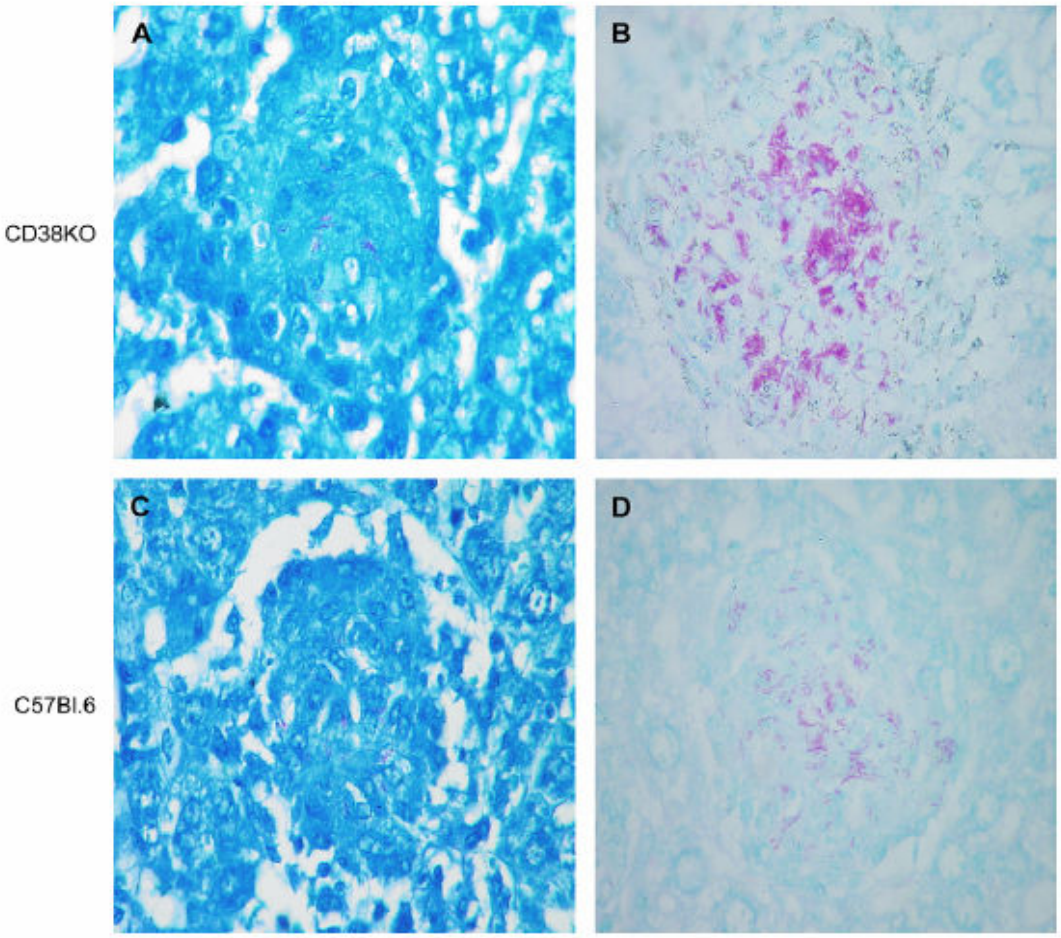


Fig. 5. CD38KO mice have an increased bacterial load inside granulomata. Photomicrographs of granulomata in livers of CD38KO (A, B) and of C57Bl.6 mice (C, D). The mice were infected i.p. with $10^6$ viable *M. avium* bacilli. At 30 days (A, C, ×630) and 45 days (B, D, ×1000), the livers were collected and stained by Zhiel-Neelsen.

immune cells to chemokines and chemotactic compounds [11,20]. These properties of CD38 may also impact the migration of cells to secondary lymphoid organs, such as the spleen, and mycobacteria target organs, the spleen and liver, and, as a consequence, the formation and organization of granulomata. Additionally, absence of CD38 may impair or reduce IFN-γ production, which is necessary for the activation of infected macrophages and elimination of mycobacteria, either directly [3] or by altered DC maturation and deficient IL-12 secretion [21].

We have found that, in contrast to C57Bl.6 mice, in CD38KO mice logarithmic bacillary growth in the spleen and liver persisted until day 45 post-infection. These findings suggest that CD38KO mice are unable to control mycobacterial growth and are more susceptible to *M. avium* infection than C57Bl.6 mice and unravel a role for CD38 during immune responses against mycobacteria. These data were further supported by the finding of increased bacterial numbers in granulomata of CD38KO mice with regards to C57Bl.6 mice. However, when we have quantified CFU in the spleen and liver at an early time point (one day of infection), we have observed that C57Bl.6 mice had a higher mycobacterial burden than CD38KO mice, an observation that may reflect an earlier bacterial dissemination. This may contribute to the higher resistance of these hosts to mycobacterial infection. Indeed, Chackerian and co-workers [22] showed that early dissemination of *Mycobacterium tuberculosis* in C57Bl.6 mice relates to an early initiation of immunity, instead of disease progression, and that it always preceded activation of specific T cells. Thus, delayed mycobacterial dissemination in CD38KO mice may compromise an early activation of mycobacteria-specific T cells, this way contributing to the higher susceptibility of these mice to *M. avium* infection.

Mycobacterial dissemination is believed to be mediated by macrophages and DC [22]. CD38 was shown to be functionally important in these cells [4,20]. CD38 is able to interfere with chemokine signalling, this way affecting migration of DC [20]. Partida-Sanchez and co-workers [23] demonstrated that CD38 is able to regulate CCR2, which is the receptor for chemokines, such as MCP-1. These chemokines are important for the migration of monocytes and derived cells to the site of mycobacterial infection [24,25]. Finally, it should be reminded that CD38 may act as an adhesion molecule through interaction with CD31 present on endothelial cells [1], and may, in this way, participate in cell migration to infection sites. Indeed, it was shown that CD31 is important for transendothelial migration of several cell subsets, namely lymphocytes, NK cells and neutrophils [1]. So, inefficient migration to the

infection site due to CD38 deficiency may contribute to the reduced bacterial dissemination observed in CD38KO mice.

As mentioned above, mycobacterial infections elicit the development of granulomata, which are a hallmark of protective immune responses, as they are essential for pathogen containment and for reducing nutrient supply to bacteria [13]. We have found that in both strains the number of these structures increased as infection progressed. However, this increase was more pronounced in CD38KO mice than in C57Bl.6 mice. This higher number of granulomata may be due to the inability of CD38KO mice to control mycobacterial growth and to contain mycobacteria inside the granuloma. These data suggest a role for CD38 on the maintenance of the integrity of granulomata and, as such, on the physical containment of mycobacteria. Indeed, we have found that CD38KO mice, in contrast to C57Bl.6 mice, were unable to form a closed fibrotic rim around granulomata. Granulomata rupture promotes bacterial dissemination to nearby areas and formation of additional lesions at distinct tissue sites [26]. Inability to form closed granulomata may also affect the proliferation rate of mycobacteria. Mycobacteria were found to be actively replicating in open lesions, whereas those in closed lesions were non-dividing [27]. Thus, inability of CD38KO mice to contain mycobacteria in a restricted area may contribute, not only to the observed higher number of granulomata, but also to the increased mycobacterial burden present in the spleen and liver.

One of the most important cytokines in the normal development and organization of granulomata is TNF-α. One could hypothesize that production of this cytokine was deficient in CD38KO mice. However, measurement of TNF-α in supernatants of splenocytes cultures, after antigenic challenge, has shown that cells from CD38KO mice are able to secrete TNF-α in levels similar to those produced by cells of C57Bl.6 mice (our unpublished data). Alternatively, macrophages present in granulomata of CD38KO mice may not be activated by T cells and will thus be unable to control mycobacterial growth or eliminate mycobacteria. This may be due to the type of immune response developed. While a Th1 immune response dictates resistance to mycobacterial infection, a Th2 response relates to susceptibility [12]. We have shown that splenic cells from CD38KO mice secreted increased quantities of IL-4 and reduced amounts of IFN-γ in response to *M. avium* antigens when compared to cells of C57Bl.6 mice. These data suggest that Th cells of CD38KO mice may differentiate in an IL-4-rich microenvironment, which inhibits Th1 skewing, and may cause problems in the induction of cell-mediated immune responses against intracellular pathogens. Insufficient differentiation of Th1 cells and reduced secretion of IFN-γ may thus be related to the inability of CD38KO mice to control *M. avium* infection as there will be no (or not enough) IFN-γ available for activation of mycobacteria-infected macrophages. Consequently, macrophages will not be able to kill intracellular bacteria and mycobacterial growth will continue.

Additionally, the decreased IFN-γ production observed in CD38KO mice may be due to the absence of CD38, as activation of T cells via CD38 induces production of this cytokine [3]. It may also be due to absence of CD38 on DC. Recent evidence demonstrated a role for DC in infection by mycobacteria, namely as central regulators of the acquired immune response [26]. Stimulation of DC precursors by mycobacterial components through Toll-like receptors (TLR) results in development of DC1 that produce IL-12 and promote Th1 differentiation [26]. However, DC1 development may be compromised in CD38KO mice. Fedele and co-workers [21] have shown that CD38 determines the intensity of the processes involved in DC maturation, namely the up-regulation of CD83 and the secretion of IL-12. Thus, the inability of CD38KO mice to polarize their acquired immune response into a Th1's may be a consequence of impaired DC1 differentiation and decreased IFN-γ secretion.

Interestingly, we have found that while, by 30 days of infection, C57Bl.6 mice were able to secrete significant amounts of IFN-γ, which may explain the lower mycobacterial burden observed in these animals when compared to CD38KO mice; by day 45, C57Bl.6 mice had lost this ability and their immune response was Th2-polarized. This is in agreement with previously published data on C57Bl.6 mice, which have shown that IFN-γ production is down-regulated after induction of bacteriostasis [12]. Wagner and co-workers [28] have also shown that IL-12 secretion, which is essential for IFN-γ production by Th cells, was more pronounced in the beginning of infection, but decreased later on, probably due to the action of mycobacteria. Dampening of a Th1 response may be due to the increased IL-10 secretion observed in C57Bl.6 mice (our unpublished data) and may be responsible for the inability of C57Bl.6 mice to control *M. avium* infection, which is reflected by increased bacterial growth by day 90 of infection (data not shown). Indeed, when C57Bl.6 mice are i.p. infected with high doses ($10^6$ CFU) of the virulent *M. avium* strain ATCC 25291, there is a progressive uncontrolled infection despite the emergence, around 3 weeks of infection, of an immune response characterized by prominent production of IFN-γ and extensive macrophage activation [18,29]. As CD38KO mice present a Th2 polarization throughout infection, it is possible that CD38 may have a role in the secretion of IFN-γ during the initial stages of the immune response against mycobacteria. During these stages, several IFN-γ secreting cells are activated by products of mycobacterial origin through TLR. These include NK cells, NK T cells and γδ T cells. CD38 was shown to be important for the activation of NK cells [7]. It is thus conceivable that it may also play a role in the activation and induction of IFN-γ secretion in NK T cells and γδ T cells. Absence of CD38 would lead to impaired secretion of this cytokine, as observed in CD38KO mice, and to an increased susceptibility to mycobacterial infection, as IFN-γ produced in the intermediate stage of infection is essential for stimulation of macrophages and DC to produce IL-12 and to further promote Th1 differentiation and stable Th1 polarization [26].

In conclusion, our data suggest that CD38 is involved in development of protective immune responses against mycobacteria, possibly in: (1) Th1 polarization of immune responses, which is essential for the control of mycobacterial infections; (2) bacterial dissemination to target organs in the initial phase

of infection; and (3) the maintenance of granulomatous barrier integrity. We provide evidence that besides being important for the development of T-cell dependent humoral immune responses, CD38 may also have a role on cell-mediated immunity, which is essential for resistance to infection by intracellular bacteria.

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2. 2 **Viegas MS, Martins TC** CD38 is required for leukocyte recruitment and macrophage activation in response to *Mycobacterium avium* infection. (Submitted).

CD38 is required for leukocyte recruitment and macrophage activation in response to *Mycobacterium avium* infection.

Marta S. Viegas[a*], Teresa C. Martins[a,b]

[a]Molecular Pathology Laboratory, Portuguese Institute for Oncology at Coimbra Francisco Gentil, EPE (IPOCFG, EPE), Av. Bissaya Barreto, 98 - Apartado 2005, 3000-075 Coimbra, Portugal

[b]Centre for Neuroscience and Cell Biology (CNC), University of Coimbra, 3004-517 Coimbra, Portugal

*Corresponding author:

Marta S. Viegas

Molecular Pathology Laboratory

Portuguese Institute for Oncology (IPOCFG-EPE)

Av. Bissaya Barreto, 98 - Apartado 2005

3000-075 Coimbra, Portugal

Phone: 351 239 400329

Fax: 351 239 484317

e-mail: marta.s.viegas@gmail.com

**Abstract**

CD38 belongs to a family of multifunctional ectoenzymes possessing signaling properties and enzymatic activity. CD38 is expressed by several cell types, including T cells and macrophages, modulating their function. It is involved in different types of immune responses, playing an important role in *Streptococcus pneumoniae* infections. CD38KO mice are more susceptible to mycobacterial infection due to deficient Th1 differentiation. They are unable to form a granulomatous barrier, allowing unrestrained mycobacterial growth and dissemination. Mycobacteria are intracellular pathogens that infect macrophages. Control of mycobacterial infection depends on Th1 responses and activation of infected macrophages. We have now investigated if the increased susceptibility of CD38KO mice to *Mycobacterium avium* involved deficiencies at the level of macrophages. CD38KO and C57Bl.6 bone marrow derived macrophages were cultured and their response to mycobacterial infection compared. We have found that CD38 was required for full activation of macrophages' microbicidal mechanisms, a feature possibly related to the ability of CD38 to induce IFN$\gamma$ production by T cells. We show that CD38 is necessary for recruitment of leukocytes to the infection site. We provide evidence that CD38 is required for the effective control of infection by intracellular pathogens, which requires cell-mediated immune responses.

Keywords: CD38; *M. AVIUM*; MACROPHAGES

## 1. Introduction

CD38 is a member of the ADP-ribosyl cyclase family of multifunctional ectoenzymes expressed by several immune cell subsets, modulating their function [1-4]. CD38KO mice have an increased susceptibility to infection by *Streptococcus pneumoniae,* due to reduced migration of neutrophils to the infection site [5]. Mycobacteria are intracellular pathogens that mainly infect macrophages [6]. We have shown that CD38KO mice were more susceptible to *Mycobacterium avium* infection [7], due to ineffective Th1 polarization, which is essential for activation of infected macrophages. Absence of CD38 compromised establishment of the granulomatous barrier allowing dissemination and unrestrained growth of mycobacteria.

Absence of CD38 may directly impact monocyte/macrophage function. CD38 synergises with MHC II molecules, augmenting macrophages' responses to superantigens [4], and modulates the response to respiratory burst activators [8]. We have now investigated whether absence of CD38 compromised macrophages' responses to mycobacteria. For this, CD38KO and C57Bl.6 bone marrow derived macrophages (BMDM) were cultured and their response to mycobacterial infection compared. Our results suggest that CD38 may be essential for macrophages full activation and production of high amounts of NO, possibly through its role on IFN$\gamma$ secretion by T cells and/or differentiation of immunogenic APC. CD38 is also required for leukocyte chemotaxis and chemokine production in response to *M. avium*, two features that are important for timed development of protective immune responses.

## 2. Material and Methods

### Mice

C57Bl.6 (Harlan Iberica, Spain) and B6.129P2-*Cd38*$^{tm1Lnd}$ (CD38KO) mice (Jackson Laboratories, USA) share the same genetic background. Mice were bred and kept in animal facilities of the Institute for Molecular and Cell Biology (IBMC, Porto, Portugal) under SPF conditions. This research was approved by the Portuguese Veterinary Council.

### Mycobacteria

*M. avium* strain ATCC 25291, serotype 2, was grown in liquid culture at 37 ºC in Middlebrook 7H9 broth (Difco Labs) containing 0.04 % Tween-80 (Sigma-Aldrich). Mycobacteria were harvested by centrifugation (6000 g), washed three times in phosphate buffered saline (PBS) and frozen at -80 ºC [9].

### Mycobacterial infection

Bacteria were diluted in saline containing 0.04 % Tween-80 to a concentration of $2x10^6$ viable bacilli per millilitre. Two-month old C57Bl.6 and CD38KO mice were intraperitoneally (i.p.) infected with $10^6$ *M. avium* bacilli.

### Bone Marrow Derived Macrophage (BMDM) cultures

Macrophage precursors were collected from murine femurs, placed in a Petri dish and incubated for 16-18 hours at 37 ºC. Non-adherent cells were recovered and washed in cold PBS. $5x10^5$ cells were plated in 24-well plates, and L929 cell culture supernatant, a source of macrophage colony stimulating factor (M-CSF), was added every other day to induce macrophage differentiation. By day 10, $10^6$ viable *M. avium* bacilli were added to each well and incubated for four hours at 37 ºC. The wells were then washed with warm PBS to stop infection.

Colony Forming Units (CFU)

3, 5 and 7 days after infection, macrophages were recovered, lysed and homogenized in 1 ml of deionised water containing 0.05 % Tween-80. Serial dilutions were performed and 10 μl of each cell suspension were plated onto Middlebrook 7H10 agar (Difco Labs). Mycobacteria were allowed to grow at 37 ºC. 15 days later, CFU were counted.

BMDM/Splenocyte co-cultures

Infected BMDM (as described above) were co-cultured with $10^6$ splenocytes recovered from 30-day infected or non-infected CD38KO and C57Bl.6 mice. CD38KO BMDM were co-cultured with splenocytes from CD38KO (MKOTKO) or C57Bl.6 (MKOTB6) mice; C57Bl.6 BMDM were co-cultured with splenocytes from C57Bl.6 (MB6TB6) or CD38KO (MB6TKO) mice. At designated times points, the supernatants were collected for cytokine and chemokine quantification.

Cytokine and chemokine quantification

Cytokines were quantified by ELISA, using purified and biotynilated anti-mouse IL-12 (clones RedTG297-289 and C17.8, respectively), IL-4 (1D11 and 24G2) and IFNγ (R4.6A2 and XMG1.2) antibodies (BD Pharmingen); purified and biotynilated anti-mouse IL-10 (MAB417 and BAF417) and TNFα (MAB410 and BAF410) antibodies (R&D Systems). Standard dilution curves were obtained using recombinant cytokines. Avidin-HRP and OPD (Sigma-Aldrich) were used for color development. The plate was read at 450 nm.

Macrophage culture supernatants were assayed for chemokine production using Mouse Chemokine 6plex kit (Bender MedSystems) according to the manufacturer's instructions.

pH measurements

$10^8$ viable *M. avium* bacilli were incubated with fluorescein for 30 min on ice and used to infect BMDM (1 hour at 37 ºC). After 6 washes with PBS, 150 μl of $Na^+$ medium (140 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM HEPES) were added to the plate. 2 μl of nigericin (200 mM) and 2 μl of 2-(N-morpholino) ethanosulforic acid (0.1 N) were added to each well and the pH measured using a fluorimeter. The standard curve was obtained using $Na^+$ medium.

Quantification of Nitric Oxide by Griess reaction

75 μl of supernatants from BMDM cultures were placed in a 96-well plate. 100 μl of 1 % *p*-aminobenzenesulfonamide in 2.5 % phosphoric acid was added to each sample, followed by of 100 μl of 0.1 % N-naphthyl-ethylenediamine in 2.5 % phosphoric acid. Optical density was determined at 540 nm. The molar concentrations of nitrite in samples were determined from standard curves generated using known concentrations of sodium nitrite in culture medium.

Quantification of leukocytes in the peritoneal exudate

4 hours, 1 week and 2 weeks after *M. avium* infection, mice were sacrificed and the peritoneal exudates recovered in 5 ml of 5 % sacharose in PBS. Cell suspensions were spun down onto slides and Wright stained, and granulocytes and mononuclear cells counted.

Statistical analysis

Statistical analysis was performed using the Student *t* test, for the comparison of two different conditions, or One-way ANOVA, when comparing more than two experimental groups. Statistical analysis of the results obtained in co-culture experiments was performed using full Factorial ANOVA. Numerical populations were considered to be significantly different when $p < 0.05$.

## 3. Results

Mycobacterial burden in BMDM cultures

To assess whether absence of CD38 on macrophages compromised ingestion and control of mycobacteria, we have quantified bacteria in BMDM cultures. A sustained mycobacterial growth was observed in macrophages of both strains of mice (Fig. 1A). However, by day 5, CD38KO macrophages had lower CFU counts than C57Bl.6's (Fig. 1A). To assess whether the ability of CD38KO macrophages to control mycobacterial growth was affected by the presence of CD38 on splenic cells, we have quantified mycobacteria in BMDM co-cultured with splenocytes from non-infected mice. Again, a sustained mycobacterial growth was observed until day 9, no differences being found between the different experimental conditions, except for day 3. At this time point, statistical analysis revealed a significant interaction between the two variables: the ability of CD38KO macrophages to control mycobacterial growth was dependent on the splenocytes' origin, that is, on whether they expressed CD38 ($p<0.001$). Co-culture of CD38KO macrophages with C57Bl.6 splenocytes was associated with a significantly increased mycobacterial burden, whereas co-culture of CD38KO macrophages with CD38KO splenocytes resulted in a significantly decreased mycobacterial growth (Fig. 1B). In contrast, the ability of C57Bl.6 macrophages to control mycobacterial growth was independent of the presence of CD38 on splenocytes ($p=0.373$). Similar CFU counts were obtained when C57Bl.6 macrophages were co-cultured with either C57Bl.6 or CD38KO splenocytes (Fig. 1B).

Phagolysosome Acidification

To control mycobacterial growth macrophages need to acidify the phagolysosome (pH 4-5). We have found that *M. avium*-infected CD38KO and C57Bl.6 macrophages were unable to acidify the phagolysosome (Fig. 2A). CD38KO macrophages' phagolysosome pH was consistently higher (≈ 7) than that of C57Bl.6 macrophages (≈ 6). This difference attained statistical significance by 60 minutes. Mycobacteria are able to inhibit phagolysosome acidification. For elimination of mycobacteria, macrophages need to be exogenously activated. When IFN$\gamma$ was added to cultures, we observed a slight decrease in the phagolysosome pH of CD38KO macrophages (Fig. 2A, B).

Production of NO

To destroy ingested bacteria, macrophages produce reactive species, namely NO. We have quantified NO production in BMDM cultures. In the absence of exogenous activation, *M. avium*-infected CD38KO and C57Bl.6 macrophages produced vestigial amounts of NO (Fig. 3A). When macrophages were exogenously activated by IFNγ, NO production was significantly increased in macrophages of both strains. By days 3 and 5, CD38KO macrophages produced significantly higher amounts of NO than C57Bl.6 macrophages (Fig. 3B). We have also quantified NO in BMDM co-cultured with splenocytes from non-infected and *M. avium*-infected mice. When macrophages were co-cultured with splenocytes from non-infected mice, only vestigial amounts of NO were detected, no differences being seen between the different experimental settings (Fig. 3C). Regarding co-cultures with splenocytes from infected mice, reduced amounts of NO were produced when CD38KO splenocytes were present in culture, regardless of the BMDM origin (Fig. 3D). In contrast, when C57Bl.6 splenocytes were added to the culture, NO production was increased despite the BMDM origin (Fig. 3D).

IL-12 and TNFα production

IL-12 and TNFα production is crucial for the control of mycobacterial infection. IL-12 is required for Th1 differentiation while TNFα is involved in granulomata formation. We have quantified these cytokines in BMDM cultures. By day 3, we have found a peak of IL-12 production by CD38KO macrophages that was not observed in C57Bl.6's. From day 5 onwards, a steady increase in IL-12 production was observed in both strains (Fig. 4A). With regards to TNFα, no differences were found (Fig. 4B).

We have also quantified these cytokines in BMDM co-cultured with splenocytes from non-infected and infected mice. In BMDM co-cultures with splenocytes from non-infected CD38KO and C57Bl.6 mice, similar amounts of IL-12 were detected (Fig. 4C). Increased amounts of TNFα were produced, by day 7, when CD38KO macrophages were co-cultured with C57Bl.6 splenocytes (Fig. 4D). Statistical analysis revealed a significant interaction between the two variables: when CD38KO macrophages were present in culture, TNFα production was dependent on the splenocytes' origin ($p<0.001$). In contrast, this was not important when C57Bl.6 macrophages were concerned ($p=0.693$). When we used BMDM co-cultured with splenocytes from infected mice, we have found that, by day 7, reduced amounts of TNFα were produced in cultures of CD38KO macrophages regardless of the splenocytes' origin (Fig. 4E).

IFNγ, IL-4 and IL-10 production

IFNγ and IL-4 distinguish Th1 and Th2 immune responses. We have quantified their production in co-cultures of BMDM and splenocytes from non-infected and *M. avium*-infected mice. We have detected two peaks of IFNγ secretion (days 3 and 9) in cultures of CD38KO macrophages with splenocytes from non-infected C57Bl.6 mice (Fig. 5A). No differences were observed between the other experimental conditions (Fig. 5A). Statistical analysis revealed a significant interaction between the two variables: when CD38KO macrophages were present in cultures, IFNγ production was dependent on the splenocytes' origin ($p<0.05$). In contrast, this was not important when C57Bl.6 macrophages were concerned ($p>0.8$). Production of this cytokine required expression of CD38 on splenocytes.

We have observed a statistically significant increase in IL-4 by day 9 when C57Bl.6 macrophages were co-cultured with splenocytes from non-infected CD38KO mice, no differences being seen in the other experimental conditions (Fig. 5B). Statistical analysis revealed a significant interaction between the two variables: when C57Bl.6 macrophages were present in cultures, IL-4 production was dependent on the splenocytes' origin ($p<0.001$). In contrast, this was not important when CD38KO macrophages were concerned ($p=0.846$).

Regarding BMDM co-cultured with splenocytes from infected mice, we have found a steady increase in IFNγ production whenever C57Bl.6 splenocytes were present in culture, despite macrophages' origin (Fig. 5C). This increase was more pronounced when CD38KO macrophages were co-cultured with C57Bl.6 splenocytes. The ability to produce IFNγ early in infection required expression of CD38 on splenocytes while, as infection progressed, expression of CD38 by macrophages also became relevant. As for IL-4, we have found two secretion peaks (days 3 and 7) when CD38KO macrophages were co-cultured with C57Bl.6 splenocytes, no differences being seen between the other experimental conditions (Fig. 5D). Statistical analysis revealed a significant interaction between the two variables: when C57Bl.6 macrophages were present in cultures, IL-4 production was dependent on the splenocytes' origin ($p<0.001$). In contrast, this was not important when CD38KO macrophages were concerned ($p>0.5$).

IL-10 is necessary for the control of immune responses, namely to their termination or limitation of collateral damage. Production of this cytokine is detrimental for the effective elimination of mycobacteria. By day 3, we have observed a statistically significant increase in IL-10 production when macrophages were co-cultured with CD38KO

splenocytes (Fig. 5E). Thereafter, IL-10 secretion decreased reaching amounts similar to those observed in cultures of C57Bl.6 splenocytes (Fig. 5E). When C57Bl.6 macrophages were co-cultured with C57Bl.6 splenocytes, we have found that IL-10 production remained fairly constant throughout infection (Fig. 5E). This modulation of IL-10 secretion was significantly associated with expression of CD38 by splenocytes ($p<0.001$). By day 7, we have found that reduced amounts of IL-10 were produced when CD38KO macrophages were co-cultured with C57Bl.6 splenocytes (Fig. 5E). Statistical analysis revealed a significant interaction between the two variables: when CD38KO macrophages were present in culture, IL-10 production was dependent on the splenocytes' origin ($p=0.007$). In contrast, this was not important when C57Bl.6 macrophages were concerned.

Leukocyte recruitment to the infection site

To assess whether absence of CD38 compromised recruitment of leukocytes to the infection site, we have i.p. infected mice and quantified the number of leukocytes present in the peritoneal exudate at different time points. We have found that CD38KO mice consistently presented lower numbers of granulocytes than C57Bl.6 mice (Fig. 6A-a). Regarding mononuclear cells, similar numbers were found in non-infected CD38KO and C57Bl.6 mice (Fig. 6A-b). Four hours after infection, a decrease in the number of mononuclear cells was observed in both strains. This decrease was significantly more pronounced in C57Bl.6 mice (Fig. 6A-b), but was followed by a significant increase by one week after infection. In contrast, the number of mononuclear cells in the peritoneal cavity of CD38KO mice was not altered and was significantly smaller than that of C57Bl.6 mice (Fig. 6A-b). By two weeks after infection, the number of mononuclear cells of C57Bl.6 mice decreased to values similar to those of CD38KO mice (Fig. 6A-b).

Chemokine secretion

Chemokines are involved in leukocyte trafficking. We have quantified production of monocyte chemoattractant proteins (MCP)-1/3, macrophage inflammatory protein (MIP)-1$\alpha/\beta$, and RANTES. Infected CD38KO and C57Bl.6 macrophages produced similar amounts of MCP-3, MCP-1, MIP-1$\alpha$, MIP-1$\beta$ and RANTES (data not shown). These observations paralleled those made for BMDM co-cultured with splenocytes from non-infected mice (data not shown). On day 3, decreased amounts of MCP-3 (Fig. 6B-a), MCP-1 (Fig. 6B-b) and RANTES (Fig. 6B-e) were detected in cultures of BMDM with

infected-CD38KO splenocytes with regards to those of C57Bl.6's, despite the origin of macrophages. Increased amounts of MIP-1$\alpha$ (days 7 and 9; Fig. 6B-c) and MIP-1$\beta$ (day 3; Fig. 6B-d) were produced when C57Bl.6 macrophages were co-cultured with C57Bl.6 splenocytes, no differences being found between the other experimental conditions.

## 4. Discussion

CD38 modulates function of several immune cells [3-5, 8, 10] and participates in immune responses against different pathogens. CD38KO mice are more susceptible to *S. pneumoniae* [5] and to *M. avium* [7]. Absence of CD38 compromises development of protective Th1 immune responses and formation of closed granulomata [7]. We have now investigated whether the increased susceptibility of CD38KO mice to mycobacteria could involve a compromised innate immune response.

We have assessed the ability of macrophages to control mycobacterial growth and found that, at initial stages of infection, macrophages of both strains were equally unable to control mycobacterial growth. However, by days 5 (BMDM cultures) and 3 (BMDM co-cultures), CD38KO macrophages had a lower mycobacterial burden than C57Bl.6 macrophages. These results paralleled our *in vivo* observations: one day after *M. avium* infection, CD38KO mice presented a lower mycobacterial burden than C57Bl.6 mice in the spleen and liver [7]. However, as infection progressed CD38KO mice presented higher CFU counts than C57Bl.6 mice [7], an indicator of the increased susceptibility of CD38KO mice to mycobacteria.

The initial phase of anti-mycobacterial responses involves macrophages' microbicidal mechanisms, including phagolysosome acidification and reactive species production [11]. We have found that CD38KO macrophages, similarly to C57Bl.6's, were unable to acidify the phagolysosome, possibly due to mycobacterial survival strategies, such as prevention of vesicular proton ATPase incorporation [12], rather than to intrinsic defects of macrophages. Effective phagolysosome acidification requires exogenous macrophage activation, namely by IFNγ. Although no differences were found between the two strains, IFNγ addition induced a slight decrease in the phagolysosome pH of CD38KO macrophages.

As CD38 is involved in superoxide production [8], we have hypothesized that it could also modulate NO production. We have found that *M. avium*-infected CD38KO and C57Bl.6 macrophages were only able to produce significant amounts of NO after exogenous activation by IFNγ or splenocytes of infected mice. IFNγ-activated *M. avium*-infected CD38KO macrophages were able to produce levels of NO that were even superior to those of C57Bl.6 macrophages. These data suggest that presence of CD38 on splenocytes, possibly activated T cells, is required for the effective activation of infected macrophages and NO production. Reduced NO production may be due to decreased IFNγ secretion. We have shown that *M. avium*-infected CD38KO mice produced reduced

amounts of IFNγ and were switched to Th2 responses [7]. We have now observed that decreased amounts of IFNγ and increased levels of IL-4 were produced when BMDM were co-cultured with CD38KO splenic cells from infected mice. Induction of Th1 responses demands an IL-12-rich microenvironment. As CD38 is required for human DC maturation and IL-12 secretion [13], we hypothesized that it could have a similar role in macrophages. However, when we quantified IL-12 production in BMDM and BMDM/splenocyte cultures, no differences were found between CD38KO and C57Bl.6 macrophages. Reduced IFNγ production by CD38KO splenic cells was not due to decreased IL-12 production. Thus, the pivotal role of CD38 in anti- mycobacterial immunity may be the induction of IFNγ secretion by T cells, CD38KO splenocytes being biased towards Th2 differentiation.

TNFα is a Th1 cytokine that is essential for granulomata formation and containment of mycobacteria [14]. We have shown that CD38KO mice are unable to form closed granulomata [7]. This could be due to reduced production of TNFα. Indeed, we have observed that TNFα secretion was significantly reduced in co-cultures of BMDM with CD38KO splenocytes from *M. avium*-infected mice with regards to C57Bl.6 splenocyte cultures. Paradoxically, we have found that, *in vivo*, splenocytes obtained from 30-day infected CD38KO and C57Bl.6 mice produced similar amounts of TNFα after antigenic challenge (unpublished data). However, it is important to note that we have measured TNFα production by splenocytes and not macrophages [7]. It is possible that CD38KO granulomata macrophages are unable to upregulate TNFα, due to decreased IFNγ production by T cells. Alternatively, absence of CD38 on T cells may, by itself, lead to reduced production of TNFα [16].

Increased susceptibility may also relate to production of suppressive cytokines. We have found that increased amounts of IL-10 were produced whenever CD38KO splenic cells were present in the culture. Altogether, our data suggest that, in the absence of CD38, activated $CD4^+$ T cells are biased towards a Th2 phenotype, with concomitant production of increased amounts of IL-4 and IL-10 and decreased secretion of IFNγ and TNFα, rendering CD38KO mice more susceptible to *M. avium* infection.

CD38 mediates immune cell chemotaxis, due to its role as an adhesion molecule and to modulation of CCR7-, CXCR4- and CCR2-signaling [5, 17-18]. The increased susceptibility of CD38KO mice to *S. pneumoniae* is the result of reduced chemotaxis of neutrophils to bacterial peptides [5]. After inoculation in the peritoneal cavity, mycobacteria are uptaken by resident macrophages, which start producing cytokines and

chemokines that attract blood leukocytes to the infection site to help in clearance of the invading pathogen [11]. We have found that CD38KO mice consistently presented lower numbers of granulocytes in the peritoneal cavity than C57Bl.6 mice. Decreased numbers of granulocytes may lead to reduced control of mycobacterial growth, as these cells enhance macrophages' bacteriostatic activity and help in containment of mycobacteria [19, 20, 21]. CD38KO and C57Bl.6 mice had similar numbers of resident macrophages in the peritoneal cavity, which were equally able to leave the infection site after ingestion of mycobacteria. However, we have found that CD38KO mice were deficient in the recruitment of blood mononuclear cells to the infection site, which may compromise the control of mycobacterial growth. It may be due to disrupted CD38/CD31 interactions or altered chemokine signaling or production. We have found that reduced amounts of MCP-3, MCP-1 and RANTES were produced whenever CD38KO splenocytes were present in BMDM cultures. MCP-1 and -3 are responsible for the recruitment of monocytes, activated T cells and immature DC to the infection site, and RANTES for that of monocytes and memory T cells. Reduced production of these chemokines may underlie the impaired recruitment of mononuclear cells to the peritoneal cavity.

The data presented herein support the involvement of CD38 in the second stage of the immune response against mycobacteria, when T-cell immunity is triggered. Activation of $CD4^+$ T cells in the absence of CD38 promotes their differentiation into Th2 cells, with production of high amounts of IL-4 and IL-10 and reduced secretion of IFN$\gamma$ and TNF$\alpha$. As a consequence, IFN$\gamma$ will be insufficient to fully activate macrophages' microbicidal mechanisms, such as production of NO. Consequently, killing of mycobacteria is compromised, rendering CD38KO mice more susceptible to *M. avium* infection. Finally, CD38 appears to be required for leukocyte chemotaxis to the infection site, due to modulation of chemokine production.

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**Legends of figures**

**Figure 1** CD38KO and C57Bl.6 macrophages had comparable CFU counts. BMDM were cultured and infected with $10^6$ viable *M. avium* bacilli. Macrophages were cultured either alone (A) or with splenocytes recovered from non-infected mice (B). At designated time points, the macrophages were recovered. Each dot represents the mean value of groups of six animals. Standard deviation of the mean is also presented. A two-tailed *t* test with a 95% confidence interval was performed; **, $p < 0.005$.

**Figure 2.** CD38KO macrophages were unable to acidify the phagolysosome even in the presence of IFNγ. BMDM were cultured and infected with $10^6$ viable *M. avium* bacilli in the absence (A) or presence of IFNγ (B). At designated time points pH measurements were performed. Each dot represents the mean value of 3 independent experiments. Standard deviation of the mean is also presented. A two-tailed *t* test with a 95% confidence interval was performed; ***, $p < 0.0005$.

**Figure 3.** CD38KO macrophages produced high amounts of NO when exogenously activated. BMDM were cultured and infected with $10^6$ viable *M. avium* bacilli. BMDM were cultured either in the absence (A) or presence of IFNγ (B). BMDM were cultured with splenocytes recovered from non-infected (C) or *M. avium*-infected mice (D). At designated time points, the supernatants were recovered and NO production quantified. Each dot represents the mean value of two independent experiments. Standard deviation of the mean is also presented. A two-tailed *t* test or a One-way ANOVA with a 95% confidence interval was performed; **, $p < 0{,}005$.

**Figure 4.** Reduced amounts of TNFα were detected in CD38KO BMDM/splenocyte cultures with regards to C57Bl.6 BMDM/splenocyte cultures. (A-B) BMDM macrophages were infected with *M. avium* bacilli. BMDM were co-cultured with (C-D) splenocytes recovered from non-infected or (E) from *M. avium*-infected mice. At designated time points, the supernatants were recovered and IL-12 and TNFα production quantified. Each dot represents the mean value of two independent experiments. Standard deviation of the

mean is also presented. A two-tailed *t* test or a One-way ANOVA with a 95% confidence interval was performed; **, $p < 0.005$.

**Figure 5.** CD38KO splenocytes secreted reduced amounts of IFNγ and high levels of IL-4 and IL-10 in response to *M. avium* infection. BMDM were co-cultured with (A) splenocytes recovered from non-infected or (B) from *M. avium*-infected mice. At designated time points, the supernatants were recovered and IFNγ, IL-4 and IL-10 production quantified. Each dot represents the mean value of two independent experiments. Standard deviation of the mean is also presented. A One-way ANOVA with a 95% confidence interval was performed; *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$.

**Figure 6.** Absence of CD38 compromised recruitment of leukocytes to the infection site and production of MCP-3, MCP-1 and RANTES. CD38KO and C57Bl.6 mice were i.p. infected with $10^6$ viable *M.avium* bacilli. A) At designated time points, mice were sacrificed, the peritoneal exudates recovered and the number of granulocytes (a) and mononuclear cells (b) quantified. Each dot represents the mean value of groups of six animals. Standard deviation of the mean is also presented. A two-tailed *t* test with a 95% confidence interval was performed; *, $p < 0.05$; **, $p < 0.005$.

B) BMDM were co-cultured with splenocytes recovered from *M. avium* infected mice. At designated time points, the supernatants were recovered and MCP-3 (a), MCP-1 (b), MIP-1α (c), MIP-1β (d) and RANTES (e) production quantified. Each dot represents the mean value of groups of two independent experiments. Standard deviation of the mean is also presented. A One-way ANOVA with a 95% confidence interval was performed; *, $p < 0.05$.

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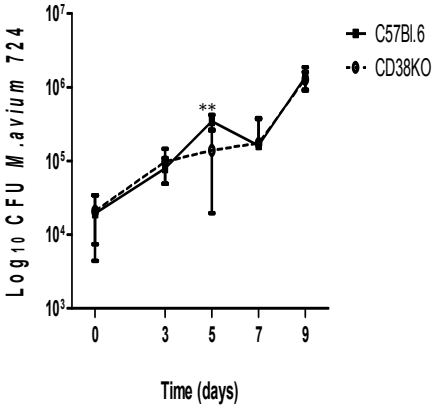

Log10 CFU M.avium 724
10^7
10^6
10^5
10^4
10^3
**
0
3
5
7
9
Time (days)
C57Bl.6
CD38KO

B)

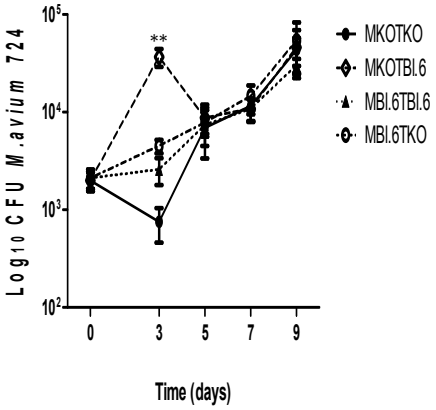
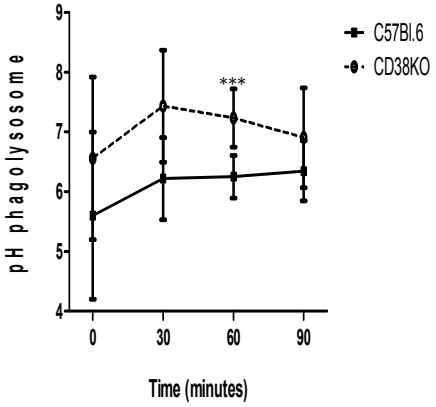

Log10 CFU M.avium 724
10^5
10^4
10^3
10^2
**
0
3
5
7
9
Time (days)
MKOTKO
MKOTBl.6
MBl.6TBl.6
MBl.6TKO

Figure 1.

A)


pH phagolysosome
9
8
7
6
5
4
0
30
60
90
Time (minutes)
***
C57Bl.6
CD38KO

B)

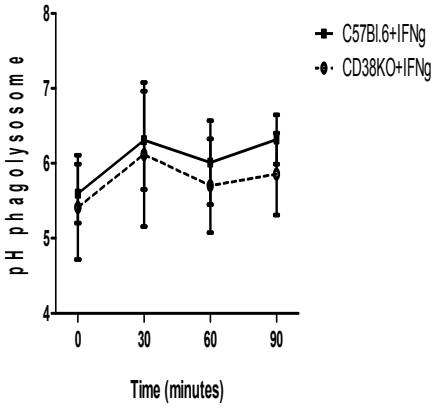
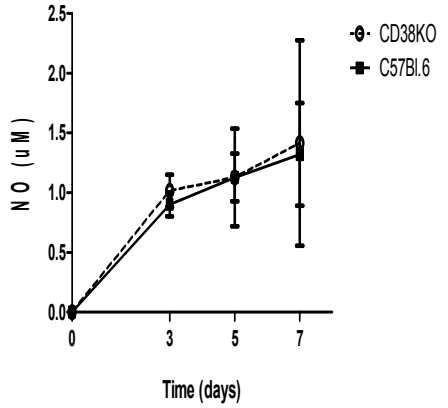

pH phagolysosome
8
7
6
5
4
0
30
60
90
Time (minutes)
C57Bl.6+IFNg
CD38KO+IFNg

Figure 2.

A)

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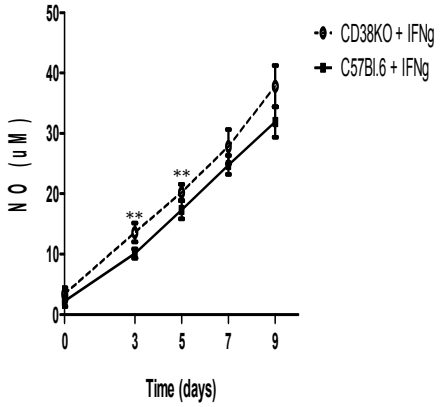


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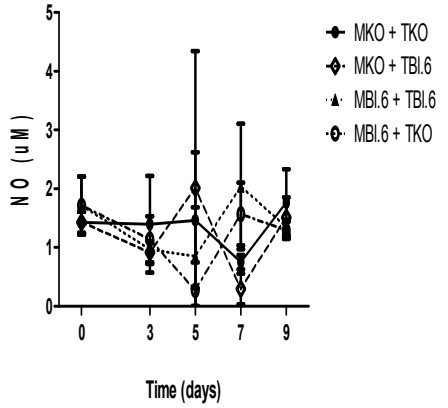


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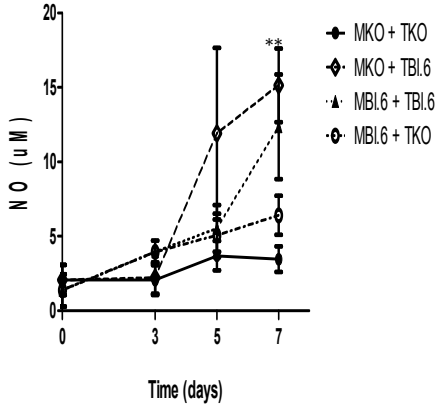


Figure 3.

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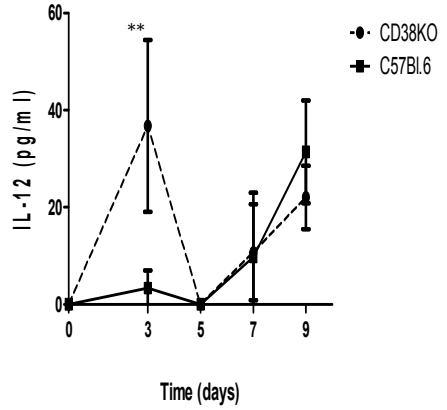


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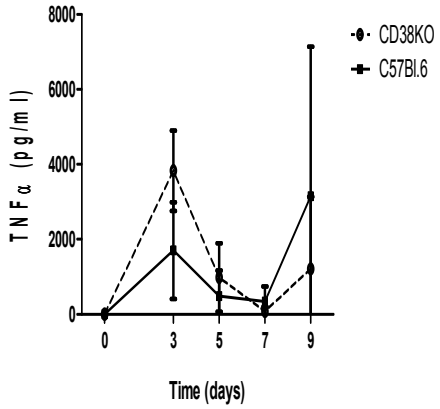


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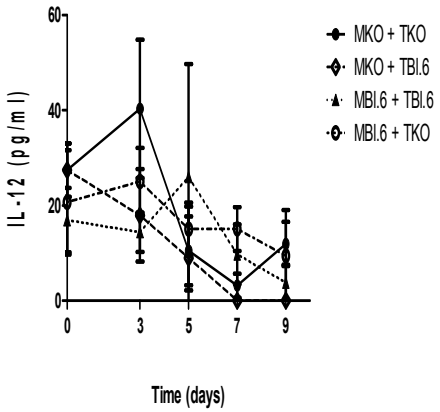


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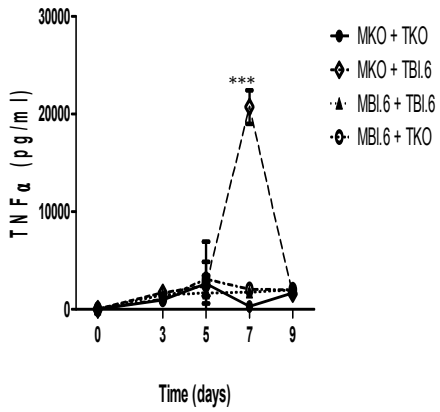


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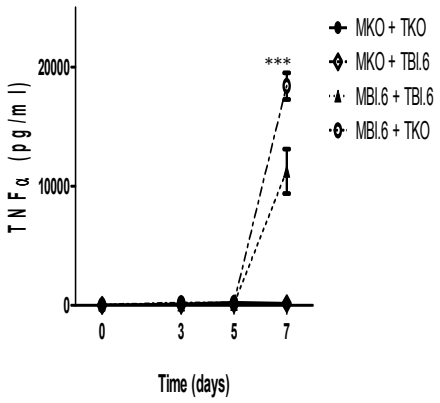


Figure 4.

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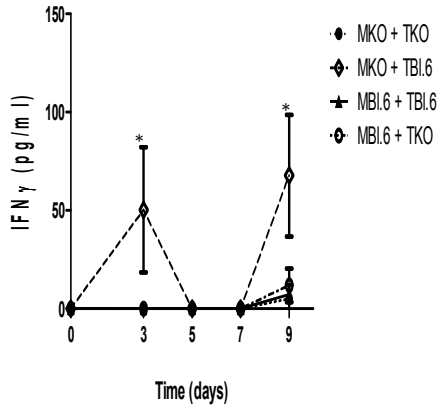


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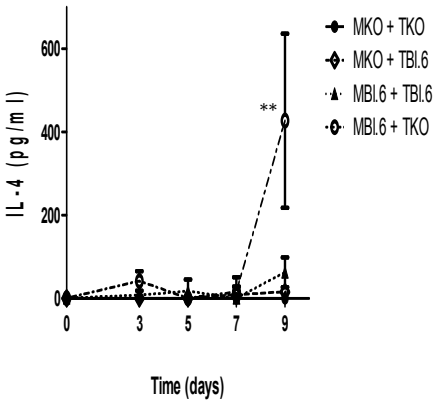


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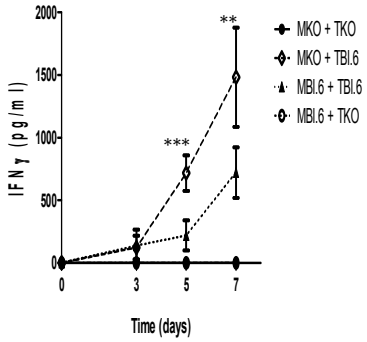


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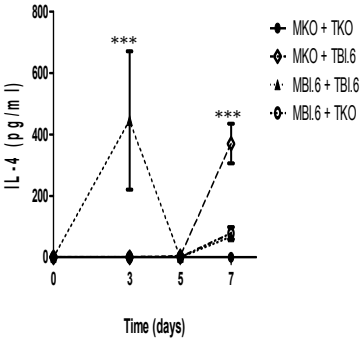


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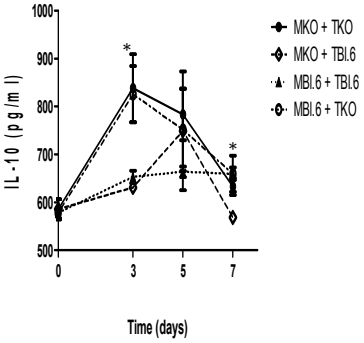


Figure 5

A

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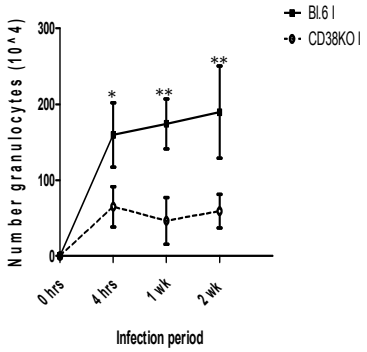


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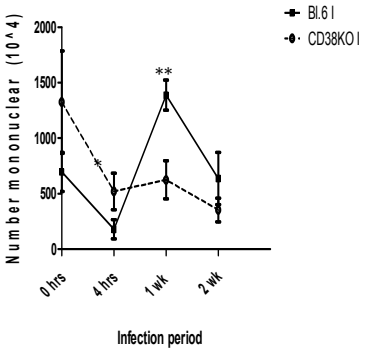


B

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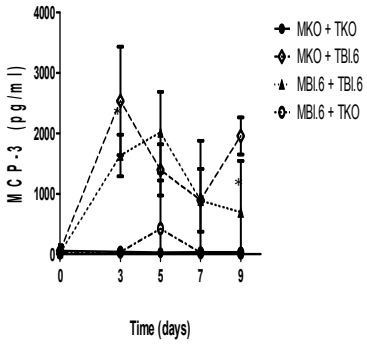


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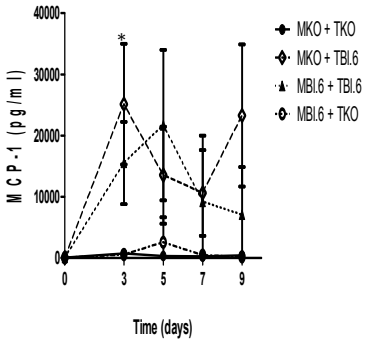


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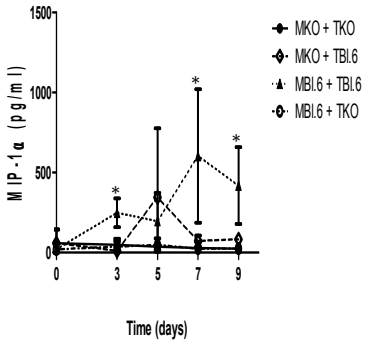


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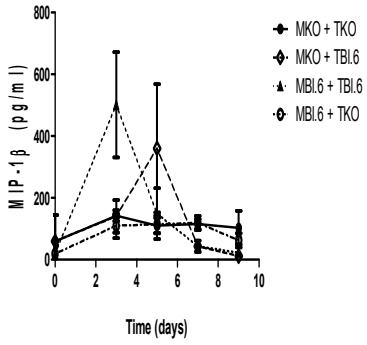


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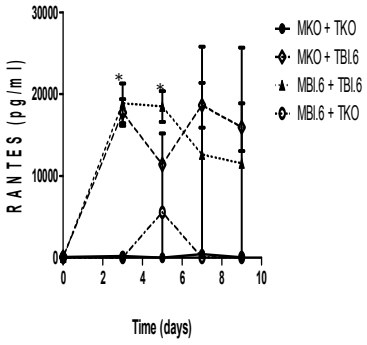


Figure 6

2.3 **Viegas MS, Lund FE, Thankappan A, Cooper A, Martins TC** CD38 is required for dendritic cell priming of Th1 responses to *Mycobacterium avium* infection. (manuscript in preparation)

CD38 is required for dendritic cell priming of Th1 responses to *Mycobacterium avium* infection.

Marta S. Viegas[a*], Frances E Lund[b,c], Anil Thankappan[b], Andrea Cooper[b], Teresa C. Martins[a,d]

[a]Molecular Pathology Laboratory, Portuguese Institute for Oncology at Coimbra Francisco Gentil, EPE (IPOCFG, EPE), Av. Bissaya Barreto, 98 - Apartado 2005, 3000-075 Coimbra, Portugal

[b]Trudeau Institute, Saranac Lake, NY, USA

[c]Div. of Allergy, Immunology and Rheumatology, Dept of Medicine, University of Rochester Medical Center, NY, USA

[d]Centre for Neuroscience and Cell Biology (CNC), University of Coimbra, 3004-517 Coimbra, Portugal

**Abstract**

CD38 belongs to a family of multifunctional ectoenzymes possessing signaling properties as well as extracellular and intracellular enzymatic activity. CD38 is expressed by several immune cell subsets and has been shown to have important immunological functions. In human dendritic cells, the cascade elicited by CD38 ligation leads to intracellular calcium mobilization, up-regulation of CD83 and production of IL-12. CD38 modulates DC migration through the control of CCR2-, CXCR4- and CCR7-mediated signaling. CD38 was shown to be required for effective immune responses against *Streptococcus pneumoniae* and *Mycobacterium avium*. Mycobacteria are intracellular pathogens that mainly infect macrophages. Control of mycobacterial infection depends on development of Th1-mediated immune responses and activation of infected macrophages. For this, DC must present mycobacterial antigens and activate T cells inducing their differentiation into Th1 cells. In this work, we have investigated whether absence of CD38 compromised the ability of dendritic cells to initiate a protective immune response against mycobacteria. For this, CD38KO and C57Bl.6 bone marrow derived dendritic cells were cultured and their response to mycobacterial infection compared. We provide evidence that concomitant expression of CD38 in the myeloid and lymphoid cell lineages is required for IFN$\gamma$ production by T cells and that the frequency of DC subsets in the spleen of mycobacteria-infected CD38KO mice is altered. Our data shows that CD38 expression on DC is required for effective priming of Th1 immune responses, which are protective against mycobacterial infections.

**Introduction**

CD38 belongs to a family of multifunctional ectoenzymes possessing signaling and cell adhesion properties, as well as extracellular and intracellular enzymatic activity (1). In mice, CD38 is widely expressed in many cell types, including B and T lymphocytes (2, 3). In these cells, CD38 physically associates with other cell surface receptors that are able to mediate signaling cascades (4-8). CD38 ligation induces activation of transcription factors that are involved in development of inflammatory responses, such as NF-κB (9). In addition, activation of cells via CD38 leads to increased production of various cytokines, namely IFNγ and GM-CSF. CD38 was shown to be functionally important on dendritic cells (DC). Activation of human DC via CD38 leads to intracellular calcium mobilization, up-regulation of CD83 and production of IL-12 (10). CD38 modulates DC migration towards CCL2, CCL19, CCL21 and CXCL12, through the control of CCR2, CXCR4 and CCR7-mediated signaling (11, 12).

Partida-Sànchez and co-workers (13) showed that CD38KO mice were more susceptible to infection by an extracellular bacterium, *Streptococcus pneumoniae*, due to diminished neutrophil chemotaxis to peptides of bacterial origin. We have shown that CD38 is also necessary for effective immune responses against *Mycobacterium avium,* an intracellular bacterium that mainly infects macrophages (14). Effective immune responses against mycobacteria involve Th1 cells, production of high amounts of IFNγ, which is required for full activation of mycobacteria-infected macrophages, and development of granulomatous responses (15). We have shown that the increased susceptibility of CD38KO mice to mycobacterial infection was associated with production of reduced amounts of IFNγ and that CD38 was required for the formation of a closed fibrotic rim around granulomata (14).

The inability of CD38KO mice to develop protective Th1 responses may result from a compromised cross-talk between the innate and the acquired immune systems. In this work, we have investigated whether the increased susceptibility of CD38KO mice to mycobacterial infection could be associated with defects on DC. We have found that absence of CD38 altered DC frequencies in the spleen. In addition, absence of CD38 compromised the ability of DC to drive Th1 differentiation. We provide evidence that concomitant expression of CD38 in the myeloid and lymphoid lineages is required for the production of IFNγ by T cells.

**Material and Methods**

Mice

C57Bl.6 mice breeding pairs were purchased from Harlan Iberica (Barcelona, Spain). B6.129P2-*Cd38*$^{tm1Lnd}$ (CD38KO) mice breeding pair was purchased from the Jackson Laboratories (Maine, USA). Both mice share the same genetic background. Mice were bred and kept in the animal housing facilities of the Institute for Molecular and Cell Biology (IBMC, Porto, Portugal) under SPF conditions. C57Bl.6-Tg (Tcr$\alpha$Tcr$\beta$) 425 Cbn/J (C57Bl.6 OT II) and CD38KO-Tg (Tcr$\alpha$Tcr$\beta$) 425 Cbn/J (CD38KO OT II) mice were bred and kept in the animal housing facilities of Trudeau Institute (Saranac Lake, NY, USA), under SPF conditions.

Mycobacteria

*Mycobacterium avium* strain ATCC 25291, serotype 2, was grown in liquid culture at 37ºC in Middlebrook 7H9 broth (Difco Labs) containing 0.04% Tween-80 (Sigma-Aldrich). Mycobacteria were harvested from liquid culture by centrifugation (6000g), washed three times in PBS, as described before (16), and kept frozen at -80ºC.

Mycobacterial infection

Bacteria were suspended in saline containing 0.04 % Tween-80 (Sigma-Aldrich) and diluted to a concentration of $2x10^6$ viable bacilli of *M. avium* per millilitre. Two-month old C57Bl.6 and CD38KO mice were intravenously (i.v.) infected with $10^6$ CFU of *M. avium* suspension. Aged-matched animals were inoculated with 0.04 % Tween-80 in saline.

Flow cytometric analysis of cell surface markers

The antibodies used in this work were as follows: i) PE-labelled anti-CD8 and anti-CD11c; ii) FITC-labelled anti-CD8 and anti-CD11b; and iii) CyChrome$^{TM}$-labelled anti-CD4 and anti-CD8 (all from BD Pharmingen). $10^6$ spleen cells from each sample were incubated with 50$\mu$l of the antibody suspension, at 4ºC, in the dark, for 15 minutes. Their staining pattern was analysed using a Becton Dickinson FACSort flow cytometer interfaced to Macintosh computer. Dead cells and erythrocytes were excluded from the analysis using a combination of forward light scatter and propidium iodide gating, as previously described (17).

Kinetics of *M. avium* infection

7, 15 and 20 days after i.v. inoculation with $10^6$ viable bacilli of *M. avium*, mice were sacrificed and the liver collected and homogenized in 10 ml of deionised water containing 0.05 % Tween-80 (Sigma-Aldrich). Serial dilutions were performed and 10 μl of each cell suspension were plated onto Middlebrook 7H10 agar (Difco Labs). Mycobacteria were allowed to grow at 37 ºC. After a period of 15 days, CFU were counted.

Mycobacterial antigens

Cell suspensions of *M. avium* were disrupted at 0 $^oC$ by ultrasonication for six periods of 30 s in a Branson sonifier set at 100 W. Protein concentration was determined by a modified Lowry procedure (18).

Purification of $CD4^+$ T cells

$CD4^+$ T cells were isolated from single-cell suspensions of the spleen of *M. avium*-infected mice using anti-$CD4^+$ magnetic beads (clone GK1.5; Miltenyi Biotec) on an AutoMACS machine following the manufacturer's instructions.

Detection of IFNγ-producing cells and IFNγ-producing $CD4^+$ T cells by ELISPOT

Splenic antigen-specific IFNγ-producing cells or IFNγ-producing $CD4^+$ T cells from infected mice were quantified using peptide-driven ELISPOT. Cells were ressuspended to $5x10^6$/ml in complete RPMI 1640 supplemented with 10% fetal bovine serum and serially diluted in MultiScreen HA 96-well plates coated with anti-IFNγ antibody (clone R46A2, eBioscience). $10^6$ irradiated syngeneic splenocytes were added to each well and stimulated with IL-2 (10U/ml) and *M. avium* Ag85 (0.5μg/ml). Cells were incubated for 24 hours at 37ºC in 5% $CO_2$ whereupon they were washed and biotinylated anti-IFNγ antibody (clone XMG 1.2, eBioScience) added. The presence of bound anti-IFNγ was detected using NBT as the substrate and the number of spots per well was determined using a dissecting microscope. The frequency of responding cells was calculated and extrapolated to the number of cells per sample to generate the total number of responding cells per organ. Neither samples cultured in the absence of antigen nor samples from uninfected mice produced detectable spots.

Chimera studies

Rag2KO male mice were lethally irradiated (450 rad) twice, with a four-hour interval, and reconstituted with a mixture of bone marrow cells from C57Bl.6, CD38KO, C57Bl.6Rag2KO and CD38Rag2KO mice. Bone marrow cells from donor mice were

injected into recipient mice through the retro-orbital venous plexus 1 hour after irradiation. Depletion of circulating cells and reconstitution with donor cells was confirmed in all chimeric mice by flow cytometry. Chimeric animals were allowed to recover for 6 weeks before infection with $10^6$ viable *M. avium* bacilli, as described. 15 days after infection, the mice were sacrificed and mycobacterial burden and the number of IFN$\gamma$-producing cells and IFN$\gamma$-producing $CD4^+$ T cells quantified. Chimeras were composed of: 25% CD38KO BM + 75% Rag2KO BM (MyeWT/LymKO), 25% CD38KO BM + 75% CD38Rag2KO BM (MyeKO/LymKO), 25% C57Bl.6 BM + 75% Rag2KO BM (MyeWT/LymWT) and 25% C57Bl.6 BM + 75% CD38Rag2KO BM (MyeKO/LymWT).

Bone Marrow Derived Dendritic Cell (BMDDC) culture

Dendritic cell precursors were recovered from murine femurs. $2x10^6$ cells were placed in a Petri dish in complete DMEM supplemented with GM-CSF (20ng/ml) and incubated at 37ºC. Every other day, GM-CSF was added to the culture to induce DC differentiation. At day 7 of culture, TNF$\alpha$ (10ng/ml) was added to the culture to induce DC maturation. $10^6$ DC were recovered and placed in a 24-well plate. $2x10^6$ viable bacilli were added to each well and incubated at 37ºC for 24 hours. After a 72-hour incubation period, the supernatants were collected and cytokine production quantified.

Cytokine quantification

DC culture supernatants were assayed for cytokine production using Mouse Th1/Th2 cytokine kit BD$^{TM}$ Cytometric Bead Array (CBA) (BD Biosciences) according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using the Student *t* test with a 95% confidence interval, for the comparison of two different conditions; and One-way ANOVA followed by Tukey HSD *a posteriori* multiple comparison tests with a 95% confidence interval, when comparing more than two experimental groups, or the corresponding non-parametric tests. Numerical populations were considered to be statistically different when $p < 0.05$.

**Results**

Absence of CD38 leads to a reduction in the number of IFNγ-producing cells.

We have previously shown that the increased susceptibility of CD38KO mice to mycobacteria was the result of decreased IFNγ secretion (14). We have now quantified the number of IFNγ-producing cells in *M. avium*-infected mice. We have found that, throughout infection, CD38KO mice consistently presented decreased numbers of these cells with regards to C57Bl.6 mice (Fig. 1A). As several cellular populations are able to produce IFNγ, we have also quantified antigen-specific IFNγ-producing $CD4^+$ T cells. Again, we have observed that CD38KO mice consistently presented lower numbers of IFNγ-producing $CD4^+$ T cells than C57Bl.6 mice (Fig. 1B).

Absence of CD38 in myeloid or lymphoid lineage leads to reduced numbers of IFNγ-producing $CD4^+$ T cells.

We have then investigated whether the increased susceptibility of CD38KO mice to mycobacteria was due to absence of CD38 in either the myeloid or the lymphoid lineage. For that, we have developed bone marrow chimeric mice in which we have selectively eliminated CD38 in either cell lineage. No differences were seen in the bacterial burden between the different chimeras (Fig 2A). Immunity to mycobacterial infection requires production of IFNγ. We began by determining whether absence of CD38 in either lineage impacted the number of IFNγ-producing cells. We have found that MyeKO/LymKO, MyeKO/LymWT and MyeWT/LymKO chimeric mice had a decreased number of IFNγ-producing cells with regards to MyeWT/LymWT chimeric mice (Fig. 2B). As many cell subsets are able to produce IFNγ, we have also quantified the number of IFNγ-producing $CD4^+$ T cells. We have found that MyeKO/LymKO chimeric mice had a lower number of these cells (6.5-fold reduction) than MyeWT/LymWT chimeric mice (Fig. 2C). We did not detect any IFNγ-producing $CD4^+$ T cells in MyeWT/LymKO and MyeKO/LymWT chimeric mice (Fig. 2C).

Absence of CD38 on DC or $CD4^+$ T cells leads to reduced production of IFNγ but not TNFα.

CD38 activation of human DC leads to up-regulation of CD83 and production of IL-12, suggesting that CD38 may play a role in DC maturation and polarization of Th1 immune responses. To study whether absence of CD38 compromised DC ability to activate T cells, we have isolated BMDDC and co-cultured them with purified $CD4^+$ T cells isolated from CD38KO OT II and C57Bl.6 OT II mice. DC were previously pulsed with

OVA as the TCR of OT II mice are restricted to this peptide. We have then quantified the production of TNFα and IFNγ. Similar amounts of TNFα were produced on CD38KO and C57Bl.6 DC cultures regardless of the origin of $CD4^+$ T cells (Fig. 3A). When CD38KO DC were co-cultured with CD38KO $CD4^+$ T cells, reduced amounts of IFNγ were produced, whereas increased levels of this cytokine were detected when C57Bl.6 DC were co-cultured with C57Bl.6 $CD4^+$ T cells (Fig. 3B). Intermediate levels of IFNγ were detected when CD38KO DC were co-cultured with C57Bl.6 $CD4^+$ T cells and C57Bl.6 DC were co-cultured with CD38KO $CD4^+$ T cells (Fig. 3B).

Absence of CD38 alters DC frequency in *M. avium*-infected mice.

Murine $CD8^+$ DC are thought to be responsible for the induction of Th1 mediated immune responses, whereas $CD8^-$ DCs induce a Th2 bias (19). We have quantified different subsets of DC, based on the expression of CD8, CD11b and CD11c. We have found that mycobacterial infection induced no differences in the percentage of $CD8^+$ DC in CD38KO mice, but an increase was observed in C57Bl.6 mice (Fig. 4A). No differences were seen when infected animals of both strains were compared (Fig. 4A). As for $CD8^-$ DC, we have found that infected CD38KO mice had a significantly higher percentage of these cells than control CD38KO mice. Similar observations were made for C57Bl.6 mice. Again, no differences were seen when infected animals of both strains were compared (Fig. 4B).

## Discussion

CD38 belongs to a family of multifunctional ectoenzymes, which possess ADP-ribosyl cyclase activity. CD38 may also behave as a selectin through interaction with CD31 (1). CD38 is expressed by several cell populations within the haematopoietic system and was reported to be functionally important in these cells (2, 3). CD38KO mice were shown to be more susceptible to infection by *Streptococcus pneumoniae,* an extracellular bacterium. This increased susceptibility was due to deficient neutrophil chemotaxis to peptides of bacterial origin, a consequence of reduced production of cADPR (13). We have shown that CD38 was also required for protection against *Mycobacterium avium* (14). We have found that splenic cells from *M. avium*-infected CD38KO mice produced decreased amounts of IFNγ with regards to C57Bl.6 splenocytes. As this effect could be due to the presence of reduced numbers of IFNγ-producing cells, we have quantified these cells. We have found that, from 15 days onwards, CD38KO mice consistently presented lower numbers of these cells than C57Bl.6 mice. Similar observations were made with regards to IFNγ-producing $CD4^+$ T cells. These data suggest that the decreased amounts of IFNγ produced by CD38KO mice result from reduced numbers of IFNγ-producing cells, namely $CD4^+$ T cells. In addition, it is important to note that absence of CD38 on T cells may, by itself, be responsible, at least to some degree, for the decreased production of IFNγ, as activation of T cells via CD38 induces production of this cytokine (3).

Reduced Th1 differentiation may be associated with altered DC priming. CD38 has a functional role in DC. In human DC, CD38 is required for CD83 expression and IL-12 production (10), thus playing a role in Th1 differentiation. When we quantified splenic DC subsets, we have found that *M. avium* infection induced an increase in the percentage of both $CD8^+$ and $CD8^-$ DC in C57Bl.6 mice, whereas in CD38KO mice only the frequency of $CD8^-$ DC was increased. This lead to an increased $CD8^-/CD8^+$ ratio in CD38KO mice (≈ 3) with regards to C57Bl.6 mice (≈ 2). Increased frequencies of $CD8^-$ DC in mycobacteria-infected CD38KO mice may result in a bias towards Th2 differentiation. Indeed, it is well established that murine $CD8^+$ DC preferentially induce IFNγ by activated T cells, whereas $CD8^-$ DC skew T cells to a Th2 phenotype (21, 22, 23).

To determine whether inability of CD38KO mice to develop Th1 immune responses was due to an intrinsic defect in the myeloid or lymphoid lineage or to inefficient priming by DC, we have developed bone marrow chimeric mice. We have quantified the number of IFNγ-producing cells in response to *M. avium* infection. We have found that knocking out of CD38 in either the myeloid, lymphoid or both lineages lead to a reduction on the number of IFNγ-producing cells. Similar observations were made when

IFNγ-producing $CD4^+$ T cells were quantified. These data suggest that expression of CD38 in both lineages is necessary for development of protective Th1 responses. Furthermore, absence of CD38 in either lineage lead to a reduction in the number of cells capable of producing IFNγ and possibly to a reduction in the amounts of IFNγ produced in response to *M. avium* infection. In fact, reduced amounts of IFNγ were detected when CD38KO BMDDC were co-cultured with CD38KO OT II $CD4^+$ T cells. In contrast, increased amounts of IFNγ were produced when C57Bl.6 BMDDC were co-cultured with C57Bl.6 OT II $CD4^+$ T cells. Altogether, these data suggest that absence of CD38 compromises DC ability to drive Th1 differentiation.

In conclusion, our data suggest that CD38KO mice are unable to mount a Th1-mediated response against *M. avium* infection and that this is probably due to ineffective priming by DC. Compromised Th1 differentiation leads to reduced production of IFNγ, which renders CD38KO mice more susceptible to *M. avium* infection, as IFNγ is required for full activation of infected macrophages and elimination of mycobacteria. We provide evidence that development of effective immune responses against mycobacteria requires expression of CD38 in cells of both the myeloid (e.g., DC) and the lymphoid (e.g., T cells) lineages.

**Acknowledgments**

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**Figure Legends**

**Figure 1.** *M. avium*-infected CD38KO mice consistently presented lower numbers of IFNγ-producing cells than C57Bl.6 mice. Mice were i.v. infected with $10^6$ viable *M. avium* bacilli. Control mice were injected with 0.04 % Tween-80 in saline. 7, 15 and 20 days after infection, spleens were collected and ELISPOT assay performed. A) Number of IFNγ-producing cells. Data represent the mean value of groups of 4 mice and the vertical line the standard deviation of the mean. B) Number of IFNγ-producing $CD4^+$ T cells. Data were obtained from a pool of 4 mice.

**Figure 2.** *M. avium*-infected CD38KO chimeric mice had decreased numbers of IFNγ-producing $CD4^+$ T cells. Chimeric mice were i.v. infected with $10^6$ viable *M. avium* bacilli. Control mice were injected with 0.04 % Tween-80 in saline. 15 days after infection, A) livers were collected and CFU quantified; B) spleens were collected, and the number of IFNγ-producing cells quantified by ELISPOT assay. Data represent the mean value of groups of 15 mice and the vertical line the standard deviation of the mean. C) 15 days after infection, spleens were collected, $CD4^+$ T cells were purified and the number of IFNγ-producing $CD4^+$ T cells quantified by ELISPOT assay. Data were obtained from a pool of 4 mice.

**Figure 3.** Expression of CD38 on DC and $CD4^+$ T cells was necessary for production of IFNγ. BMDDC were cultured and infected with $10^6$ viable *M. avium* bacilli to induce their maturation. DC were pulsed with OVA peptide and incubated with $CD4^+$ T cells recovered from either CD38KO OT II or C57Bl.6 OT II mice. After a 72-hour incubation, the supernatants were recovered and TNFα (A) and IFNγ (B) production quantified. Each column represents the mean value of two independent experiments and the bars the standard deviation.

**Figure.4.** In *M. avium*-infected CD38KO mice the frequency of $CD8^+$ DC remained unchanged in contrast to C57Bl.6 mice. Mice were i.p infected with $10^6$ viable *M. avium* bacilli. Control mice were injected with 0.04% Tween-80 in saline alone. 30 days after infection, spleens were collected and cells stained for flow cytometry. The identification of

the DC subsets was based on three distinct cell surface markers: CD8, CD11b and CD11c. $CD11c^+$ DC were gated and DC subsets $CD8^+CD11b^-$ ($CD8^+$ DC) and $CD8^-CD11b^+$ ($CD8^-$ DC) analyzed. Each bar represents the mean value of groups of 6 animals and standard deviation of the mean is also represented.

A) B)

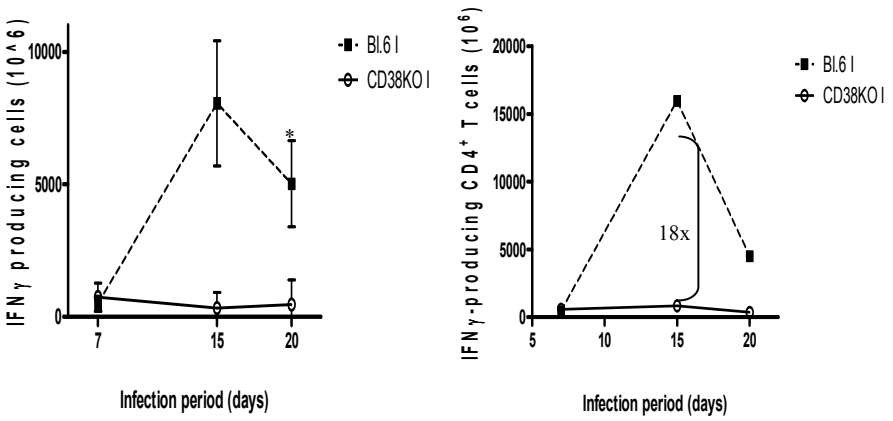

IFNγ producing cells (10^6)
10000
5000
0
7
15
20
*
Infection period (days)
Bl.6 I
CD38KO I
IFNγ-producing CD4+ T cells (10^6)
20000
15000
10000
5000
0
5
10
15
20
18x
Infection period (days)
Bl.6 I
CD38KO I

**Figure 1**

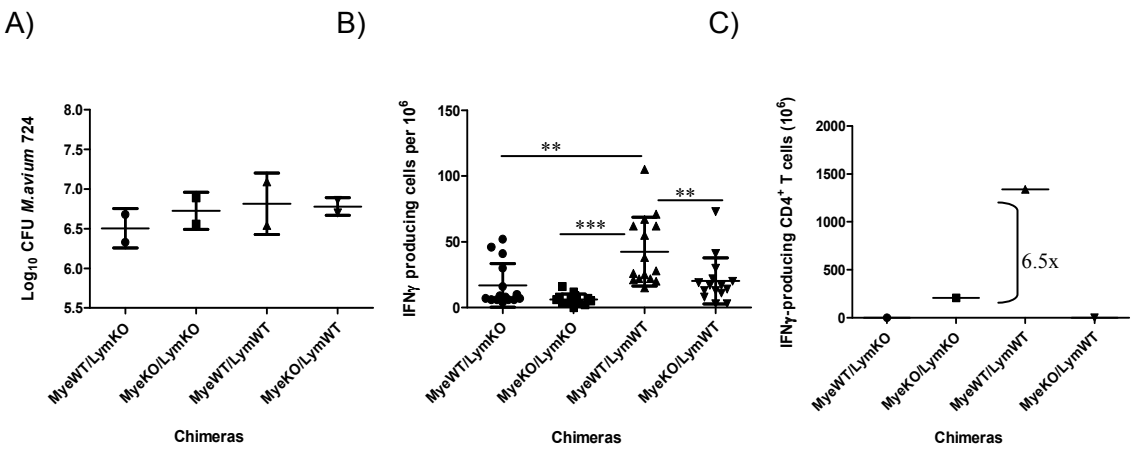
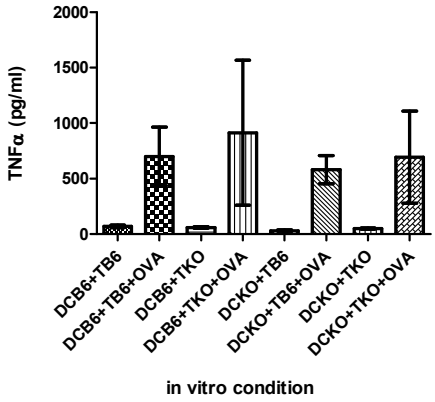

A)
B)
C)
Log10 CFU M.avium 724
8.0
7.5
7.0
6.5
6.0
5.5
MyeWT/LymKO
MyeKO/LymKO
MyeWT/LymWT
MyeKO/LymWT
Chimeras
IFNγ producing cells per 10^6
150
100
50
0
**
***
**
MyeWT/LymKO
MyeKO/LymKO
MyeWT/LymWT
MyeKO/LymWT
Chimeras
IFNγ-producing CD4+ T cells (10^6)
2000
1500
1000
500
0
6.5x
MyeWT/LymKO
MyeKO/LymKO
MyeWT/LymWT
MyeKO/LymWT
Chimeras

**Figure 2.**

A)


TNFα (pg/ml)
2000
1500
1000
500
0
DCB6+TB6
DCB6+TB6+OVA
DCB6+TKO
DCB6+TKO+OVA
DCKO+TB6
DCKO+TB6+OVA
DCKO+TKO
DCKO+TKO+OVA
in vitro condition

B)

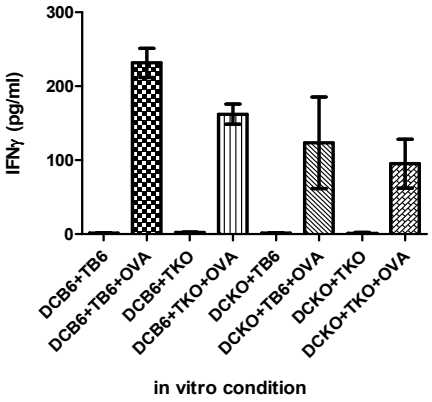
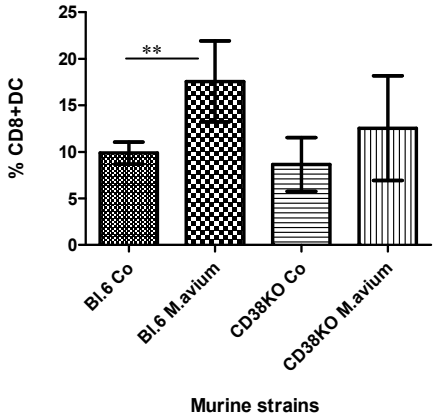

IFNγ (pg/ml)
300
200
100
0
DCB6+TB6
DCB6+TB6+OVA
DCB6+TKO
DCB6+TKO+OVA
DCKO+TB6
DCKO+TB6+OVA
DCKO+TKO
DCKO+TKO+OVA
in vitro condition

**Figure 3**

A)


% CD8+DC
25
20
15
10
5
0
**
Bl.6 Co
Bl.6 M.avium
CD38KO Co
CD38KO M.avium
Murine strains

B)

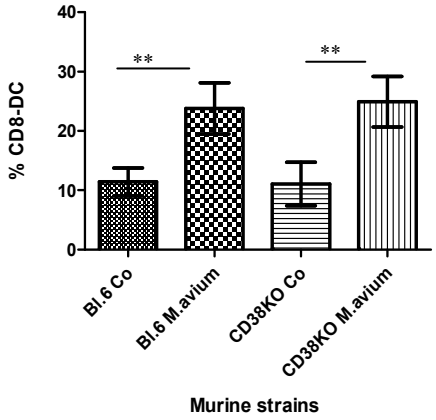

% CD8-DC
40
30
20
10
0
**
**
Bl.6 Co
Bl.6 M.avium
CD38KO Co
CD38KO M.avium
Murine strains

**Figure 4**

2.4 **Viegas MS, Pearl J, Cooper A, Lund FE, Martins TC** CD38 is required for development of protective Th1 responses against *Mycobacterium tuberculosis*. (manuscript in preparation)

CD38 is required for development of protective Th1 responses against *Mycobacterium tuberculosis*.

Marta S. Viegas[a*], Jonh Pearl[b], Andrea Cooper[b], Frances Lund[b,c], Teresa C. Martins[a,d]

[a]Molecular Pathology Laboratory, Portuguese Institute of Oncology at Coimbra Francisco Gentil, EPE (IPOCFG, EPE), Av. Bissaya Barreto, 98 Apartado 2005, 3000-075 Coimbra, Portugal

[b]Trudeau Institute, Saranac Lake, NY, USA

[c]Div. of Allergy, Immunology and Rheumatology, Dept of Medicine, University of Rochester Medical Center, NY, USA

[d]Center for Neurosciences and Cell Biology (CNC), University of Coimbra, Portugal

**Abstract**

CD38 is a multifunctional ectoenzyme. It may behave as an enzyme, a cell adhesion molecule or a cell surface receptor. Several cell lineages express CD38, including B and T lymphocytes, and macrophages. Besides being necessary for protection against extracellular bacteria, CD38 is also required for immunity against intracellular pathogens. Absence of CD38 leads to an increased susceptibility to *Mycobacterium avium* infection, a feature that was associated with decreased Th1 differentiation and decreased IFNγ production. *Mycobacterium tuberculosis* is among the human most life threatening intracellular bacteria and is becoming a growing problem due to the increased resistance of this bacterium to the available drugs. The present work aimed at investigating whether CD38 was required for the immune response against *M. tuberculosis*. After aerosol infection, the immune response of CD38KO mice was compared to that of their parental strain, C57Bl.6 mice. We have found that absence of CD38 reduced the ability of mice to control mycobacterial growth in the second phase of infection, that is, when T-cell mediated immune responses are triggered. In addition, priming of mycobacteria-specific T cells, in the absence of CD38, lead to a reduced production of IFNγ. Moreover, absence of CD38 was associated with reduced iNOS mRNA levels. We provide evidence that CD38 plays a role in development of Th1 immune responses, which are essential for immunity to mycobacteria.

## Introduction

CD38 belongs to a family of multifunctional ectoenzymes possessing signaling properties as well as extracellular and intracellular enzymatic activity (1). As an enzyme, CD38 is able to convert $NAD^+$ to ADPR and the latter to cADPR (1). The expression of CD38 is successively switched on and off during the ontogeny of lymphocytes, suggesting a role for this ectoenzyme in this process (2). Activation of lymphocytes through CD38 leads to $Ca^{2+}$ mobilization, cell activation and proliferation, cytokine secretion (IL-6, IL-10, IFN and GM-CSF), immunoglobulin class switching and modulation of apoptosis and of expression of different molecules (CD5, CD69, CD28, CD95, MHCII, B7.2) (3-7). In addition, CD38 synergizes with MHC class II molecules, augmenting macrophages' responses to superantigens (8). Partida-Sànchez and co-workers (9) showed that CD38KO mice were more susceptible to infection by *Streptococcus pneumoniae*, due to diminished neutrophil chemotaxis to the site of infection. We have previously shown that absence of CD38 rendered mice more susceptible to *Mycobacterium avium* infection. This susceptibility was due to reduced Th1 responses, which are essential for the control of infection. In addition, absence of CD38 compromised maintenance of the granulomatous barrier, leading to unrestrained growth and dissemination of mycobacteria (10). *Mycobacterium tuberculosis* (Mtb) is among the most life threatening intracellular bacteria for humans. It mainly infects macrophages, guaranteeing its survival by circumventing macrophages' microbicidal strategies (11). Mtb infection is acquired through the inhalation of infectious aerosols. Mycobacteria then travel to the terminal airways and establish infection. Effective immune responses to Mtb involve activation of Th1 cells and production of high amounts of IFNγ (12), as well as development of granulomatous responses (13). In this work, we have investigated whether absence of CD38 compromised the immune response against Mtb. Altogether our data suggest that CD38 plays a role in sustained immunity against this pathogen. In the absence of CD38, mycobacteria-specific T cells are compromised with regards to IFNγ production. Moreover, we have found that CD38 may play a role in NO production through enhancement of iNOS expression and may modulate apoptosis of mycobacterial-infected macrophages, two features that are essential for the control of mycobacterial infections.

**Material and Methods**

Mice

C57Bl.6 mice breeding pairs, purchased from The Jackson Laboratories (Maine, USA), and B6.129P2-*Cd38*$^{tm1Lnd}$ (CD38KO) mice (14) were bred and maintained in the Trudeau Institute Animal Breeding Facility.

Mycobacterial infection

The HRv37 strain of Mtb was grown to mid-log phase in Proskauer Beck medium containing 0.05% Tween 80 and frozen in 1 ml aliquots at -70ºC. C57Bl.6 and CD38KO mice were aerosol infected with 100 viable Mtb bacilli using a Glas-Col (Terre Haute, IN) airborne infection system as described previously (15).

Colony Forming Units (CFU)

1, 20, 30, 60, 90, 150, 200 days after infection, mice were killed by $CO_2$ inhalation, and lungs, spleen, liver and mediastinal lymph nodes (MLN) were collected and homogenized in physiological saline: 5ml for lungs, spleen and MLN, and 10ml for liver. Serial dilutions of organ homogenates were plated on nutrient 7H11 agar. Bacterial colony formation was quantified after 3 weeks of incubation at 37ºC.

Lung and MLN cell suspensions

Lung tissue was prepared as described previously (15). Lung cell suspensions were prepared by perfusing cold saline containing heparin through the heart until the lungs appeared white, whereupon they were collected. Dissected lung tissue was then incubated in collagenase IX (0.7mg/ml, Sigma-Aldrich) and DNAse (30μg/ml, Sigma-Aldrich). Single cell suspensions from either digested lung tissue or MLN were dispersed through a 70μm nylon tissue strainer (BD Falcon). The resultant suspension was treated with Geys solution, to remove any residual red blood cell, and washed twice, and cells were counted in a haemocytometer (15).

Purification of $CD4^+$ T cells

$CD4^+$ T cells were isolated from single-cell suspensions of lungs and MLN of Mtb infected mice using anti-$CD4^+$ magnetic beads (clone GK1.5; Miltenyi Biotec) on an AutoMACS machine following the manufacturer's instructions.

Detection of IFNγ-producing cells by ELISPOT

Lung and MLN antigen-specific IFNγ-producing cells and purified IFNγ-producing $CD4^+$ T cells from Mtb-infected mice were counted using peptide-driven ELISPOT. Cells were resuspended to $5x10^6$/ml in complete RPMI 1640 supplemented with 10% fetal bovine serum, serially diluted and plated in MultiScreen HA 96-well plates coated with anti-IFNγ antibody (clone R46A2, eBioscience, San Diego, CA). $10^6$ irradiated syngeneic splenocytes were added to each well along with IL-2 (10U/ml) and ESAT-6 (10μg/ml). Cells were cultured for 24 hours at 37ºC in 5% $CO_2$, the wells were washed and biotinylated anti-IFNγ antibody (clone XMG 1.2, eBioScience) was added. The presence of bound anti-IFNγ was detected using NBT as the substrate. The number of spots per well was determined using a dissecting microscope. The frequency of responding cells was calculated and extrapolated to the number of cells per sample to generate the total number of responding cells per organ. Neither samples cultured in the absence of antigen nor samples from uninfected mice produced detectable spots.

Histological analysis of granulomata

Two-month old female CD38KO and C57Bl.6 mice were aerosol infected with 100 Mtb bacteria. 20 and 60 days after infection, mice were sacrificed and the lungs recovered, fixed in 10% paraformaldehyde and embedded in paraffin. Histological slides were prepared and stained with haematoxylin-eosin (H-E) for morphologic evaluation of granulomata. An Axiophot microscope (Zeiss, Germany) with a digital still camera (DSC-S85, Sony, Japan) interfaced with a PC was used for image acquisition.

TUNEL assay

For *in situ* apoptosis assessment, the TUNEL assay was performed on lung tissue sections according to the manufacturer's instructions. A fluorescence microscope (Leica, DMI 6000B) with a still camera (Leica, DFC350FX) was used for image acquisition.

Cytokine quantification by Real-Time PCR

Lungs from 20, 60 and 90 days Mtb-infected CD38KO and C57Bl.6 mice were recovered and homogenized in RNAeasy Lysis Buffer. Total RNA was extracted according to the manufacturer's instructions (RNAeasy Mini Kit, Quiagen). RNA samples were treated with DNAse (Ambion) and were reverse transcribed using TaqMan reagents (Applied Biosystems). cDNA was then amplified using BioRad reagents on the IQ5 sequence detection system (BioRad). The endogenous control used to normalize the samples was *GAPDH*, the expression of which did not change in response to infection.

Fold increase was determined using ΔΔct calculation, as recommended by BioRad. The primers and probe sequences for murine IFNγ, iNOS and IL12p40 are published elsewhere (16).

Statistical analysis

Statistical analysis was performed using the Student *t* test with a 95% confidence interval. Numerical populations were considered to be significantly different when $p < 0.05$.

## Results

Bacterial burden in Mtb target organs.

To assess the susceptibility/resistance of CD38KO mice to Mtb infection, we have quantified the mycobacterial burden present in target organs (lungs, liver, spleen and MLN) of CD38KO and C57Bl.6 mice. At day 10 post-infection, CFU counts in the MLN of CD38KO were higher than in C57Bl.6 mice (Fig. 1). Regarding the liver, no differences were seen between the two strains (Fig. 1). We have also found that, at 200 days post-infection, mycobacterial growth in the lungs and spleen of CD38KO mice was increased with regards to the one observed in C57Bl.6 mice (Fig. 1).

Absence of CD38 did not compromise accumulation of $CD4^+$ T cells in mycobacteria target organs.

To determine whether absence of CD38 compromised accumulation of $CD4^+$ T cells in the lungs and MLN, we have quantified the number of these cells in these organs. We have found that, by day 20 and day 30 post-infection, infected CD38KO and C57Bl.6 mice had comparable numbers of $CD4^+$ T cells in the lungs (Fig. 2A). Of note, we have found that Mtb infection induced a higher increase in the number of $CD4^+$ T cells in C57Bl.6 mice than in CD38KO mice (Fig. 2A), which attained statistical significance by day 30. Regarding MLN, we have observed that CD38KO and C57Bl.6 mice had comparable numbers of $CD4^+$ T cells throughout the infection period (Fig. 2B). However, in CD38KO mice, the number of these cells declined as infection progressed, whereas in C57Bl.6 mice an increase was observed (Fig. 2B).

Absence of CD38 was not associated with a reduced number of IFNγ-producing cells.

Development of Th1 responses and consequent production of IFNγ is essential for protection against Mtb. We have thus quantified the number of IFNγ-producing cells specific for the immunodominant mycobacterial antigen, ESAT6, in the lungs and MLN of CD38KO and C57Bl.6 mice. We have found that CD38KO and C57Bl.6 mice had similar numbers of IFNγ-producing cells throughout infection in the lungs (Fig. 3A) and MLN (Fig. 3B). Of note, in the lungs, while in C57Bl.6 mice the number of these cells increased as infection progressed, in CD38KO mice the number of these cells remained unchanged (Fig. 3A). As for MLN, the numbers of these cells decreased in both strains as infection progressed, the decrease being more pronounced in CD38KO mice than in C57Bl.6 mice (Fig. 3B).

Absence of CD38 was not associated with reduced numbers of IFNγ-producing $CD4^+$ T cells.

After development of an acquired immune response, activated $CD4^+$ T cells become the main source of IFNγ. We have thus quantified the number of mycobacteria-specific $CD4^+$ T cells that were producing IFNγ in the lungs and MLN. Although no significant differences were observed between CD38KO and C57Bl.6 mice, the former consistently presented lower numbers of IFNγ-producing $CD4^+$ T cells than the latter, in the lungs (Fig. 4A) and MLN (Fig. 4B).

Absence of CD38 lead to reduced transcription of *IFNγ* and *iNOS* but not of *IL-12p40.*

We have previously reported that the increased susceptibility of CD38KO mice to *M. avium* was associated with decreased production of IFNγ (10) and to reduced numbers of IFNγ-producing cells (manuscript in preparation). Even though we herein present evidence that, in Mtb infection, the number of IFNγ-producing cells is similar in both strains, absence of CD38 may still affect the amount of IFNγ produced by each cell. As such, we have quantified the amount of IFNγ mRNA in mycobacteria-infected lung samples. As IFNγ production by T cells requires an IL-12-rich microenvironment, we have also quantified IL-12p40 mRNA. Since production of NO by activated macrophages is essential for the effective elimination of mycobacteria and *iNOS* is an IFNγ-inducible gene, quantification of iNOS mRNA was performed. As for IL-12p40 mRNA, no differences were observed between the two strains at any time point (Fig. 5c). By 20 and 90 days post-infection, CD38KO mice presented lower amounts of IFNγ mRNA than C57Bl.6 mice (Fig. 5a), no differences being seen by day 60 (Fig. 5a). Regarding iNOS mRNA, we have found that, by day 90 post-infection, CD38KO mice had decreased amounts of iNOS mRNA with regards to C57Bl.6 mice (Fig 5b), no differences being seen at 20 or 60 days post-infection (Fig. 5b).

Absence of CD38 did not affect development of granulomata.

Granuloma formation serves as an effective means for the containment of mycobacteria, preventing their sustained growth and dissemination. The adequate formation of granulomata depends, among other factors, on the number of macrophages and microorganisms present at the site of infection. As the host's initial resistance to Mtb infection is directly proportional to the strength of this granulomatous response, we have analyzed lung granulomata from CD38KO and C57Bl.6 mice. We have found that both strains presented well-organized, non-necrotizing granulomata with epithelioid cells,

macrophages and lymphocytes (Fig. 6). However, in CD38KO mice, there were larger areas of leukocyte accumulation than in C57Bl.6 mice (Fig. 6).

Absence of CD38 compromised induction of apoptosis in granulomata.

Apoptosis is an active process and may constitute a mechanism for the infected host to limit the outgrowth of mycobacteria, tissue damage and spread of infection. Moreover, apoptosis of mycobacteria-infected macrophages is associated with a reduced viability of intracellular mycobacteria (17). We have thus analyzed apoptosis in lung tissue sections of mycobacteria-infected mice by TUNEL. We have found that there was less apoptosis in CD38KO mice by 20 days post-infection than in C57Bl.6 mice, and that apoptotic cells were restricted to the border of the granulomata (Fig. 7A, B). In contrast, in C57Bl.6 mice apoptotic cells were homogenously distributed throughout the granulomata. These differences were not present at day 60. At this time point both strains presented similar amounts and distribution of apoptotic cells (Fig. 7C, D).

## Discussion

CD38 is a multifunctional ectoenzyme that is able to catalyze the hydrolysis of $NAD^+$ to nicotinamide and ADPR, as well as the synthesis and hydrolysis of cADPR (1). CD38 has a number of immunologically relevant functions. For instance, it may behave as an adhesion molecule, it can induce cytokine secretion, and it is able to enhance macrophage antigen-presenting function (14). CD38KO mice were found to be more susceptible to infection by extracellular bacteria, such as *S. pneumoniae*, due to reduced neutrophil migration to peptides of bacterial origin (9). CD38KO mice were also found to be more susceptible to infection by intracellular bacteria, namely *M. avium*, due to ineffective Th1 polarization and the inability to form closed granulomata (10).

Among the human most life threatening intracellular bacteria is *Mycobacterium tuberculosis.* In contrast to *M. avium*, Mtb infection is acquired through the inhalation of infectious aerosols. Mycobacteria then travel to the terminal airways and establish infection. The first stage of Mtb infection begins with entry of mycobacteria into the lungs. Macrophages and dendritic cells (DC) present at the site of infection ingest the bacilli. At this stage, the destruction of mycobacteria will depend on the intrinsic microbicidal capacity of the host phagocytes and virulence factors of the ingested mycobacteria. At two to three weeks after infection, T-cell immunity develops. Antigen-specific T cells arrive to and proliferate within early granulomata, and activate macrophages, which are then able to kill intracellular mycobacteria (17). In this work we have investigated whether CD38 was required for effective immune responses against Mtb. For this, CD38KO mice were aerosol infected with viable Mtb bacilli and their immune response compared to that developed by C57Bl.6 mice. We began by comparing the mycobacterial burden in CD38KO and C57Bl.6 mice at different time points. We have found that, early in infection (10 days), CD38KO mice presented an increased mycobacterial burden in MLN with regards to C57Bl.6 mice, suggesting an accelerated dissemination of mycobacteria to these organs. It has been shown that immunity to mycobacteria is associated with early mycobacterial dissemination to the MLN, reflecting a rapid initiation of cellular immune responses, rather than an increased susceptibility to the pathogen (18). However, we have found that CD38KO mice presented a higher mycobacterial burden than C57Bl.6 mice at late stages of infection (200 days), that is, after initiation of T cell mediated immune responses. These data are in agreement with our previous data on *M. avium* infection (10) and suggest that CD38 may be required for development/maintenance of effective cell-mediated immune responses against Mtb.

It is well established that Mtb dissemination to MLN always precedes and is required for development of T cell responses (18, 19). DC packed with mycobacterial

antigens arrive at T-cell areas in MLN, where they engage in antigen presentation. Mtb-specific $CD4^+$ T cells will be activated, differentiate and proliferate. Activated $CD4^+$ T cells leave the MLN and travel to the site of infection (lungs in this case) where they help in containment of mycobacteria (20). When we quantified the number of $CD4^+$ T cells in the MLN of Mtb-infected mice, we have found that, by day 20 and although not statistically significant, CD38KO mice had a higher number of $CD4^+$ T cells than C57Bl.6 mice. Furthermore, we have found that in CD38KO mice the number of $CD4^+$ T cells decreased as infection progressed. As for C57Bl.6 mice, we have found that the number of these cells increased as infection progressed. These data suggest that in CD38KO mice the early dissemination of mycobacteria to MLN may lead to a more rapid activation and proliferation of $CD4^+$ T cells than in C57Bl.6 mice; the reduced dissemination of mycobacteria to the MLN would delay this process in the latter. Once activated, $CD4^+$ T cells leave the MLN and migrate to the lungs. Indeed, we have found that, in both strains, the number of these cells increased as infection progressed.

Immunity to mycobacteria requires that activated T cells are biased towards a Th1 phenotype (25). We have previously shown that CD38KO mice, in response to *M. avium* infection, produced decreased amounts of IFNγ with regards to C57Bl.6 mice (10) and had reduced numbers of IFNγ-producing cells (manuscript in preparation). We have thus decided to quantify the number of IFNγ-producing cells and IFNγ-producing $CD4^+$ T cells in the MLN and lungs of Mtb-infected mice. We have found that CD38KO and C57Bl.6 mice had comparable numbers of these cells in both organs. Even though the number of IFNγ-production cells, in response to Mtb infection, is similar in both strains, absence of CD38 may still impact the amount of IFNγ produced by each cell. Indeed, activation of T cells through CD38 is associated with production of different cytokines, particularly Th1 cytokines (1, 5, 26). When we quantified the levels of IFNγ mRNA in the lungs of Mtb-infected mice, we have found that CD38KO mice had lower amounts of IFNγ mRNA than C57Bl.6 mice. These data suggest that activation of $CD4^+$ T cells, in the absence of CD38, leads to a reduction on the amounts of IFNγ secreted by these cells. This decrease in production of IFNγ could be due to reduced production of IL-12, as this cytokine is essential for Th1 priming. CD38 was shown to be required for IL-12 production by human DC (27). We have hypothesized that CD38 could have a similar role in murine APC and quantified the levels of IL12p40 mRNA in lungs of Mtb-infected CD38KO and C57Bl.6 mice. No differences were seen between the two strains, suggesting that CD38 is not required for production of this cytokine by murine APC. These data are in agreement with our *in vitro* data: we have found that CD38KO and C57Bl.6 macrophages were equally able to produce IL-12 upon *M. avium* infection (manuscript in preparation).

Mycobacteria-infected macrophages, unless fully activated, are unable to kill intracellular mycobacteria. Macrophages' activation can be performed by IFNγ (17). One of the microbicidal mechanisms used by activated macrophages is the production of reactive species, such as NO. NO is formed upon cleavage of L-arginine by nitric oxide synthases. Upon microbial challenge, the inducible NOS (iNOS) isoform, an IFNγ-inducible gene, is expressed and catalyses production of large amounts of NO over a period of days (28). In mycobacterial infections, iNOS assumes a relevant role, particularly during the late phases of infection (36). We have quantified the levels of iNOS mRNA and found that, at late stages of infection, CD38KO mice presented reduced levels of iNOS mRNA with regards to C57Bl.6 mice. The reduced levels of iNOS may be associated with reduced activation of mycobacteria-infected macrophages due to decreased production of IFNγ by activated CD38KO $CD4^+$ T cells. Indeed, we have found that *M. avium*-infected CD38KO and C57Bl.6 macrophages produced high amounts of NO provided IFNγ was present in culture (manuscript in preparation).

Mycobacterial infections elicit the development of granulomatous responses. Granulomata are host organized immune structures, composed of tightly interposed macrophages and T cells that are essential for host containment of mycobacterial infection (29, 30). With arrival of antigen-specific T cells, the lesion develops into a mature granuloma where activation of macrophages by IFNγ and TNFα results in inhibition of microbial growth. The fully matured granuloma protects the host not only by promoting mycobacterial containment, but also by reducing nutrient supply to the pathogens (31). In addition, mycobacterial growth is controlled through apoptosis of mycobacteria-infected macrophages inside granulomata. Apoptosis prevents spreading of mycobacteria, limits tissue damage and provides mycobacterial antigens for presentation to T cells (32). The presence of apoptotic cells in these structures is associated with resistance to infection by mycobacteria (11). When we quantified apoptosis, we have found that, at 20 days after infection, CD38KO mice had fewer apoptotic cells than C57Bl.6 mice. Of note, while in CD38KO mice apoptotic cells were restricted to the border of the granulomata dispersed apoptotic cells were found in C57Bl.6 mice. By day 60 after infection, CD38KO and C57Bl.6 mice had comparable numbers and distribution of apoptotic cells. These data suggest that absence of CD38 delayed apoptosis induction. This may compromise the control of mycobacterial growth.

Induction of macrophage apoptosis by mycobacteria requires production of TNFα by T cells (33) and NO production by Mtb-infected macrophages (34). Our previous data suggest that CD38 is required for production of TNFα, as its absence lead to decreased secretion of this cytokine (manuscript in preparation). Furthermore, we have found that

Mtb- and *M. avium*-infected CD38KO macrophages had reduced levels of iNOS mRNA (data presented herein) and produced decreased amounts of NO (manuscript in preparation), respectively, with regards to C57Bl.6 macrophages. We propose that CD38, due to its role in TNF$\alpha$ and NO production, may indirectly modulate apoptosis induction.

Failure to restrain mycobacterial growth within the granulomata may lead to disruption of this structure leading to bacterial dissemination to nearby areas and formation of additional lesions at distinct tissue sites (35). In fact, although *M. avium*-infected CD38KO mice form well-organized, non-necrotizing granulomata, they are unable to form closed fibrotic rims around them (10). Coalescent granulomata are often found in mycobacterial target organs in these mice. Similar observations were made in Mtb-infected CD38KO. Indeed, we have found that CD38KO granulomata occupied a larger area of the lungs with regards to that occupied by C57Bl.6's. This effect may be associated with the reduced killing of mycobacteria by Mtb-infected macrophages.

Altogether, our data suggest that CD38 assumes an important role during the second phase of mycobacterial infections, being pivotal for development of effective Th1-mediated immune responses. In addition, we show that CD38 is necessary for production of IFN$\gamma$ by Th1 cells, a feature that was not associated with reduced production of IL-12 by murine APC. Reduced production of IFN$\gamma$ may compromise macrophages' activation, which is essential for mycobacterial control, namely through iNOS upregulation and NO production. Through its ability to control NO and TNF$\alpha$ production, CD38 may indirectly modulate apoptosis induction of mycobacteria-infected macrophages, a feature that is associated with resistance to Mtb infection. In conclusion, we provide evidence that CD38 is required for immunity against *Mycobacterium tuberculosis* during the second stage of infection, that is, when T cell responses are triggered.

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**Figure legends**

**Figure 1.** CD38KO had an increased mycobacterial burden in lungs and spleen than C57Bl.6 mice. The animals were aerosol -infected with 100 viable *M. tuberculosis* bacilli. Target organs were collected at several time points and CFU counted. Data represent the mean value of groups of 4 mice and the vertical line the standard deviation of the mean (SD). *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0005$.

**Figure 2.** *M. tuberculosis*–infected CD38KO mice and C57Bl.6 mice had similar numbers of $CD4^+$ T cells in the lungs and MLN. The animals were aerosol -infected with 100 viable *M. tuberculosis* bacilli. Lungs (A) and MLN (B) were collected at day 20 and day 30 after infection and the number of $CD4^+$ T cells present was quantified by flow cytometry. For lungs, each dot represents one animal; for MLN, each dot represents the pool of 4 mice. The vertical line represents the SD. *, $p < 0.05$ non-infected Bl.6 vs infected Bl.6.

**Figure 3.** *M. tuberculosis*–infected CD38KO and C57Bl.6 mice had similar numbers of antigen-specific IFNγ-producing cells. The animals were aerosol -infected with 100 viable *M. tuberculosis* bacilli. At day 20 and day 30, lungs (A) and MLN (B) were collected and total cells were stimulated overnight with ESAT6. For lungs, each dot represents one animal; for MLN, each dot represents the pool of 4 mice. The vertical line represents the SD.

**Figure 4.** *M. tuberculosis*–infected CD38KO mice presented decreased numbers of IFNγ-producing $CD4^+$ T cells with regards to C57Bl.6 mice. The animals were aerosol -infected with 100 viable *M. tuberculosis* bacilli. At day 20 and day 30, lungs (A) and MLN (B) were collected and purified $CD4^+$ T cells stimulated overnight with ESAT6. For lungs, each dot represents one animal; for MLN, each dot represents the pool of 4 mice. The vertical line represents the SD. **, $p < 0.005$.

**Figure 5.** *M. tuberculosis*–infected CD38KO mice had decreased levels of IFNγ and iNOS mRNA with regards to C57Bl.6 mice. The animals were aerosol -infected with 100 viable

*M. tuberculosis* bacilli. At indicated time points, lungs were harvested and processed to extract RNA. The presence of specific mRNA was determined by real-time PCR. Each dot represents the mean value of 3 animals and the vertical lines the SD. *, $p < 0.05$.

**Figure 6.** *M. tuberculosis*–infected CD38KO mice develop well defined, non-necrotizing granulomata. At 20 and 60 days post-infection, lungs were collected and H-E stained for histological analysis (x100). Photomicrographs of representative granulomata in lungs of CD38KO (B, D) and of C57Bl.6 mice (A, C).

**Figure 7.** *M. tuberculosis*–infected CD38KO mice had reduced numbers of apoptotic cells at early time points of infection with regards to C57Bl.6 mice. The animals were aerosol - infected with 100 viable *M. tuberculosis* bacilli. At 20 and 60 days post-infection, lungs were collected and TUNEL assay was performed (x1000). Photomicrographs of representative granulomata in lungs of CD38KO (B, D) and C57Bl.6 mice (A, C).

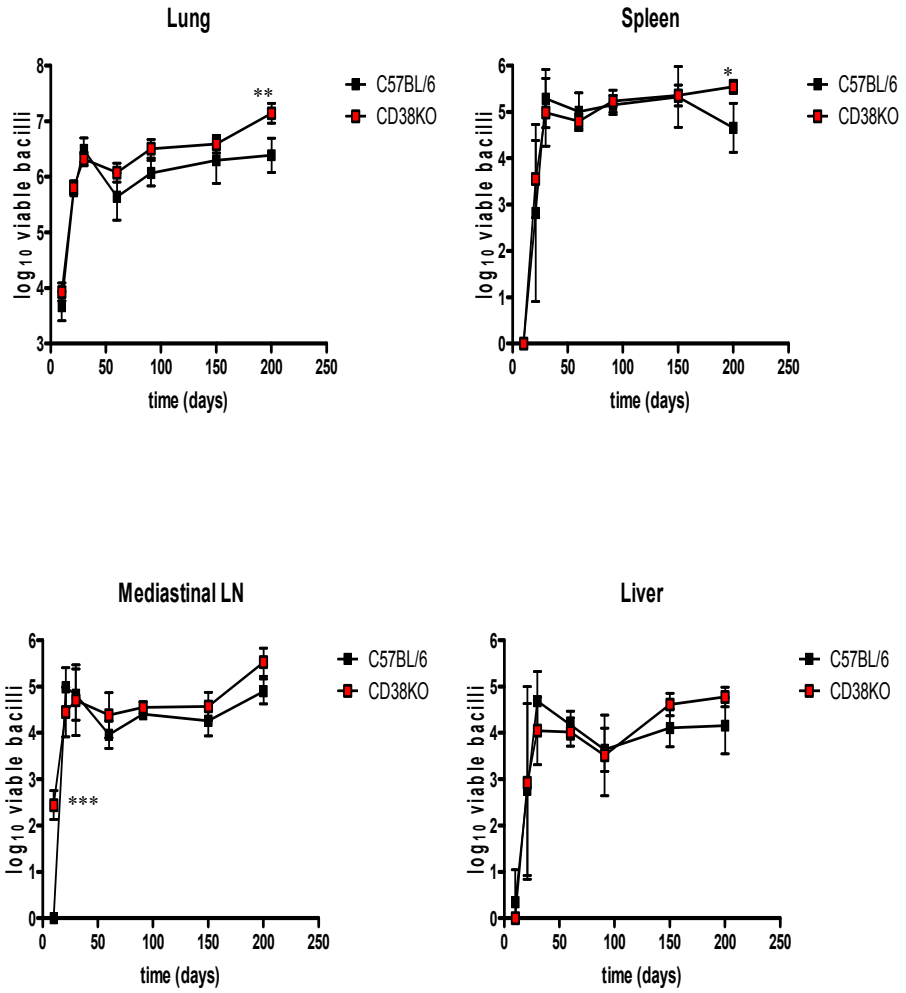
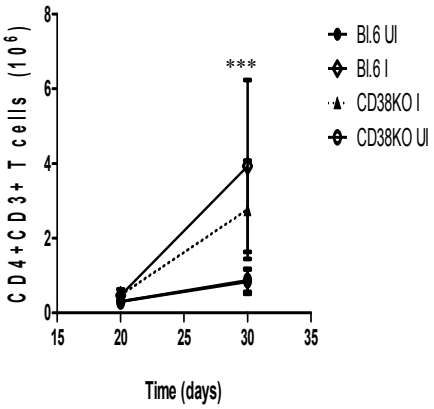

Lung
Spleen
Mediastinal LN
Liver
log10 viable bacilli
time (days)
C57BL/6
CD38KO
**
*
***

**Figure 1**

A)


CD4+CD3+ T cells (10^6)
***
Bl.6 UI
Bl.6 I
CD38KO I
CD38KO UI
Time (days)

B)

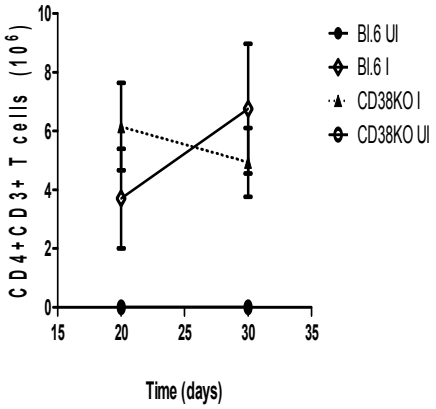
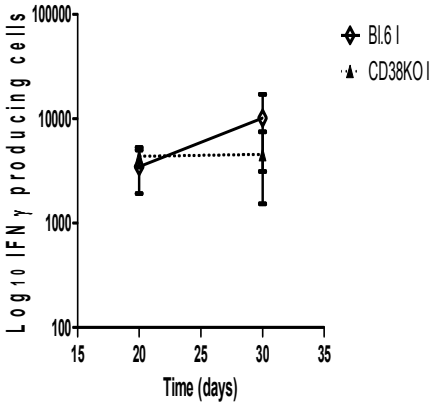

CD4+CD3+ T cells (10^6)
Bl.6 UI
Bl.6 I
CD38KO I
CD38KO UI
Time (days)

**Figure 2**

A)


Log10 IFNγ producing cells
100000
10000
1000
100
15
20
25
30
35
Time (days)
Bl.6 I
CD38KO I

B)

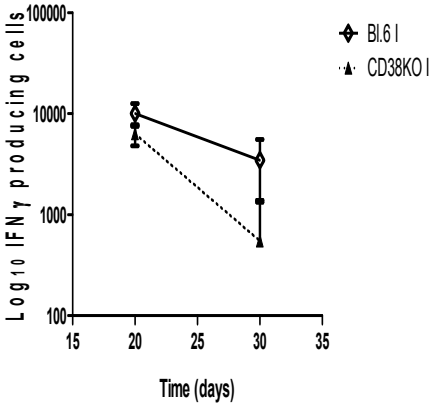
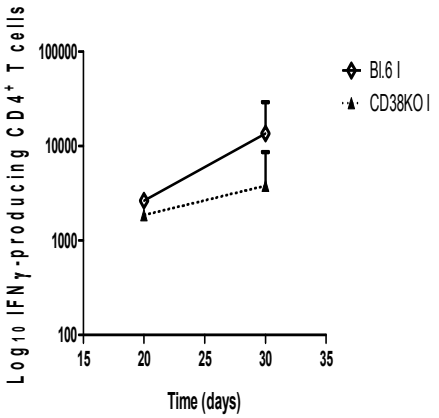

Log10 IFNγ producing cells
100000
10000
1000
100
15
20
25
30
35
Time (days)
Bl.6 I
CD38KO I

**Figure 3**

A)


Log10 IFNγ-producing CD4+ T cells
100000
10000
1000
100
15
20
25
30
35
Time (days)
Bl.6 l
CD38KO l

B)

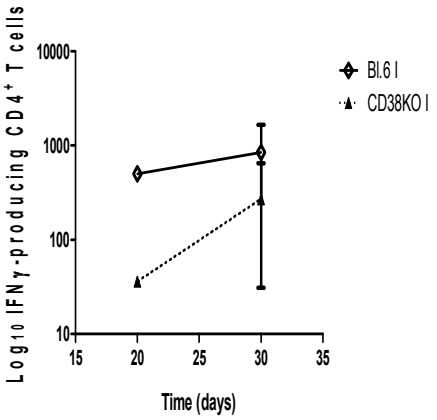
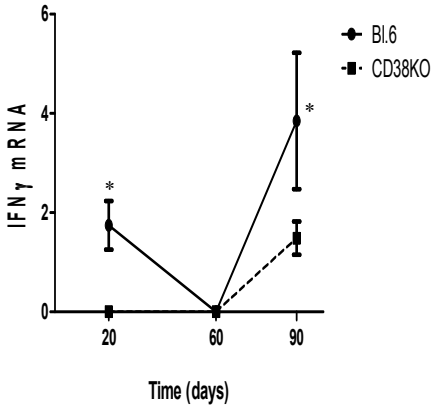
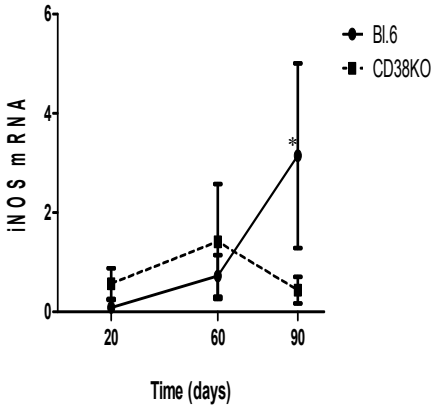

Log10 IFNγ-producing CD4+ T cells
10000
1000
100
10
15
20
25
30
35
Time (days)
Bl.6 l
CD38KO l

**Figure 4**

A)


IFNγ mRNA
6
4
2
0
20
60
90
Time (days)
Bl.6
CD38KO
*
*

B)


iNOS mRNA
6
4
2
0
20
60
90
Time (days)
Bl.6
CD38KO
*

C)

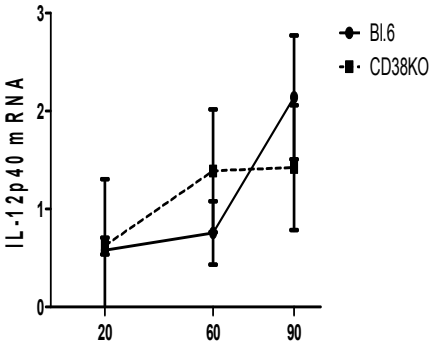
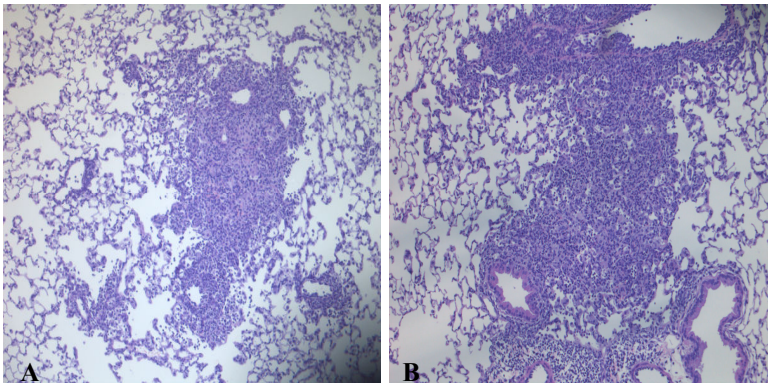

IL-12p40 mRNA
3
2
1
0
20
60
90
Bl.6
CD38KO

**Figure 5**

C57Bl.6 CD38KO

D20




D60

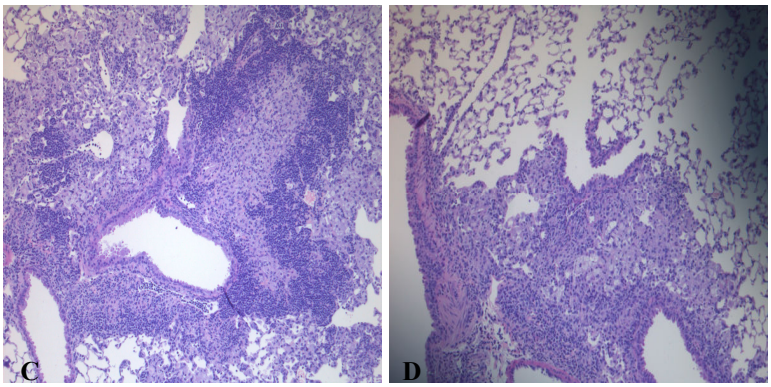



**Figure 6**

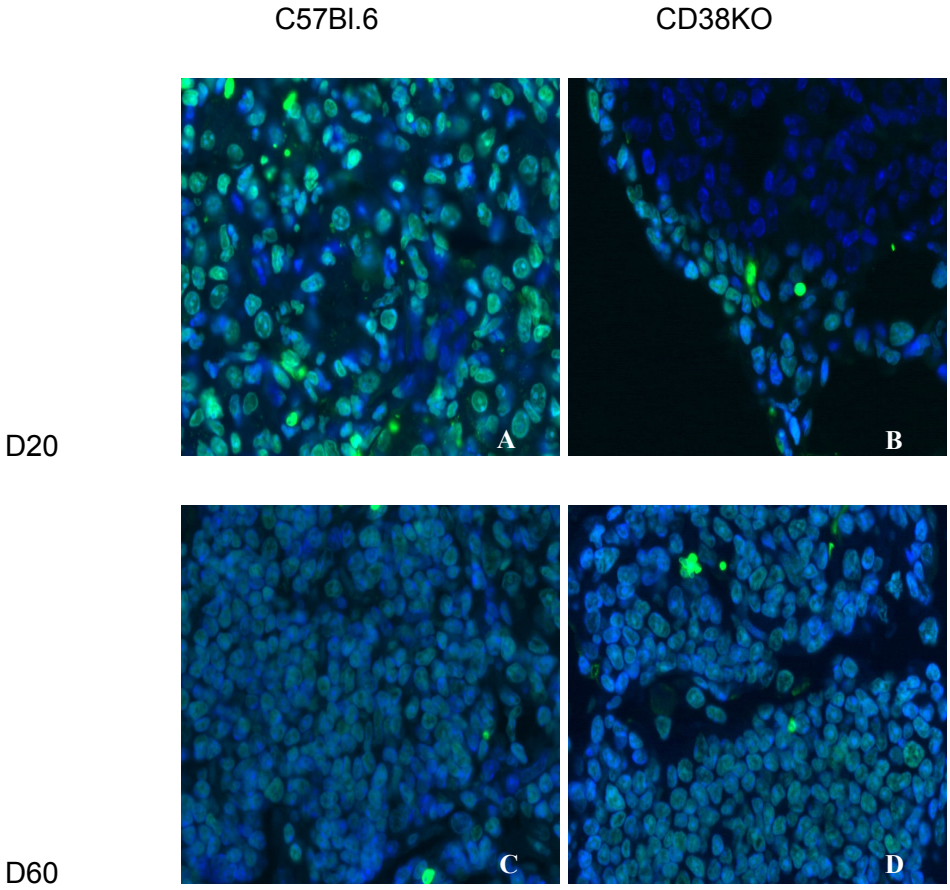



**Figure 7**

# Chapter Three

## 3. Role of CD38 in lupus.

3.1 **Viegas M, Martins TC, Seco F, Carmo A** (2007) An improved and cost effective methodology for the reduction of autofluorescence in direct immunofluorescence studies on formalin-fixed paraffin-embedded tissues. *Eur. J. Histochemistry* 51: 59-66.

# An improved and cost-effective methodology for the reduction of autofluorescence in direct immunofluorescence studies on formalin-fixed paraffin-embedded tissues

**M.S. Viegas,[1] T.C. Martins,[1,2] F. Seco,[3] A. do Carmo[2,3]**

[1]Molecular Pathology Laboratory, Portuguese Institute for Oncology of Coimbra (IPOCFG-EPE); [2]Center for Neurosciences and Cell Biology (CNC), University of Coimbra; [3]Vasco da Gama University School, Coimbra, Portugal

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Interference by autofluorescence is one of the major shortcomes of immunofluorescence analysis by confocal laser scanning microscopy (CLSM). CLSM requires minimal tissue autofluorescence and reduced unspecific fluorescence background, requisites that become more critical when direct immunofluorescence studies are concerned. To control autofluorescence, different reagents and treatments can be used. Until now, the efficacy of the processes described depended on the tissue type and on the processing technique, no general recipe for the control of autofluorescence being available. Using paraffin sections of archival formalin-fixed murine liver, kidney and pancreas, we have found that previously described techniques were not able to reduce autofluorescence to levels that allowed direct immunofluorescence labelling. In this work, we aimed at improving currently described methodologies so that they would allow reduction of the autofluorescent background without affecting tissue integrity or direct immunofluorescence labelling. We have found that the combination of short-duration, high-intensity UV irradiation and Sudan Black B was the best approach to reduce autofluorescence in highly vascularised, high lipofuscins' content tissues, such as murine liver and kidney, and poorly vascularised, low lipofuscins' content tissues such as the pancreas. In addition, we herein show that this methodology is highly effective in reducing autofluorescent background to levels that allow detection of specific signals by direct immunofluorescence.

Key words: direct immunofluorescence, paraffin sections, autofluorescence, liver, kidney, confocal laser scanning microscopy.

Correspondence: Marta Viegas,
Molecular Pathology Laboratory
Portuguese Institute for Oncology, IPOCFG-EPE
Av Bissaya Barreto, 98
Apartado 2005, 3000-075 Coimbra, Portugal
Tel: +351.239 400 200.
Fax: +351.239 484 317.
E-mail: marta.s.viegas@gmail.com

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Natural and fixative-induced tissue autofluorescence has long posed a problem in fluorescence-labelling studies, as specific signals can be masked. Fluorescence is the property of some molecules to absorb light at a particular wavelength and to subsequently emit light of a longer one. Natural fluorescence is mainly due to the presence of endogenous flavins, reduced NAD(P)H, lipofuscins, reticulin fibres, collagen and elastin. Flavins and NAD(P)H are coenzymatic redox carriers that play an important role in most metabolic pathways. NAD(P)H fluorescence results from hydrogen uptake at position 4 of its planar pyridine ring (Andersson *et al.* 1998). Lipofuscins, which are localized in lysosomes, consist of malonaldehyde which is a major product of lipid peroxidation. Malonaldehyde is known to react with amino acids and proteins to yield conjugated Schiff bases and hence fluorescent products (Baschong *et al.* 2001) (Table 1). Collagen/elastins, which are typical constituents of blood vessels, are also a source of autofluorescence (Billinton *et al.* 2001) (Table 1). Collagen is also an important component of stroma and connective tissue. Reticular fibres are also an important source of green autofluorescence. The degree of natural fluorescence relates to the tissue type. Tissues such as kidneys and liver can be very challenging, as these organs have extremely high metabolic rates with constant NADP-NADPH turnover, high accumulation of flavins and lipofuscins, and are highly vascularised, thus containing high amounts of collagen and elastin, and their parenchyma, especially that of liver, are rich in reticular fibres. These characteristics are not shared with tissues, such as the pancreas, that have low accumulation of both flavins and lipofucins, and reduced vascularisation.

Tissue fixatives, which are used for preservation of morphological details, may further augment background fluorescence. Neutral buffered formalin (the fixative used in this work) is the most widely used

**Table 1. Emission wavelength of the fluorophores responsible for intrinsic and fixative-induced autofluorescence.**

| *Autofluorescence source* | *Emission wavelength (nm)* | *Excitation wavelength (nm)* |
|---|---|---|
| Flavins | 500-560 | 360-520 |
| NAD(P)H | 440-470 | 340-460 |
| Lipofuscins | 450-650 | 345-360 |
| Collagen/elastins | 470-520 | 330-400 |
| Formaldehyde induced fluorescence | 420-470 | 355-435 |

fixative in a routine setting. Formaldehyde forms covalent bonds between adjacent amine-containing groups through Schiff acid-base reactions. As a consequence, fluorescent products are formed, resulting in an intense fluorescent background (Beisker *et al.* 1987) (Table 1). These products may also unspecifically react with antibodies.

The molecules that contribute to intrinsic and induced autofluorescence are responsible for an emission of fluorescence between 450 and 650 nm that overlaps the emission wavelength of fluorophores widely used in immunofluorescence studies, such as fluorescein isothiocianate (FITC) and phycoerithrin (PE).

As many parameters need to be taken into account, reduction of tissue autofluorescence has proven very difficult. Depending on the cause of autofluorescence, different methodologies have been used (Cowen *et al.* 1985; Mera *et al.* 1980; Neumann *et al.* 2002; Schnell *et al.* 1999). It was suggested that the use of chemical procedures, such as treatment with ammonia/ethanol, could be a good approach to reduce autofluorescence (Baschong *et al.* 2001). Autofluorescence quenching using dyes, such as Sudan Black B, Pontamine Sky Blue and Trypan Blue, among others, may also constitute a good alternative (Baschong *et al.* 2001, Cowen T *et al.*, 1985, Mosiman VL *et al.*, 1997, Schnell *et al.* 1999). A third approach is the use of photochemical methods, such as photobleaching (Billinton *et al.* 2001), through which, the molecular structure of a fluorophore is changed so that it loses its ability to fluoresce. The use of instruments, such as optimised filter sets, confocal laser scanning microscopy (CLSM) and post-measurement image correction also allow discrimination of autofluorescence through the use of mathematical models (Steinkamp *et al.* 1986; Van de Lest *et al.* 1995). The use of optimised filter sets is particularly suited for the reduction of NAD(P)H-associated autofluorescence background. So far, tissue type and processing techniques have dictated the effectiveness of the methodologies used for quenching of autofluorescence. Until now, no methodology has been shown to sufficiently reduce the autofluorescent background in organs with different characteristics, for the detection of direct immunofluorescence signals. In fact, the application of the previously described methods to kidney, liver and pancreas tissue sections did not allow the detection of specific immunofluorescent signals due to the autofluorescent background still observed. In this work, we aimed at the improvement of these methodologies so that they would permit drastic reduction of autofluorescence without compromising direct immunofluorescence labelling in formalin-fixed, paraffin-embedded murine kidney, liver and pancreas. A total reduction of the autofluorescent background was attained when kidney, liver and pancreas tissue sections were pre-treated with short-duration (2 hours), high-intensity (30 W) photobleaching and Sudan Black B.

## Materials and Methods

### *Tissue sections*

Murine kidneys, livers, and pancreata were collected from six-month old C57Bl.6*lpr/lpr* mice, fixed in 10% paraformaldehyde and embedded in paraffin. Tissue sections of 5 μm were cut and mounted onto glass slides that were previously coated with poly-L-lysine (5 mg/mL) (Sigma). For removal of paraffin, the slides were immersed in xylene (twice for 10 min), rehydrated with graded ethanol (5 min in 100% and 5 min in 95% ethanol) and transferred to deionised water.

All mice used in this work were bred and kept in the animal housing facilities of the Institute for Molecular and Cell Biology (Porto, Portugal), under specific pathogen free conditions. This work was approved by the Animal Welfare Division of the Portuguese Veterinary Council.

### *Ammonia/ethanol*

The slides were incubated with 0.25% ammonia in 70% ethanol for 1 hour at room temperature, in a wet chamber. For rehydration, the slides were immersed in 50% ethanol for 10 min, at room temperature, in a wet chamber and transferred to MHB buffer (calcium free Hank's buffer containing 2 mM EGTA, 5 mM 2-morpholino-ethenesulfonic acid), pH 6.2-6.4, for 20 min, at room temperature.

### *Sudan Black B*

Tissue sections were immersed in 0.1% Sudan Black B (Sigma) in 70% ethanol for 20 min, at room temperature, in a wet chamber. For the removal of Sudan Black B excess, the slides were washed 3 times, for 5 min, in phosphate buffered saline (PBS) 0.02% Tween 20. A final jet wash with PBS 0.02% Tween 20 was required for the complete removal of Sudan Black B excess.

### *Photobleaching*

Before removal of paraffin, tissue sections were irradiated with UV light (30 W, 253 nm to 400 nm discrete emission, Philips), at room temperature, and hourly monitored for reduction of the autofluorescent background, after which they were transferred to PBS.

### *Proteolytic digestion*

Tissue sections were incubated with proteinase K (20 µg/mL) (Quiagen) in PBS, for 10 min, in a wet chamber, at room temperature. After antigen retrieval, the slides were washed in PBS 0.02% Tween 20.

### *Immunofluorescence labelling*

Kidneys from autoimmune glomerulonephritis-prone mice were collected, fixed in 10% paraformaldehyde and embedded in paraffin. Tissue sections were photobleached (30 W) for 2 hours at room temperature. After removal of paraffin and proteolytic digestion, tissue sections were blocked in 10% foetal bovine serum (FBS) in PBS, for 1 hour, at room temperature, and incubated with 0,1% Sudan Black B in 70% ethanol, for 20 min, in a wet chamber, at room temperature. Afterwards, the slides were incubated with FITC-labelled goat anti-mouse $IgG_1$ (cat. nº 1070-02), goat anti-mouse $IgG_2$ (cat. nº 1090-02) or goat anti-mouse $IgG_3$ (cat. nº 1100-02) monoclonal antibodies (all from Southern Biotechnologies) overnight, at 4 °C, in a wet chamber. These antibodies react with the heavy chain of mouse $IgG_1$, $IgG_2$ and $IgG_3$, respectively. As a control, slides were incubated in PBS alone. The slides were then washed 3 times in PBS 0.02% Tween 20 for 5 min and mounted with VectaShield immunofluorescence mounting medium (Vector Laboratories, Inc).

### *Evaluation of autofluorescence and of immunolabelling*

For image acquisition, a confocal laser scanning microscope (BioRad, MRC60C) was used. The settings for contrast, brightness, pinhole, acquisition mode and scanning time were maintained throughout the work. The illumination system consisted of a Kr-Ar laser, a 20x plan-neofluor objective (Olympus) and two filter sets. One of the filter sets included an excitation filter of 488 nm and an emission filter at 520 nm. The second filter set included an excitation filter of 568 nm and a barrier filter of 585 nm. Tissue sections were evaluated under transmission light to check for any structural abnormality. Epifluorescence was used to evaluate autofluorescence and immunolabelling. All the tissue sections were evaluated with each of the two filters sets.

## Results

### *Control of autofluorescence background*

*Natural autofluorescence of different tissues*

We have found that formalin-fixed murine kidney, liver and pancreas tissue sections presented an intense autofluorescence, irrespective of the filter set used (Figure 1A-C). Of notice, the autofluorescent background of pancreas tissue sections was not as bright as that observed for kidney and liver tissue sections.

*Ammonia/ethanol*

Ammonia/ethanol has been used to decrease fixative-induced fluorescence in decalcified bone marrow preparations (Baschong *et al.* 2001). When applied to kidney, liver and pancreas tissue sections the autofluorescence was not reduced regardless of the filter set used for excitation (Figure 1D-F).

*Sudan Black B*

Sudan Black B is widely used to quench natural autofluorescence, namely that of lipofuscin (Baschong *et al.* 2001). When excitation was performed at 488 nm, we have observed a drastic reduction of the autofluorescent background in kidney, liver and pancreas tissue sections. However, when tissue sections were exposed to light of 568 nm excitation wavelength the autofluorescent background was not decreased in any of the tissue sections under study (Figure 1G-I).

*Combined use of ammonia/ethanol and Sudan Black B*

When ammonia/ethanol and Sudan Black B were combinedly applied to kidney tissue sections, auto-

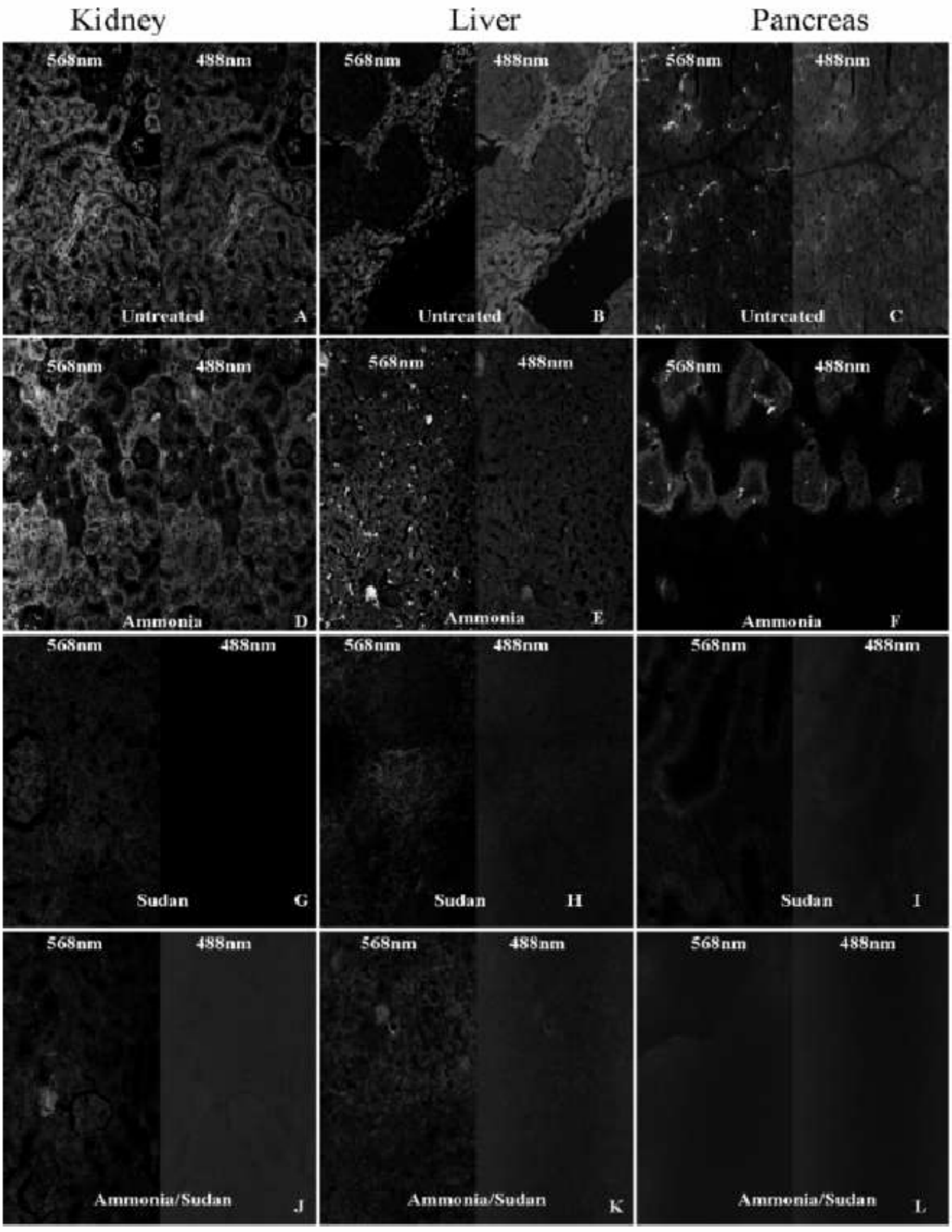


Figure 1. Influence of tissue type and processing in histochemical control of autofluorescence on formalin-fixed, paraffin-embedded tissues. Untreated controls: kidney (A), liver (B) and pancreas (C). Treatment with ammonia/ethanol did not reduce autofluorescence regardless of the wavelength of excitation (D, E, F). After treatment with Sudan Black B, reduction of autofluorescent background was attained when excitation was performed at 488 nm, but not at 568 nm (G, H, I). Treatment with ammonia/ethanol and Sudan Black B was inefficient in kidney (J), but reduced autofluorescence in pancreas (L). No effect was observed when this treatment was applied to liver tissue sections (K). (x200, CLSM).

fluorescence was increased regardless of the filter set used. In contrast, in pancreas tissue sections treatment with ammonia/ethanol and Sudan Black B reduced the autofluorescent background to some extent. As for the liver, treatment with ammonia/ethanol and Sudan Black B had no effect in reduction of the autofluorescent background (Figure 1J-L).

*Photobleaching*

Photobleaching has been used to decrease fixative-induced fluorescence. It was reported that the fluorescent background decreased as irradiation time increased (Neumann *et al.* 2002). Kidney, liver and pancreas tissue sections were submitted to UV irradiation and reduction of fluorescence was hourly monitored. We have found that in kidney, liver and pancreas tissue sections reduction of the background fluorescence reached its peak after two hours of UV irradiation (Figure 2A-B). Nevertheless, some fluorescence was still observed. Interestingly, with regards to pancreas tissue sec-

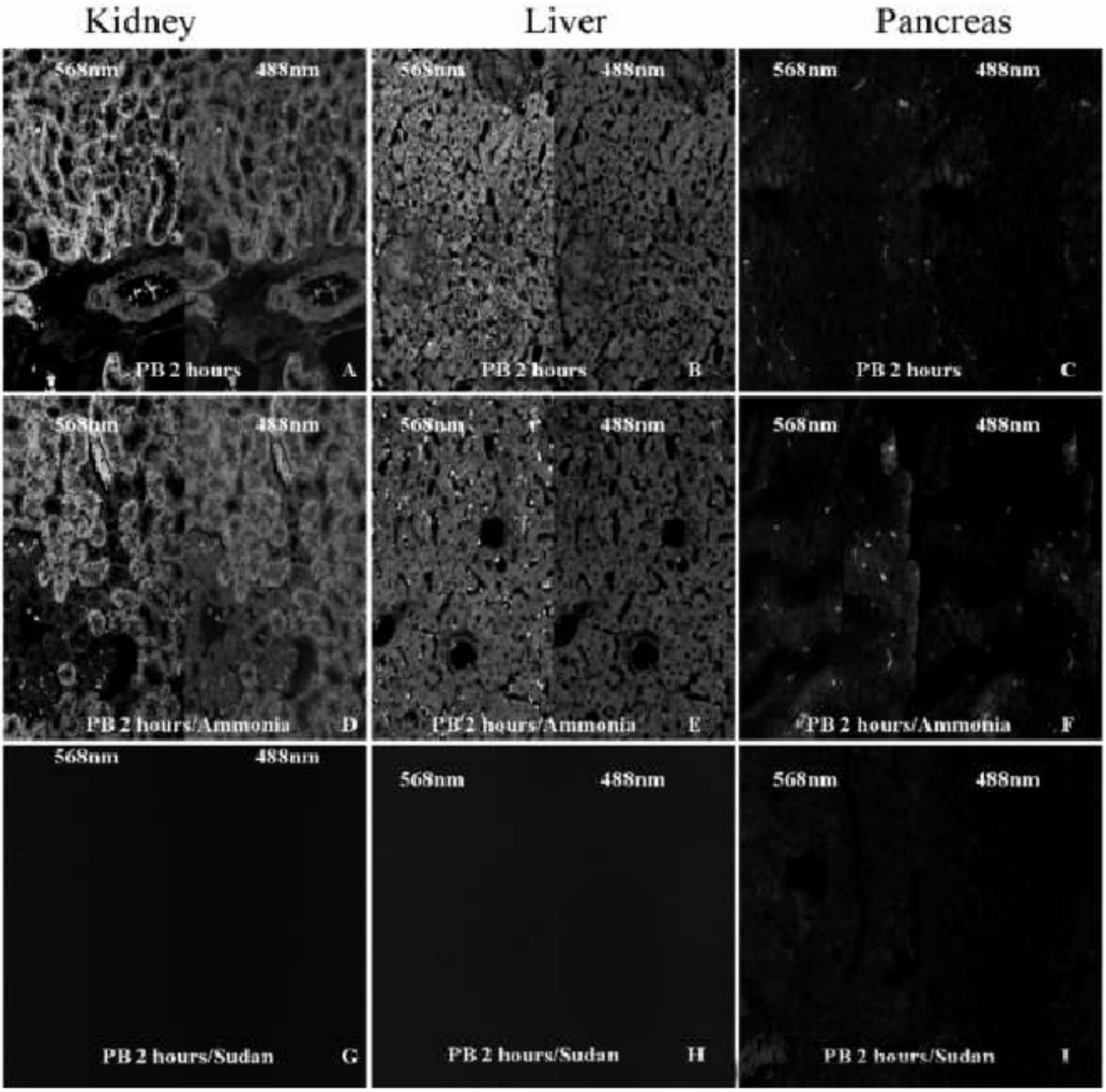


**Figure 2. Histochemical control of autofluorescence on formalin-fixed, paraffin-embedded kidney, liver and pancreas tissue sections. Reduction of fixative-induced autofluorescence in kidney, liver and pancreas tissue sections reached its maximum after 2 hours of high-intensity (30 W) photobleaching (A, B, C). Combined use of photobleaching and ammonia/ethanol was inefficient in all tissue sections under study (D, E, F). Photobleaching of kidney, liver and pancreas tissue sections and treatment with Sudan Black B quenched the autofluorescent background (G, H, I). (x200, CLSM).**

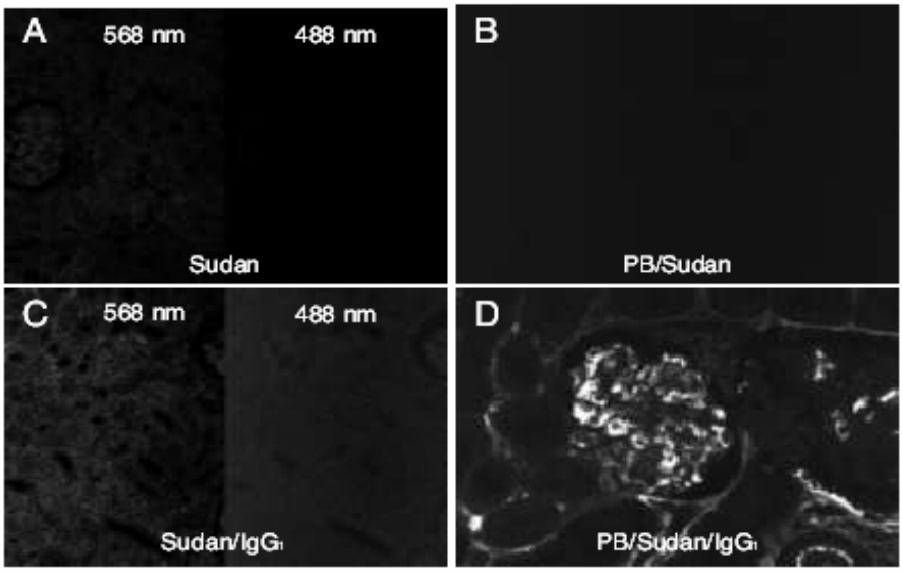


**Figure 3. Immunofluorescence labelling of formalin-fixed, paraffin-embedded kidneys of autoimmune glomerulonephritis-prone mice. Kidney tissue sections were pretreated with Sudan Black B either alone (A) or in combination with photobleaching (B). For immunolabelling, a FITC-labelled goat anti-mouse $IgG_1$ monoclonal antibody was used (C,D). Specific green fluorescence (488nm) indicates the presence of $IgG_1$ mediated immune complexes. Immune complex deposits could only be detected after treatment of tissue sections with a combination of photobleaching and Sudan Black B (D). (x200, CLSM).**

tions a near to total reduction of the fluorescent background was achieved after 2 hours of UV irradiation (Figure 2C). We have observed that tissue integrity was not affected by photobleaching (data not shown).

*Combined use of Photobleaching and Ammonia/Ethanol*

When photobleaching and ammonia/ethanol were applied to the tissue sections under study, we have found that they did not reduce the autofluorescent background regardless of the filter set used. In fact, in kidney, liver and pancreas tissue sections the autofluorescent background resembled that of the untreated controls (Figure 2D-F).

*Combined use of Photobleaching and Sudan Black B*

We have found that the combined use of irradiation with UV light (30 W) for 2 hours and incubation with Sudan Black B resulted in a total reduction of the autofluorescent background in kidney, liver and pancreas tissue sections, regardless of the excitation wavelength used (Figure 2G-I).

### *Immunofluorescence labelling*

To evaluate whether the pretreatments to which the tissue sections were subjected compromised direct immunofluorescence labelling, we have selected kidneys from autoimmune glomerulonephritis-prone mice and detected immune complexes on kidney tissue sections using FITC-labelled monoclonal antibodies. Treatment of kidney tissue sections with ammonia/ethanol, combined with either photobleaching or Sudan Black B, did not reduce the autofluorescent background to levels that would allow detection of immunofluorescence labelling (data not shown). Similar observations were made when kidney tissue sections were pre-treated with Sudan Black B alone (Figure 3A, 3C). In contrast, we have found that the combined use of photobleaching and Sudan Black B reduced the autofluorescent background to levels that allowed detection of specific fluorescence labelling signals (Figure 3B, 3D). In addition, we have found that the combined use of photobleaching and Sudan Black B did not compromise the immune staining as the staining pattern observed was similar to that widely illustrated in the literature.

## Discussion

Autofluorescence has long posed a problem when immunofluorescent labelling studies are required, especially in the case of direct staining. The fluorescent background can result from natural factors or be induced by fixation. A third source of background fluorescence can result from unspecific binding of antibodies to Fc receptors. As the latter is well documented and routinely dealt with in all immunofluorescence methodologies (Lu *et al.* 1998), it will not be discussed here. The majority of the immunofluorescence protocols described so far used indirect labelling of antigens, which allows amplification of the fluorescent signal and requires a lesser degree of reduction of autofluorescence. Furthermore, they are limited to tissue type and often require a combination of various procedures, as well as constant adaptations. Moreover, the reduction of the autoflu-

orescent background is not always satisfactory. Indeed, when applied to detection of antigens in tissue sections of murine kidney, liver and pancreas, by direct immunofluorescence, these methodologies (Baschong *et al.* 2001, Neumann *et al.* 2002) were far from being effective and autofluorescence still masked immunolabelling. As quenching of autofluorescence in kidney and liver can be very challenging due to their high metabolic rates, high content of flavins, lipofuscins, reticulin fibres and high vascularisation, we have decided to optimise a methodology that would allow detection of direct fluorescent labelling signals and through which the autofluorescent background would always be effectively reduced without the need for constant adjustments.

As mentioned before, tissue autofluorescence may be due to intrinsic factors. In kidney and liver, natural fluorescence is mainly due to NAD(P)H, flavins, lipofuscins and reticulin fibres. NAD(P)H autofluorescence results from hydrogen uptake, whereas that of lipofuscins is due to the conjugated Schiff bases formed between groups of amino acids and proteins (Baschong *et al.* 2001). These natural fluorophores are responsible for the emission of light ranging from 450 nm to 650 nm, wavelengths that overlap those of the fluorophores used in immunofluorescence studies (from 488 nm to 568 nm). One of the approaches used to quench intrinsic autofluorescence is the use of dyes whose absorbance spectrum overlaps the autofluorescence emission. A wide range of dyes can be used to quench background autofluorescence. The choice for the most appropriate dye should be done according to tissue characteristics and assays to be performed. For instance, trypan blue is mostly used to quench autofluorescence when performing flow cytometry assays (Mosiman VL *et al.*, 1997). Likewise, Pontamine Sky Blue is used when selective quenching of mesenteric vessels and carotid arteries autofluorescence is required (Cowen T *et al.*, 1985). Sudan Black B, among other Sudan dyes, is the most appropriate dye to quench autofluorescence of lipofuscins, fats, triglycerides and lipoproteins (Baschong *et al.* 2001, Schnell *et al.* 1999). A drastic reduction of the autofluorescent background was observed when Sudan Black B was applied to kidney, liver and pancreas tissue sections. However, this reduction was still not sufficient to allow detection of signals by direct immunofluorescence.

Another source of background fluorescence is the use of fixatives, namely neutral buffered formalin, which is the fixative routinely used in the clinical setting. Formaldehyde forms cross-linking methylene bridges and Schiff bases between basic amino acid and protein residues. Although this cross-linking is important for the *in situ* stabilization of proteins, when immunofluorescence studies are concerned, these bridges are a problem due to their fluorescence, which is difficult to eliminate. The emission of light of these fluorescent products occurs near the emission wavelength of FITC. Indeed, we have observed that the fluorescent background of the untreated sections was high. Treatment with ammonia/ethanol (Baschong *et al.* 2001) or photobleaching (Billinton *et al.* 2001) were considered good approaches to quenching fixative-induced fluorescence. Ammonia/ethanol react with free formaldehyde residues consequently reducing the autofluorescent background. Furthermore, these compounds appear to dissolve charged lipid derivatives, phenols or polyphenols, and to hydrolyse weak esters (Baschong *et al.* 2001). Nevertheless, and as shown by Baschong and co-workers (2001), ammonia/ethanol was only efficient in reducing the autofluorescent background in formaldehyde-fixed, decalcified bone marrow preparations but not in non-decalcified bone marrow slides. They propose ammonia/ethanol to be able to thoroughly dissolve deposits formed during the decalcification process by trichloroacetic acid. In addition, degradation of pH-sensitive fluorophores further augments ammonia/ethanol efficacy. So, it was not surprising that ammonia/ethanol was unable to reduce the autofluorescent background in kidney, liver and pancreas tissue sections. In fact, the autofluorescent background resembled that observed in untreated tissue sections. As these approaches did not reduce the autofluorescent background, we have decided to treat tissue sections with ammonia/ ethanol and Sudan Black B, a combination that was described to be effective in reducing autofluorescence in bone marrow tissue sections (Baschong *et al.*, 2001). Nonetheless, we have found that when these treatments were applied to the tissues under study, some autofluorescence was still observed. Although the achieved reduction of autofluorescence may be sufficient for indirect immunofluorescence studies, it was still not adequate for direct immunolabelling. Photobleaching reduces fixative-induced fluorescence through continuous and prolonged illumination of tissue sections. Newmann and co-workers (2002) described that quenching of fixative-induced

autofluorescence by UV irradiation was time dependent: longer UV irradiation resulted in greater quenching of autofluorescence. We have found that when kidney, liver and pancreas tissue sections were photobleached, maximal reduction of autofluorescence was achieved after 2 hours of UV irradiation. It should be noted that the intensity of UV irradiation used by Newmann and co-workers (2002) was lower than the one used in our work. This may justify the longer irradiation time used in their work. In addition, they did not directly irradiate tissue sections as we did. Nonetheless, remaining autofluorescence did not allow detection of direct immunofluorescence signals. As such, we have decided to treat tissue sections with a combination of photobleaching and Sudan Black B. Using this approach the autofluorescent background in kidney, liver and pancreas tissue sections was totally eliminated.

To evaluate the applicability of our method in direct immunofluorescence labelling, we have selected kidneys of glomerulonephritis-prone C57Bl. *6/pr/lpr* female mice. In these mice, glomerulonephritis is the consequence of the deposition of immune complexes in the kidney, which may be detected by immunofluorescence. Thus, kidney tissue sections were labelled with FITC-labelled monoclonal antibodies directed against the heavy chain of mouse $IgG_1$, $IgG_2$ and $IgG_3$. We have found that the reduction of the autofluorescent background, achieved by treating tissue sections with a combination of photobleaching and Sudan Black B, allowed the detection of specific immunofluorescent signals. The staining patterns obtained by these procedures were similar to those described in the literature (Vidal S *et al.* 1994).

In conclusion, we herein describe an improved methodology that besides eliminating autofluorescence in formalin-fixed paraffin-embedded tissue sections, also allows the detection of antigens by direct immunofluorescence using FITC-labelled monoclonal antibodies. Treatment of kidney, liver and pancreas tissue sections with short-duration (2 hours), high-intensity (30 W) photobleaching and Sudan Black B totally eliminated the autofluorescent background, regardless of the filter set used for excitation (488 nm or 568 nm). This quenching of autofluorescence enabled detection of direct fluorescence labelling signals, which are not amplified by the use of a secondary antibody, in contrast to indirect immunofluorescence studies. Another advantage of this methodology is the reduction of the cost of the experiments as it avoids the use of indirect labelling methods. Additionally, this improved methodology allows performance of direct immunefluorescence studies in formalin-fixed paraffin-embedded archival samples. Finally, we propose that this new methodology (combined use of high-intensity, short-duration photobleaching and Sudan Black B) can be used regardless of tissue type as similar results were obtained for highly vascularised, lipofuscin-rich tissues, such as kidney and liver, and for tissues with reduced vascularisation and low lipofuscin content, such as the pancreas. This approach is even more effective when poorly vascularised and low lipofuscin content tissues, such as the pancreas, are under study.

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3.2 **Viegas MS, Silva T, Monteiro MM, Carmo A, Martins TC** Knocking out of CD38 accelerates development of a lupus-like disease in *lpr* mice. (submitted)

***Corresponding author:**

Marta S. Viegas

Molecular Pathology Laboratory

Portuguese Institute for Oncology at Coimbra Francisco Gentil (IPOCFG), E.P.E.

Av. Bissaya Barreto, 98 - Apartado 2005

3000-075 Coimbra, Portugal

Phone: 351 239 400329

Fax: 351 239 484317

e-mail: marta.s.viegas@gmail.com

All authors concur with submission and none of the data presented have been previously reported or are under consideration for publication elsewhere.

Knocking out of CD38 accelerates development of a lupus-like disease in *lpr* mice.

Marta S. Viegas[1*], Teresa Silva[2], Maria M. Monteiro[3], Anália do Carmo[4], Teresa C. Martins[1,4]

[1] Molecular Pathology Laboratory, Portuguese Institute for Oncology at Coimbra Francisco Gentil, EPE, Portugal.

[2] Vasco da Gama University School, Quinta de S. Jorge, Estrada da Conraria, 3040-714 Castelo Viegas, Portugal.

[3] Histopathology Laboratory, Portuguese Institute for Oncology at Coimbra Francisco Gentil, EPE, Portugal.

[4] Centre for Neuroscience and Cell Biology (CNBC), University of Coimbra, Portugal.

**Condensed title:** Absence of CD38 accelerates lupus

***Corresponding author:**

Marta Viegas

Molecular Pathology Laboratory

Portuguese Institute for Oncology at Coimbra Francisco Gentil (IPOCFG), E.P.E.

Av. Bissaya Barreto, 98 - Apartado 2005

3000-075 Coimbra, Portugal

Phone: 351 239 400329

Fax: 351 239 484317

e-mail: marta.s.viegas@gmail.com

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**Abstract**

CD38 participates in lymphocyte ontogeny and function and may be involved in autoimmunity. Absence of CD38 accelerates development of NOD mice diabetes, anti-CD38 antibodies being good markers of human disease. Little is known regarding systemic autoimmunity. Active systemic lupus erythematosus patients have higher numbers of $CD38^+$ T and B cells. CD38 is a candidate gene for the murine *Lmb2* lupus *locus*. We have now investigated whether CD38 was involved in systemic autoimmunity. We developed CD38KO-*lpr/lpr* mice and monitored them for development of a lupus-like disease. We found that absence of CD38 accelerated disease development: CD38KO-*lpr/lpr* mice presented severe proteinuria and glomerulonephritis earlier in life than C57Bl.6-*lpr/lpr* mice. In contrast to C57Bl.6-*lpr/lpr*, CD38KO-*lpr/lpr* mice presented deposition of immune complexes in the renal medulla, a feature that suggested increased renal destruction. Similarly to that of C57Bl.6-*lpr/lpr*, disease of CD38KO-*lpr/lpr* mice was associated with low amounts of anti-dsDNA autoantibodies and RF, and production of ANA and ANCA. Absence of CD38 in *lpr* mice altered differentiation of T cells and DC. Although the definite role of CD38 in tolerance is still to be elucidated, we provide evidence that it does play an active role in the control of systemic autoimmunity.

## Introduction

CD38 belongs to a family of multifunctional ectoenzymes possessing signalling properties and extracellular and intracellular enzymatic activity (Lund *et al*., 1998). CD38 seems to play a role in lymphocyte ontogeny and function, as its expression is successively switched on and off throughout lymphocyte development and activation (Donís-Hernandez *et al*., 2001, Deterre *et al*., 2000). Lymphocyte activation via CD38 induces calcium mobilization, proliferation, activation of transcription factors, cytokine secretion, immunoglobulin (Ig) class switching and modulation of apoptosis (Lund *et al*., 1996, Morra *et al*., 1998, Deterre *et al*., 2000). CD38 may also behave as a selectin through interaction with CD31 (Deaglio *et al*., 2000). CD38 is involved in several distinct pathological situations, including autoimmune diseases, being used as a marker for some of these conditions (Bofill and Boethwick, 2000). With regards to organ-specific autoimmune disorders, it has been shown that absence of CD38 accelerated development of type I autoimmune diabetes in NOD mice (Chen *et al*., 2006). However, little is known about its role in systemic autoimmunity. In humans, patients with active systemic lupus erythematosus (SLE) have higher numbers of CD38-expressing $CD8^+$ T cells and of Ig-secreting B cells (Grammer *et al*., 2003). CD38 was proposed as a candidate gene for the *Lmb2* lupus predisposing *locus* of *lpr* mice (Vidal *et al*., 1998).

SLE is a systemic autoimmune disease of unknown ethiology that affects multiple organ systems. Many murine strains spontaneously develop lupus-like syndromes. These models share several clinical and immunopathological features, such as B-cell hyperactivity; production of autoantibodies (autoAbs), mainly anti-double stranded DNA (anti-dsDNA), rheumatoid factor (RF), anti-nuclear autoantibodies (ANA) and anti-neutrophil cytoplasmic autoantibodies (ANCA); high levels of circulating immune complexes (IC), Ig and complement; and IC-mediated glomerulonephritis (GN) (Andrews *et al*., 1978). Murine lupus models differ in the amount and specificity of the autoAbs produced, age of onset and kinetics of disease progression (Andrews *et al*., 1978; Theofilopoulos and Dixon, 1985). To investigate whether CD38 played a role in systemic autoimmunity, we have developed CD38KO-*lpr/lpr* mice. CD38KO-*lpr/lpr* and C57Bl.6-*lpr/lpr* mice, the autoimmunity-prone parental strain, were monitored for disease development. We have found that CD38KO-*lpr/lpr* mice developed a lupus-like disease earlier in life than C57Bl.6-*lpr/lpr* mice. Disease of CD38KO-*lpr/lpr* mice was characterized by a female bias, increased proteinuria, development of IC-mediated GN, high titres of circulating IgG, production of ANA and ANCA, and reduced amounts of anti-dsDNA autoAbs and RF. Although both female and male CD38KO-*lpr/lpr* mice presented signs of disease earlier in life, the process became under control in male mice, as it is generally

observed in autoimmunity-prone murine strains. Further characterization of CD38KO-*lpr/lpr* mice showed that absence of CD38 was associated with a decreased percentage of double positive (DP) and an increased frequency of $CD4^+CD25^+$ and $CD4^+$ single positive (SP) thymocytes in FKOMlpr mice. These findings suggest that, in an autoimmunity-prone genetic background, absence of CD38 may lead to altered thymic differentiation of T cells. In addition, absence of CD38 in a *lpr* background was associated with altered frequencies of dendritic cell (DC) subsets in secondary lymphoid organs, suggesting a role for CD38 in development, differentiation and/or trafficking of these cells. Although it is not yet clear how absence of CD38 contributes to acceleration or aggravation of murine lupus, our data evidence an active role for CD38 in the control of autoreactivity.

**Results**

To investigate whether CD38 played a role in systemic autoimmunity, we have developed CD38KO-*lpr/lpr* mice using either female or male CD38KO and C57Bl.6-*lpr/lpr* mice. Resulting CD38KO-*lpr/lpr* mice, FKOMlpr (female CD38KO x male C57Bl.6-*lpr/lpr* mice) and MKOFlpr (male CD38KO x female C57Bl.6-*lpr/lpr* mice), were monitored for development of a lupus-like disease.

*CD38KO-lpr/lpr mice present increased proteinuria and severe GN earlier in life.* Murine lupus is characterized by kidney damage, which leads to increased protein excretion in urine. Values of 100mg/dl of protein in urine are generally indicative of kidney damage (Mellors, 1965). In order to assess disease development, CD38KO-*lpr/lpr* and C57Bl.6-*lpr/lpr* mice (the autoimmunity-prone parental strain) were monitored for proteinuria, every two weeks, beginning at two months of age. We have found that, at two months of age, 10% of female MKOFlpr and 40% of female FKOMlpr mice already presented pathological levels of proteinuria (≥100mg/dl) whereas C57Bl.6-*lpr/lpr* mice remained within normal levels (Fig. 1). By six months of age, 100% of female MKOFlpr mice and 75% of FKOMlpr mice showed pathological levels of proteinuria (≥100mg/dl) (Fig. 1A). Of these, 14.3% of MKOFlpr and 20% of FKOMlpr female mice had reached 500mg/dl of protein in urine (data not shown). Age-matched C57Bl.6-*lpr/lpr* mice still presented normal levels of proteinuria (≤30mg/dl) (Fig. 1A). As for male mice, we have found that, by six months of age, all CD38KO-*lpr/lpr* mice presented 100mg/dl of proteinuria while only 20% of age-matched C57Bl.6-*lpr/lpr* mice reached this value (Fig. 1B). As increased protein levels in urine are indicative of renal damage, we have performed histological analysis of the kidneys. We have found that, similarly to female C57Bl.6-*lpr/lpr* mice, six-month old female CD38KO-*lpr/lpr* mice developed membranoproliferative GN (Fig. 2). However, female CD38KO-*lpr/lpr* mice that reached the highest levels of proteinuria (≥500mg/dl) characteristically presented glomerulosclerosis (GS) (Fig 2C, D, arrows), a feature that was not seen in the parental strain. As for male mice, we have found that six-month old CD38KO-*lpr/lpr* mice, similarly to C57Bl.6-*lpr/lpr* mice, had developed GN (Fig. 2B, E, F) with crescentic formations (Fig. 2B, E, F, arrows). As lupus-associated GN may be a consequence of IC deposition in the kidneys, we have analysed kidney sections for the presence of IC. We have found that six-month old female and male CD38KO-*lpr/lpr* mice had $IgG_1$ and $IgG_3$ deposits, of variable intensity, in the renal medulla (Fig. 3C-J), whereas age-matched female C57Bl.6-*lpr/lpr* mice presented diffuse granular deposits of $IgG_1$ and $IgG_3$ restricted to the glomeruli (Fig. 3A, B). No IC deposits were detected in male

C57Bl.6-*lpr/lpr* mice (data not shown). Female and male CD38KO-*lpr/lpr* mice also presented diffuse C3 deposits in the Bowman's space (Fig. 3L-O), while in age-matched C57Bl.6-*lpr/lpr* mice C3 deposits were restricted to the glomeruli (Fig. 3K).

*CD38KO-lpr/lpr and C57Bl.6-lpr/lpr mice produce similar levels of circulating IgG.* SLE, as well as murine lupus-like syndromes, is characterized by production of increased amounts of circulating IgG. We have quantified serum IgM, $IgG_1$, $IgG_{2a}$ and $IgG_3$ levels by ELISA. We have found that all female and male *lpr* and CD38KO mice produced similar amounts of IgM (data not shown). Regarding $IgG_1$ production, we have found that all *lpr* female mice had comparable levels of $IgG_1$ (Fig. 4A-a). Concerning IgG2a (Fig. 4B-a) and IgG3 (4C-a) production, we have found that female FKOMlpr had decreased levels of these Ig with regards to C57Bl.6-*lpr/lpr* mice. No differences in the amounts of these Ig were found between female MKOFlpr and the autoimmunity-prone parental strain. Regarding male mice, we have found that FKOMlpr had significantly lower levels of $IgG_1$ than MKOFlpr mice, which were similar to those observed for C57Bl.6-*lpr/lpr* and CD38KO mice (Fig. 4A-b). Male FKOMlpr mice presented lower levels of $IgG_{2a}$ than male MKOFlpr and C57Bl.6-*lpr/lpr*, which were closer to those observed for CD38KO mice (Fig. 4B-b). We have found that male CD38KO-*lpr/lpr* mice and both parental strains had comparable levels of $IgG_3$ (Fig. 4C-b).

*CD38KO-lpr/lpr and C57Bl.6-lpr/lpr mice produced comparable amounts of anti-dsDNA autoAbs and RF.* Also characteristic of disease of *lpr* mice are the increased levels of circulating autoAbs. As such, we have quantified serum anti-dsDNA autoAbs and RF by ELISA. We have found that, by six months of age, both female and male CD38KO-*lpr/lpr* mice, similarly to C57Bl.6-*lpr/lpr* mice, had low amounts of anti-dsDNA autoAbs and RF (Fig 5). In female CD38KO-*lpr/lpr* mice, high amounts of protein in urine (≥500mg/dl) correlated with significantly increased production of these autoAbs (Fig. 5B). Production of ANA and ANCA is also a hallmark of SLE, being used as diagnostic criteria. We have readily detected ANA and ANCA in CD38KO-*lpr/lpr* mice as well as in C57Bl.6-*lpr/lpr* mice (Fig. 6). The staining pattern was similar in all murine strains with clear speckled, nuclear membrane and centromere patterns (Fig. 6).

*Immunophenotype of two-month old CD38KO-lpr/lpr mice.* To analyse the effects of the elimination of CD38 on the development of the immune system of *lpr* mice, we have

quantified the main immune cell subsets present in the thymus, spleen and lymph nodes (LN) of the three *lpr* strains at two months of age.

*Thymus.* We have found that female FKOMlpr had an increased percentage of $CD4^+$ SP (Fig. 7A) and $CD4^+CD25^+$ (Fig. 7E) and a decreased percentage of DP (Fig. 7C) T cells with regards to the other strains. We have observed that female CD38KO-*lpr/lpr* and C57Bl.6-*lpr/lpr* mice had comparable percentages of DN T cells, which were higher than those observed for CD38KO mice (Fig. 7D). No differences were observed between the four strains with regards to $CD8^+$ SP T cells (Fig. 7B). Regarding male mice, we have found that CD38KO-*lpr/lpr* and CD38KO mice had comparable frequencies of $CD4^+$ SP T cells (Fig. 7G). Although no differences were observed when male FKOMlpr and C57Bl.6-*lpr/lpr* mice were compared, we have found that MKOFlpr had significantly reduced percentages of $CD4^+$ SP T cells with regards to C57Bl.6-*lpr/lpr* mice (Fig. 7G). No differences were found between male CD38KO-*lpr/lpr* mice and the parental strains with regards to $CD8^+$ SP (Fig. 7H), DP (Fig. 7I), DN (Fig. 7J) and $CD4^+CD25^+$ (Fig. 7K) T cells.

*Spleen.* We have found that female CD38KO-*lpr/lpr* and C57Bl.6-*lpr/lpr* mice had similar percentages of $CD4^+$ T cells, which were lower than those observed for CD38KO mice (Fig. 8A). CD38KO-*lpr/lpr* mice and the parental strains had comparable frequencies of $CD8^+$ T (Fig. 8B) and B cells (Fig. 8C). Regarding DC, we have found that female FKOMlpr and C57Bl.6-*lpr/lpr* mice had comparable percentages of $CD11c^+$ DC, which were higher than those observed for female MKOFlpr and CD38KO mice (Fig. 8D). Regarding $CD8^-$ DC, we have found that female MKOFlpr had an increased percentage, whereas female FKOMlpr had a decreased frequency of these cells with regards to both parental strains (Fig. 8F). As for $CD8^+$ DC, no differences were found between the four strains (Fig. 8E). With regards to male mice, we have found that CD38KO-*lpr/lpr* and C57Bl.6-*lpr/lpr* mice had comparable frequencies of $CD4^+$ (Fig. 8G) and $CD8^+$ (Fig. 8H) T cells, which were lower than those observed for CD38KO mice. Regarding DC, male MKOFlpr had a lower percentage of $CD8^+$ DC than male FKOMlpr and both parental strains (Fig. 8J), no differences being found between the four strains with relation to $CD11c^+$ (Fig. 8I) and $CD8^-$ DC (Fig. 8K).

*LN.* We have found that female CD38KO-*lpr/lpr* and C57Bl.6-*lpr/lpr* mice had comparable frequencies of $CD4^+$, which were lower than those observed for CD38KO mice (Fig. 9A) As for $CD8^+$ T cells, we have observed that female FKOMlpr had a lower percentage of these cells than both parental strains (Fig. 9B). Female CD38KO-*lpr/lpr* and the parental strains presented similar frequencies of B cells (Fig. 9C). As for DC, we have found that female CD38KO-*lpr/lpr* mice had increased percentages of $CD11c^+$ DC with regards to the

parental strains (Fig. 9D), but comparable frequencies of $CD8^+$ and $CD8^-$ DC (Fig. 9E, F). Regarding male mice, we have observed that CD38KO-*lpr/lpr* mice and the parental strains presented comparable frequencies of $CD4^+$ T cells (Fig. 9G). CD38KO-*lpr/lpr* mice had decreased percentages of $CD8^+$ T cells with regards to C57Bl.6-*lpr/lpr,* but not CD38KO mice (Fig. 9H). No differences were seen with respect to DC (Fig. 9I, J, K).

*Immunophenotype of six-month old CD38KO-lpr/lpr mice.* To investigate how absence of CD38 could contribute to development of a lupus-like disease in *lpr* mice, we have analysed changes in the main immune cell subsets of the thymus, spleen and LN that occurred during development until disease onset in the three *lpr* strains, and that could be associated with disease pathogenesis.

*Thymus.* We have observed that female MKOFlpr had a lower frequency of DN T cells than C57Bl.6-*lpr/lpr* mice, no differences being seen between female FKOMlpr and the parental strains. No differences were observed between the four strains regarding $CD4^+$ SP, $CD8^+$ SP, DP and $CD4^+CD25^+$ T cells (data not shown). Regarding male mice, we have observed that FKOMlpr had a higher percentage of $CD8^+$ SP T cells than C57Bl.6-*lpr/lpr* mice, whereas comparable frequencies of these cells were observed in male MKOFlpr and the parental strains. We have found that all strains had similar frequencies of $CD4^+$ SP, DP, DN and $CD4^+CD25^+$ T cells (data not shown).

*Spleen.* No differences were observed between the strains with regards to $CD4^+$ T cells and DC. As for $CD8^+$ T and B cells, we have found that female MKOFlpr presented a higher percentage of these cells than C57Bl.6-*lpr/lpr* mice, no differences being seen with regards to the other strains (data not shown). Characteristic of the lupus-like disease of *lpr* mice is the accumulation, in secondary lymphoid organs, of an inert subpopulation of T cells, characterized by down-modulation of CD4 and CD8 and acquired expression of B220 (DN $B220^+$ T cells) (Mellors, 1965). We have found that female CD38KO-*lpr/lpr* and C57Bl.6-*lpr/lpr* mice had similar percentages of these cells (data not shown). Regarding males, we have found that CD38KO-*lpr/lpr* mice and the autoimmunity-prone parental strain, C57Bl.6-*lpr/lpr* mice, had similar percentages of $CD4^+$ and $CD8^+$ T cells and B cells (data not shown). Regarding DN $B220^+$ T cells, we have found that male MKOFlpr had an increased percentage of these cells with regards to C57Bl.6-*lpr/lpr* mice (data not shown). Male MKOFlpr had a higher percentage of $CD11c^+$ DC than CD38KO mice, no differences being seen between the other strains, or with relation to other DC subsets (data not shown).

*LN.* We have found that all female mice had comparable frequencies of $CD8^+$ T cells, B cells and DC. We have found that female FKOMlpr had a lower percentage of $CD4^+$ T cells than MKOFlpr, no differences being seen with regards to the parental strain (data not shown). With regards to DN $B220^+$ T cells, we have found that female MKOFlpr had a higher percentage of these cells than C57Bl.6-*lpr/lpr* mice, no differences being seen between the other strains (data not shown). No differences were observed with regards to male mice (data not shown).

## Discussion

CD38 is a type II glycoprotein, which belongs to a family of multifunctional ectoenzymes (Lund *et al*., 1998). As an enzyme, it is able to convert $NAD^+$ to cADPR and the latter to ADPR (Deaglio *et al*., 2001). CD38 also displays adhesion properties through interaction with CD31 (Deaglio *et al*., 2000). Expression of CD38 is switched on and off during lymphocyte ontogeny (Donis-Hernandez *et al*., 2001). Lymphocyte activation via CD38 triggers signalling cascades, including that of NF-κB, and mobilization of intracellular calcium. CD38 is involved in the regulation of apoptosis and proliferation, and in the production of cytokines. In B cells, it also participates in Ig class switching (Deterre *et al*., 2000). CD38 appears to play a role on distinct pathological situations, including autoimmune diseases (Bofill and Borthwick, 2000). It was shown that absence of CD38 accelerated development of autoimmune diabetes of NOD mice (Chen *et al*., 2006). However, little is known about its role in systemic autoimmunity. SLE is a heterogeneous systemic autoimmune disease that affects multiple organs. It is characterized by B cell hyperactivity, which results in the production of high amounts of autoAbs of multiple specificities. These autoAbs can lead to cell and tissue damage by either Fc receptor-mediated cytotoxicity or by direct activation of the complement cascade. In the kidneys, extracellular matrix or cell surface components may serve as potential targets for autoAbs. Direct binding of autoAbs to kidney intrinsic autoantigens (autoAgs) results in *in situ* IC formation. GN may also result from deposition of preformed IC, as a result of binding of autoAbs to circulating autoAgs (Andrews *et al*., 1978; Theofilopoulos and Dixon, 1985). There is some indirect evidence that CD38 may be involved in SLE and murine counterparts. In humans, active SLE patients have a higher number of CD38 expressing $CD8^+$ T cells and of Ig-secreting B cells (Grammer *et al*., 2003). In addition, CD38 was proposed as a candidate gene for the *Lmb2* lupus predisposing *locus* of *lpr* mice (Vidal *et al*., 1998).

In this work, we have investigated whether CD38 played a role in development of a murine lupus-like disease. For that, we have developed CD38KO-*lpr/lpr* mice. CD38KO mice were crossed with C57Bl.6-*lpr/lpr* mice, a well characterized and widely accepted murine model for SLE. The resulting CD38KO-*lpr/lpr* mice, FKOMlpr (female CD38KO x male C57Bl.6-*lpr/lpr* mice) and MKOFlpr (male CD38KO x C57Bl.6-*lpr/lpr* mice), were monitored for disease development through measurement of protein excretion in urine. We have found that, at two months of age, 10% of female MKOFlpr and 40% of female FKOMlpr mice already presented pathological levels of proteinuria (≥100mg/dl), whereas C57Bl.6-*lpr/lpr* mice still had no signs of disease. The percentage of diseased animals increased with age. At six months of age, 100% of female MKOFlpr and 75% of female

FKOMlpr reached 100mg/dl of proteinuria. Of these, 14% of female MKOFlpr and 20% of female FKOMlpr reached 500mg/dl of proteinuria. No signs of disease were detected in C57Bl.6-*lpr/lpr* mice. As for male mice, we have found that, at six months of age, 100% of CD38KO-*lpr/lpr* mice presented increased proteinuria (≥100mg/dl), while only 30% of C57Bl.6-*lpr/lpr* mice reached that value. Taken together, our data suggest that absence of CD38 in *lpr* mice leads to kidney damage earlier in life. This was confirmed by histological analysis of the kidneys. Although, at six months of age, all female *lpr* mice presented membranoproliferative GN, GS was only seen in female CD38KO-*lpr/lpr* mice that reached 500mg/dl of proteinuria. Our results are in agreement with data in the literature that correlated the highest values of proteinuria with increased renal damage (Waters *et al*., 2001). Interestingly, FKOMlpr presented a higher number of sclerotic glomeruli than female MKOFlpr, which suggests increased renal damage in the former. Male CD38KO-*lpr/lpr* mice, similarly to C57Bl.6-*lpr/lpr* mice, presented membranoproliferative GN with crescentic formations. Lupus GN results from deposition of IC in the kidneys. We have found that GN was associated with $IgG_1$ and $IgG_3$-IC deposition, which is suggestive of an ongoing Th2 immune response. Interestingly, while in C57Bl.6-*lpr/lpr* mice, IC deposition was observed in the glomeruli, as usually described, in CD38KO-*lpr/lpr* mice, IC deposits were found in the renal medulla. These findings are compatible with and may result from the increased renal damage observed, for instance, in female CD38KO-*lpr/lpr* mice with advanced disease (500mg/dl proteinuria). GS may abolish the filtrating function of the glomeruli, allowing IC to reach the renal medulla, namely the collecting ducts, where they may be retained. Our data suggest that absence of CD38 accelerates development of a lupus-like disease, female FKOMlpr and MKOFlpr being differently affected: female FKOMlpr developed a more aggravated form of the disease than female MKOFlpr. We have also found that male CD38KO-*lpr/lpr* mice were able to control disease, as described for other murine models. This could be due, as generally proposed, to hormonal factors (Hughes and Clark, 2007).

Having established that CD38KO-*lpr/lpr* mice were more susceptible to development of a lupus-like disease than C57Bl.6-*lpr/lpr* mice, we have then characterized disease in this new murine model of lupus. Murine lupus, similarly to human SLE, is characterized by spontaneous production of autoAbs, mainly against nuclear antigens, and increased levels of circulating Ig and complement, all reflecting B cell hyperactivity (Andrews *et al*., 1978). We have found that female and male CD38KO-*lpr/lpr* mice share many of the serologic characteristics of murine models of lupus and human SLE, namely high titres of circulating IgG and production of ANCA and ANA. Similarly to other murine models (Waters *et al*., 2004, Jacob *et al*., 2003), including C57Bl.6-*lpr/lpr*

mice, we have observed that, in CD38KO-*lpr/lp*r mice, damage was already present even in the absence of significant serum amounts of anti-dsDNA autoAbs and RF. Of note, female CD38KO-*lpr/lpr* that reached 500mg/dl of proteinuria produced significantly increased amounts of these autoAbs, suggesting that these antibodies (Abs) are not required for disease initiation but may play a role at later stages of disease. Our results further support the emerging idea that anti-dsDNA autoAbs and RF may not be the main actors in the pathogenesis of lupus; as such, attention should be driven towards other antigen-antibody systems. Indeed, different autoAbs have been shown to be potentially pathogenic in different murine lupus strains, such as anti-snRNP, -Ro/La, -histone, -laminin, -fibronecin, and –actinin, as well as polyreactive glomerulotrophic autoAbs (Mannic *et al*., 2003, Lefkowith and Gilkeson, 1996).

CD38KO mice, developed in a C57Bl.6 genetic background, have a normal distribution and ratio of T and B lymphocytes and the numbers of granulocytes, macrophages and neutrophils in the periphery are within normal ranges (Cockayne *et al*., 1998). The effect of CD38 deletion in an *lpr* genetic background was unknown. As changes in development of the immune system may affect later development of autoimmune diseases, we have immunophenotyped two-month old CD38KO-*lpr/lpr* mice. The main immune cell subsets present in the thymus were quantified. Interestingly, we have observed that female FKOMlpr, in contrast to female MKOFlpr, presented altered T-cell frequencies in the thymus, namely with regards to DP, $CD4^+CD25^+$ and $CD4^+$ SP T cells. The decreased percentage of DP T cells observed in female FKOMlpr may result from the arrest of thymocyte development before reaching the DP stage, or increased death of DP cells.

The thymus is the central organ in which T cells develop. T cell ontogeny can be broadly divided into three major stages based on CD4 and CD8 expression: DN, DP and SP T cells. At early stages of differentiation, pre-T cells are characterized by the absence of CD4, CD8 and TCR expression. These DN T cells can be further divided into four subpopulations (DN1, DN2, DN3 and DN4) based on the differential expression of CD4 and CD25 (Ceredig and Rolink, 2002). It is during the DN2 stage that cells begin to rearrange their TCR genes and this process continues in the DN3 stage, during which the $\beta$, $\gamma$ and $\delta$ *loci* are rearranged. Expression of CD38 is tightly controlled in the thymus, suggesting a role for this molecule in thymic selection (Bean *et al*., 1995). CD38 expression is low at DN1 stage and upregulated during DN2 and DN3 stages, suggesting that CD38 may be involved in TCR $\beta$ rearrangements. Cells that successfully rearrange the TCR $\beta$ undergo a series of events known as $\beta$-selection, during which TCR$\beta$, TCR$\gamma$

and TCRδ rearrangements are arrested, CD4 and CD8 upregulated (DP T cells) and TCRα rearrangements begin. If TCRα rearrangements are productive, DP T cells will receive survival signals and will undergo positive and negative selection processes. In contrast, if TCRα rearrangements are not productive, DP T cells do not receive survival signals and die. CD38 expression is still upregulated during this stage, suggesting that CD38 may also be an active player in this process (Bean *et al*., 1995). The signaling cascade that provides survival signals and determines β-selection is similar to that observed after activation of mature T cells through the TCR (Hengartner *et al*., 2004). On mature T cells, CD38 was shown to be constitutively associated with lipid rafts (Zubiaur *et al*., 2002) and to be able to modulate TCR signaling through its ability to interact with Lck and CD3ζ/ZAP-70 and activate the Raf1/MAPK signaling cascade. It may also facilitate interactions with other lipid rafts, such as those containing LAT (Munoz *et al*., 2003). Thus, CD38, through its functions as a receptor, may also be involved in thymic differentiation of T cells, namely at the level of TCR rearragements or by mediating survival signals necessary for the selection of lymphocytes with productive TCR. CD38 also acts as an adhesion molecule (Deaglio *et al*., 2000). Adhesion molecules play an important role in keeping two interacting cells bound long enough to allow signal transduction. If cell adhesion is not sustained, or weak signals are delivered to the cells, cell responses may not be triggered. Under these circumstances, DP T cells would not receive survival signals and could die by neglect. CD38, through its role as an adhesion molecule, may contribute to firm adhesion of DP T cells to thymic epithelial cells, assuring the delivery of sufficient survival signals to these cells. In light of this, we propose that absence of CD38 may contribute to increased thymocyte death prior to and/or during the DP stage, due to its proposed role in the assembly of productive TCRs and/or delivery of survival signals when DP thymocytes are tested for the rearranged β and α chains of the TCR.

We have also observed that female FKOMlpr mice had an increased percentage of $CD4^+$ SP T cells with regards to the autoimmunity-prone parental strain, suggesting a bias towards selection of these cells. Before release into the periphery, DP T cells must undergo positive and negative selection, two processes that are also TCR-dependent. The quantitative-avidity model proposes that the interactions between thymocytes and thymic APC dictate which cells complete these processes and are released to the periphery (Klein and Kyewski, 2000). Cells firstly undergo positive selection, which occurs in the cortex and assures that the newly assembled αβTCRs are able to interact with self-MHC molecules. Positively selected cells then undergo a series of changes that will determine whether the thymocyte will become a $CD4^+$ or a $CD8^+$ SP T cell, depending on TCR/co-receptor interactions with self-MHC molecules (class II for $CD4^+$ SP T cells, and

class I for $CD8^+$ SP T cells). The signaling cascade elicited by TCR/CD4-MHC and TCR/CD8-MHC interactions is similar, involving Lck phosphorylation and protein kinase C activation. However, Lck is differentially recruited in the two situations, with TCR/CD4-MHC recruiting more Lck to the vicinity of the immunological synapse than TCR/CD8-MHC interactions. This will determine whether a thymocyte will become a $CD4^+$ or a $CD8^+$ SP T cell. The kinetics of the interaction also influence the process: thymocytes become a $CD4^+$ SP T cell, if the signal is sustained; and a $CD8^+$ SP T cell, if the signal is transient. As stated earlier, CD38 modulates activation of T cells via the TCR through recruitment of adaptor molecules and downstream effectors. CD38 may amplify the signal elicited by TCR ligation by augmenting recruitment of Lck and downstream effectors to the lipid raft. Strong TCR-mediated signals more rapidly elicit negative feedback mechanisms, which lead to termination of TCR signaling; this could favor differentiation towards the CD8 lineage. In contrast, in the absence of CD38, the initiation of these negative feedback mechanisms would be delayed, allowing continuous engagement of the TCR/CD4 and promoting differentiation of CD4 SP T cells. Alternatively, CD38 may be able to "highjack" Lck molecules, competing with the TCR. As the amount of Lck available for TCR signaling is critical and limited, highjacking of Lck by CD38 may disrupt TCR signalling and bias differentiation towards CD8 SP T cells. In this scenario, absence of CD38 would contribute to an increased availability of Lck, allowing for sustained TCR signalling and thus favoring development of the CD4 lineage. In conclusion, CD38, by influencing the time-course of the TCR response or through the recruitment of downstream effectors, may indirectly influence the CD4/CD8 lineage commitment.

In a similar way, CD38 may also affect the peripheral T-cell repertoire by modulating death/survival decisions during the process of negative selection, which takes place in the thymic medulla. During this stage, there is also selection of SP T cells that bear highly autoreactive TCRs and will exert a regulatory action in the periphery. These regulatory T (Treg) cells are characterized by the expression of CD4, CD25 and Foxp3 and are dependent on IL-2 (Lio and Hsieh, 2008, Bassiri and Carding, 2001). Interestingly, we have observed that female FKOMlpr had a higher percentage of $CD4^+CD25^+$ T cells in the thymus than the autoimmunity-prone parental strain. Directing SP T cells to the Treg pathway or to negative selection seems to require additional yet unidentified signals. We propose that CD38 may have such a role. Indeed, it was suggested that events that dictate whether cells will become a Treg take place during β-selection, as alterations in this process lead to altered frequencies of $CD4^+CD25^+$ T cells (Bosco *et al*., 2006; Pennington *et al*., 2006). As already mentioned, CD38 is expressed at high levels during this stage of thymic differention of T cells and was suggested to be involved in TCR

rearrangements. We now propose that CD38 may direct SP thymocytes to the process of negative selection through amplification of TCR-mediated signals (indicative of strong, undesired autoreactivity). In the absence of such signals, differentiation of $CD4^+CD25^+$ Treg cells would be favored.

We have also quantified the main cell subsets present in the spleen and LN of two-month old mice. We have found that female CD38KO-*lpr/lpr* mice presented altered frequencies of different DC subsets. These data suggest a role for CD38 in development and/or differentiation of these cells. A growing body of evidence has supported a role for DC in lupus (Ueno *et al*., 2007). Increased numbers of $CD11c^+$ DC have been detected in murine models of lupus and these cells were shown to have abnormal phenotype and function, such as altered costimulatory patterns, increased production of chemokines and cytokines, and selective downregulation of CD83, a feature that was associated with altered cytokine secretion, such as increased production of IL-4 and IL-10 by $CD4^+$ T cells (Prechtel and Steinkasserer, 2007, Colona *et al*., 2006, Ishikawa *et al*., 2002). Similarly to these models, we have found that female FKOMlpr had increased frequencies of $CD11c^+$ DC with regards to the other strains. $CD8^+$ DC and $CD8^-$ DC are two well established murine DC subsets (Pulendran *et al*., 1999; Soares *et al*., 2007; Maldonado-López *et al*., 1999). $CD8^-$ DC skew T cells to produce high amounts of IL-4 and IL-10 but few IFNγ whereas $CD8^+$ DC preferentially induce IFNγ production by activated T cells as a consequence of increased secretion of IL-12. However, $CD8^+$ DC that do not secrete IL-12 also promote Th2 differentiation. We have observed that CD38KO-*lpr/lpr* had altered frequencies of $CD8^-$ DC: female FKOMlpr presented a decreased percentage whereas female MKOFlpr had an increased frequency of these cells with regards to C57Bl.6-*lpr/lpr* mice. As CD38 is required for IL-12 production and CD83 expression (Fedele *et al*., 2004), in its absence DC, including $CD8^+$ cells, may skew helper T cells into the Th2 phenotype. Supporting this idea is the observation that CD38KO-*lpr/lpr* mice presented $IgG_1$- and $IgG_3$-mediated GN, two IgG subclasses associated with Th2 responses. In addition, we have found that CD38KO mice produced higher amounts of IL-4 than C57Bl.6 mice in response to antigenic stimuli (Viegas *et al*., 2007). These data are compatible with the classically proposed model of lupus, which involves spontaneous hyperactivation of T cells and increased production of Th2 cytokines, such as IL-4. Excessive T cell help will then contribute to the observed B-cell hyperactivity and the production of increased amounts of IgG. Interestingly, we have found that female MKOFlpr had a $CD8^-$:$CD8^+$ DC ratio of 3 and female FKOMlpr of 1, whereas both parental strains had a ratio of 2. It has been proposed that alterations of DC ratios may potentate

the development of undesired autoimmune responses, as DC are at the interface of innate and acquired immune response, (Belz *et al*., 2002, den Haan *et al*., 2000; Naik, 2008).

We have also observed that six-month old female MKOFlpr had an increased percentage of $CD8^+$ T cells with regards to C57Bl.6-*lpr/lpr* mice, which suggests that these cells may be accumulating in secondary lymphoid organs as a consequence of their resistance to apoptosis associated with the *lpr* mutation. Prolonged accumulation of $CD8^+$ T cells may lead to downregulation of CD8 and generation of DN $B220^+$ T cells. In fact, we have observed an increased frequency of DN $B220^+$ T cells in six-month old female MKOFlpr with regards to C57Bl.6-*lpr/lpr* mice.

Accelerated development of lupus-like disease may result from increased effector T cell activation or from decreased Treg function. In CD38KO NOD mice, accelerated development of diabetes was associated with a Teffector: Treg cells imbalance in the periphery, due to NAD-induced selective death of Treg cells (Chen *et al*., 2006). It is possible that in CD38KO-*lpr/lpr* mice a similar imbalance occurs.

Finally, we have observed that female FKOMlpr and MKOFlpr are differently affected by the absence of CD38. This may be associated with a maternal parent-of-origin effect that proposes that gene regulation may be controlled by the expression of a single allele in a parent-of-origin-dependent manner (Zeft *et al*., 2008, Ideraabdullah *et al*., 2008).

In conclusion, we herein show that absence of CD38 accelerates development of an autoimmune disease with increased renal damage that shares several characteristics with murine models of lupus and human SLE, namely a female bias, increased proteinuria, development of IC-mediated GN, and high titres of circulating IgG, production of ANA and ANCA. Disease of CD38KO-*lpr/lpr* mice, although more severe, resembles that of C57Bl.6-*lpr/lpr* mice. CD38KO-*lpr/lpr* mice thus constitute a good model to study the role of CD38 in SLE. The protective role of CD38 may be exerted at the level of thymic selection and activation of T cells and differentiation of DC subsets. Activation of CD38KO-*lpr/lpr* T cells by DC may lead to their differentiation into Th2 cells with concomitant production of increased amounts of Th2 cytokines, such as IL-4. Hyperactivated CD38KO-*lpr/lpr* Th2 cells then provide excessive help to autoreactive CD38KO-*lpr/lpr* B cells leading to augmented production of autoantibodies, namely of $IgG_1$ and $IgG_3$ subclasses, and increased formation of IC. IC-mediated disruption of kidney function leads to development of glomerulonephritis, a characteristic feature of lupus.

## Material and Methods

*Mice*. Lupus-prone C57Bl.6-*lpr/lpr* mice, purchased from Harlan Iberica, and B6.129P2-*Cd38*$^{tm1Lnd}$ (CD38KO) mice from the Jackson Laboratories, share the same genetic background. CD38KO-*lpr/lpr* mice were generated by crossing: female CD38KO x male C57Bl.6-*lpr/lpr* (FKOMlpr) and male CD38KO x female C57Bl.6-*lpr/lpr* (MKOFlpr). Heterozygous $F_1$ mice were crossed to yield CD38KO-*lpr/lpr* homozygous animals, which were used in the following crosses. Mice were bred and kept in SPF animal facilities of the Institute for Molecular and Cell Biology (Porto, Portugal). This work was approved by the Animal Welfare Division of the Portuguese Veterinary Council.

*Selection of CD38KO-lpr/lpr Mice.* $F_2$ mice were bled and CD38 expression analysed by flow cytometry using PE-labelled anti-CD38 (BD Pharmingen). CD38KO mice were typed for the *lpr* mutation (insertion of an ETn element on intron 2 of *Fas*). Tail tips were digested in 200μl of 10mM TrisHCl, pH=8.0; 0.1M EDTA, pH=8.0; 0.5% SDS, 0.5μg/μl Proteinase K, at 56ºC, and DNA extracted and precipitated using phenol/chloroform and ethanol. Three primers were used: P1 forward (5' cctaaggacgatgcttcgtgtt 3'), specific for the 3' end sequence of the insertion; P2 reverse (5' agaacgaatgagcaggaagctcc 3') for the 5' end sequence of the insertion; and P3 reverse (5' ctcagtgcagcaaatgtacaggg 3') for the internal sequence of the insertion. P1+P2 PCR reaction amplified the WT allele, whereas P1+P3 PCR reaction amplified the *lpr* allele. PCR reactions (50μl final volume) included 50mM KCl, 10mM Tris-HCL pH=9.0, 1.5mM MgCl, 0.2mM dNTPs, 50pmol of each primer, 2.5U Taq Polimerase (Fermentas) and 100ng of target DNA. The PCR program included an initial denaturation step at 94ºC for 5'; 45 cycles of: denaturation at 95ºC for 30'', annealing at 55ºC for 30'' and extension at 72ºC for 45''; and a final extension at 72ºC for 10'. Fragment analysis was performed in a 3% agarose gel.

*Clinical and Histological Assessment.* CD38KO- and C57Bl.6-*lpr/lpr* mice were screened for proteinuria, every two weeks, using colorimetric strips (Combur test, Roche), beginning at two months of age. Kidneys from six-month old CD38KO-*lpr/lpr*, C57Bl.6-*lpr/lpr* and CD38KO mice were fixed in 10% buffered formaldehyde and embedded in paraffin. Tissue sections were stained with haematoxilin-eosin (H-E). The slides were blind analysed. An Axiophot microscope (Zeiss) with a digital still camera (DSC-S85, Sony) interfaced to a PC was used for image acquisition.

*Immune Complex Depostion.* Formalin-fixed, paraffin-embedded kidneys from six-month old CD38KO- and C57Bl.6-*lpr/lpr* mice were used for detection of immune complexes by direct immunofluorescence (IF), as described (Viegas *et al*., 2007): tissue sections were UV irradiated, deparaffined, treated with 0.1% Sudan Black B in 70% ethanol, and incubated with FITC-labelled anti-$IgG_1$, anti-$IgG_2$, anti-$IgG_3$ (Southern Biotechnology), or anti-C3 (ICN Pharmaceuticals), overnight, at 4ºC. Slides were blind analysed. For image acquisition, a confocal laser scanning microscope (Bio-Rad, MRC60C) was used. The settings for contrast, brightness, pinhole, acquisition mode and scanning time were maintained throughout the work. The filter sets used included an excitation filter of 488nm and a barrier filter at 520nm. Fluorescence images were collected, digitalized and analysed using Bio-Rad software. For C3 detection, a fluorescence microscope (Leica, DMI 6000B) with a still camera (Leica, DFC350FX) was used.

*Serum Ig and Autoantibody Quantification.* Six-month old mice were terminally bled and sera recovered. MRL-*lpr/lpr* serum was used as a positive control. For quantification of circulating IgM and IgG isotypes, 96-well plates (Maxisorp, Nunc) were coated with purified IgM, $IgG_1$, $IgG_2$, $IgG_3$ (BD Pharmingen) overnight, at 4 ºC. For detection of anti-dsDNA autoAbs, 96-well plates (Nunc, Maxisorp) were pre-treated with poly-L-lysine and coated with calf thymus DNA (Sigma) for 1 hour at 37 ºC. For detection of RF, 96-well plates (Nunc, Maxisorp) were coated with rabbit anti-mouse IgG (BD Pharmingen), overnight, at 4 ºC. Sera (1:100) were added and incubated for 2 hours. Anti-Ig-HRP (GE Healthcare) was added and incubated for 1 hour. O-phenylenediamine (Sigma) was used for colour development. The plate was read in an ELISA plate reader at 450 nm. ANA and ANCA were detected by direct IF. Human peripheral blood leukocytes were spun down onto slides and incubated with sera (1:100), overnight, at 4ºC. After washing in 0.02% Tween 20 in PBS (PBST), slides were incubated with FITC-labelled anti-mouse IgG (BD Pharmingen) for 2 hours, and then washed in PBST and mounted with VectaShield (Vector Laboratories). For image acquisition, a fluorescence microscope (Leica, DMI 6000B) with a digital still camera (Leica, DFC350FX) was used. Unless otherwise specified, all incubations were performed in a wet chamber at room temperature.

*Flow cytometry analysis of cell surface markers.* The Abs used were as follows: i) PE-labelled anti-mouse CD4, CD8, CD11c, B220; ii) FITC-labelled anti-mouse CD8, CD4, CD11b, CD25 and CD3; and iii) CyChrome$^{TM}$-labelled anti-CD4 and CD8 (all from BD Pharmingen). $10^6$ cells of thymus, spleen or lymph nodes were incubated with 50μl of

antibody suspensions, at 4ºC, in the dark, for 15 minutes. The staining pattern was analysed using a Becton Dickinson FACSort flow cytometer interfaced to Macintosh computer. Dead cells and erythrocytes were excluded from the analysis using a combination of the forward light scatter and propidium iodide gating (Bandeira *et al*., 1990).

*Statistical analysis.* Statistical analysis was performed using a One-way ANOVA followed by Tukey HSD *a posteriori* multiple comparison tests with a 95% confidence interval. Numerical populations were considered to be statistically different when $p < 0.05$.

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**Figure legends**

**Figure 1.** CD38KO-*lpr/lpr* mice developed severe proteinuria earlier in life. Starting at two months of age, female (A) and male (B) CD38KO-*lpr/lpr* and C57Bl.6-*lpr/lpr* were monitored for proteinuria (≥ 100mg/dl), every two weeks, using colorimeric strips. Female and male FKOMlpr, n= 15 and 11, respectively; female and male MKOFlpr, n=8 and 4, respectively; female and male C57Bl.6-*lpr/lpr* n=6 and 16, respectively.

**Figure 2.** CD38KO-*lpr/lpr* mice developed membranoproliferative GN. Evidence of GS was only observed in female CD38KO-*lpr/lpr* that reached 500mg/dl of proteinuria (C, E arrows). In male mice, crescent formations were observed (B, D, F arrows). Kidneys from six-month old mice were recovered, formalin-fixed and paraffin-embedded. Representative histological slides are shown (H-E, x400). Representative of two independent experiments.

**Figure 3.** CD38KO-*lpr/lpr* mice presented $IgG_1$- and $IgG_3$-IC deposits in the renal medulla and C3-deposits in the Bowman's space. In contrast, C57Bl.6-*lpr/lpr* presented $IgG_1$- and $IgG_3$-IC and C3-deposits the glomeruli. No IC deposition was detected in male C57Bl.6-*lpr/lpr* mice. Kidneys from six-month old mice were recovered, formalin-fixed and paraffin-embedded. Kidney sections were stained with FITC-labelled $IgG_1$ (A, C, E, G, I), $IgG_3$ (B, D, F, H, J) and C3 (K-O). Representative slides are shown ($IgG_1$ and $IgG_3$, x400; C3, x630). Representative of two independent experiments.

**Figure 4.** CD38KO-*lpr/lpr* and C57Bl.6-*lpr/lpr* mice produced similar levels of circulating $IgG_1$ (A), $IgG_{2a}$ (B) and $IgG_3$ (C). Female CD38KO-*lpr/lpr* mice that reached 500mg/dl of proteinuria had significantly increased amounts of circulating IgG. A comparative ELISA was performed using sera from terminally bled six-month old mice. Female and male FKOMlpr, n= 16; female and male MKOFlpr, n=11 and 8, respectively; female and male C57Bl.6-*lpr/lpr* n=8; and female and male CD38KO, n= 11 and 2, respectively. Each dot represents one animal; the mean value is presented (-). Data for CD38KO mice is shown as a reference for the non-autoimmunity-prone parental strain.

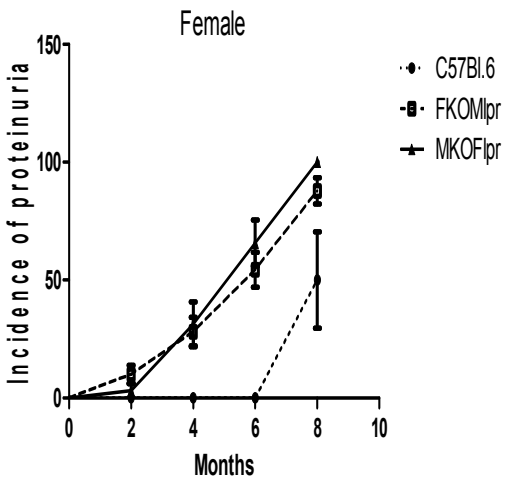
**Figure 5.** CD38KO-*lpr/lpr* and C57Bl.6-*lpr/lpr* mice produced similar amounts of anti-dsDNA autoAbs (A) and RF (B). Female CD38KO-*lpr/lpr* mice that reached 500mg/dl of proteinuria had significantly increased amounts of anti-dsDNA autoAbs and RF. A comparative ELISA was performed using sera from terminally bled six-month old mice. Female and male FKOMlpr, n= 16; female and male MKOFlpr, n=11 and 8, respectively; female and male C57Bl.6-*lpr/lpr* n=8 and female and male CD38KO, n= 11 and 2, respectively. Each dot represents one animal; the mean value is also presented (-). Data for CD38KO mice is shown as a reference for the non-autoimmunity-prone parental strain.

**Figure 6.** CD38KO-*lpr/lpr* mice produced ANA and ANCA similarly to C57Bl.6-*lpr/lpr* mice. Different reactivity patterns were observed: homogeneous, speckeled, centromere and perinuclear. Nuclei were incubated with sera from terminally bled six-month old mice followed by FITC-labelled anti-IgG (x1000). Representative of two independent experiments.

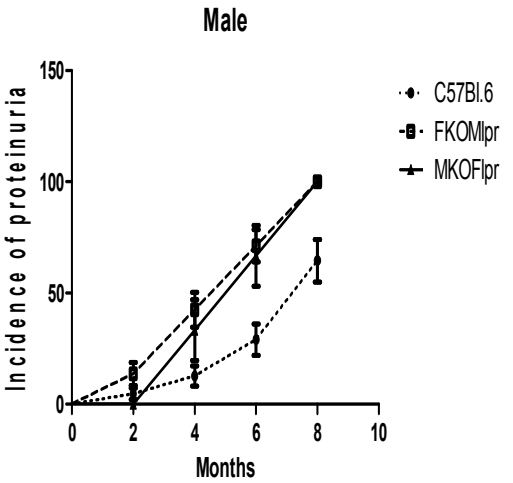
**Figure 7.** Absence of CD38 was associated with decreased percentages of DN and increased percentages of $CD4^+$ SP and $CD4^+CD25^+$ T cells in female FKOMlpr. Two-month old female and male mice were sacrificed and thymuses recovered and stained for flow cytometry. Female and male FKOMlpr, n= 8 and 6; female and male MKOFlpr, n=9 and 7, respectively; female and male C57Bl.6-*lpr/lpr* n=6 and female and male CD38KO, n= 4, respectively. Standard deviation of the mean is also presented. Data for CD38KO mice is shown as a reference for the non-autoimmunity-prone parental strain.

**Figure 8.** Absence of CD38 altered DC differentiation in female and male *lpr* mice. Two-month old female and male mice were sacrificed and spleen recovered and stained for flow cytometry. The identification of the DC subsets was based on three distinct cell surface markers: CD8, CD11b and CD11c. $CD11c^+$ DC were gated and DC subsets $CD8^+CD11b^-$ ($CD8^+$ DC) and $CD8^-CD11b^+$ ($CD8^-$ DC) analyzed. Female and male FKOMlpr, n= 8 and 6; female and male MKOFlpr, n=9 and 7, respectively; female and male C57Bl.6-*lpr/lpr* n=6 and female and male CD38KO, n= 4, respectively. Standard deviation of the mean is also presented. Data for CD38KO mice is shown as a reference for the non-autoimmunity-prone parental strain.

**Figure 9.** Absence of CD38 is associated with increased percentages of $CD11c^+$ DC in female CD38KO-*lpr/lpr* mice. Two-month old female and male mice were sacrificed and LN recovered and stained for flow cytometry. The identification of the DC subsets was based on three distinct cell surface markers: CD8, CD11b and CD11c. $CD11c^+$ DC were gated and DC subsets $CD8^+CD11b^-$ ($CD8^+$ DC) and $CD8^-CD11b^+$ ($CD8^-$ DC) analyzed. Female and male FKOMlpr, n= 8 and 6; female and male MKOFlpr, n=9 and 7, respectively; female and male C57Bl.6-*lpr/lpr* n=6 and female and male CD38KO, n= 4, respectively. Standard deviation of the mean is also presented. Data for CD38KO mice is shown as a reference for the non-autoimmunity-prone parental strain.


Female
Incidence of proteinuria
150
100
50
0
0
2
4
6
8
10
Months
C57Bl.6
FKOMIpr
MKOFIpr

A


Male
Incidence of proteinuria
150
100
50
0
0
2
4
6
8
10
Months
C57Bl.6
FKOMIpr
MKOFIpr

B

**Figure 1**

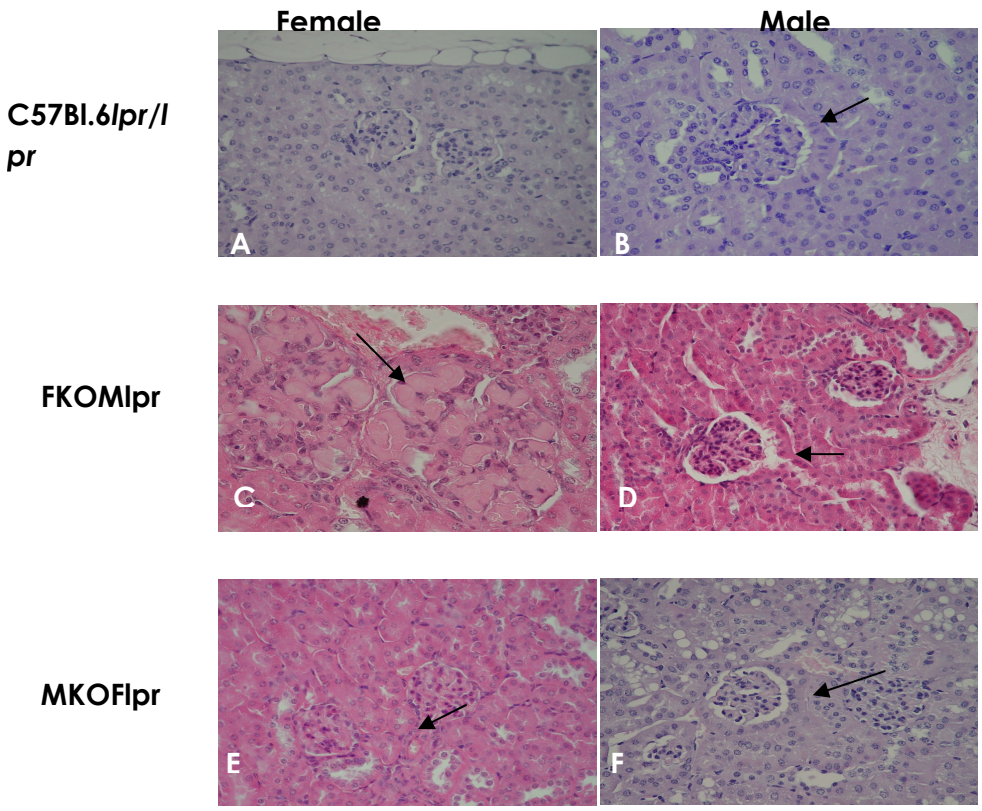



**Figure 2**

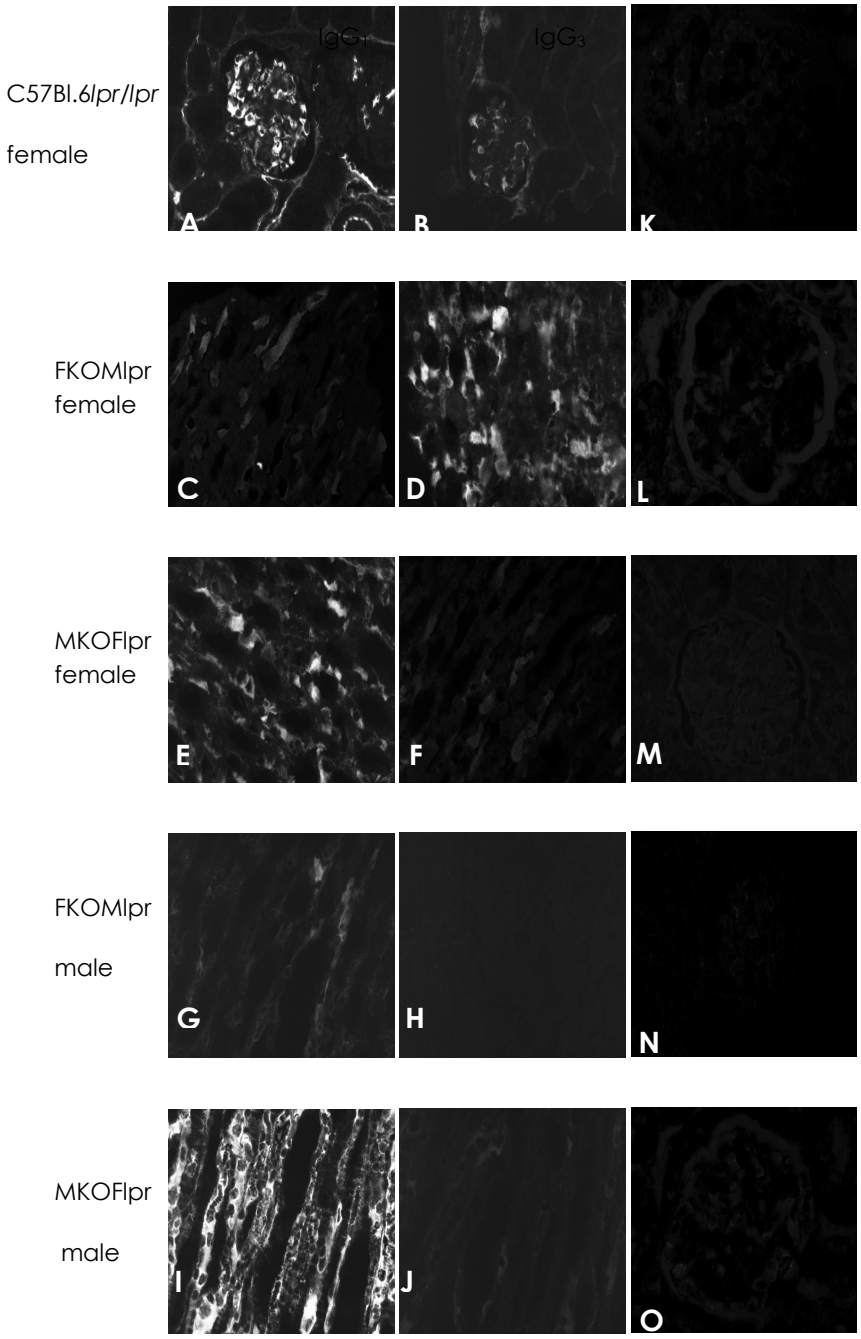

C57Bl.6lpr/lpr
female
IgG1
IgG3
A
B
K
FKOMlpr
female
C
D
L
MKOFlpr
female
E
F
M
FKOMlpr
male
G
H
N
MKOFlpr
male
I
J
O

**Figure 3**

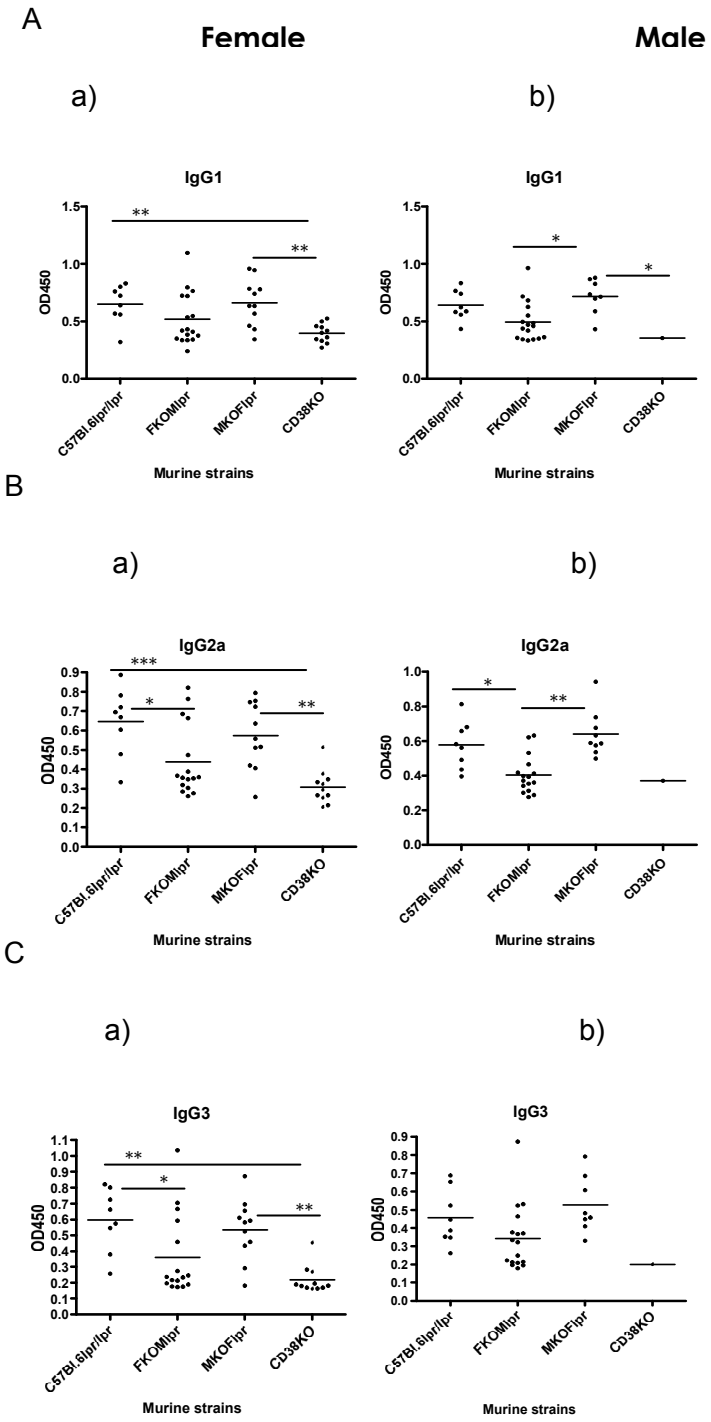

A
Female
Male
a)
b)
IgG1
OD450
Murine strains
C57Bl.6lpr/lpr
FKOMIpr
MKOFIpr
CD38KO
B
IgG2a
C
IgG3

**Figure 4**

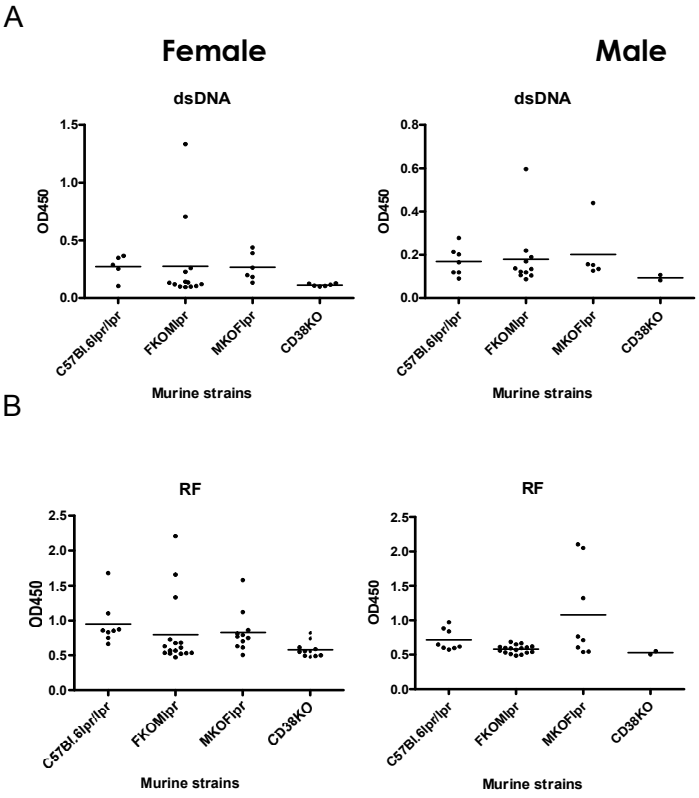

A
Female
Male
dsDNA
dsDNA
OD450
1.5
1.0
0.5
0.0
0.8
0.6
0.4
0.2
0.0
C57Bl.6lpr/lpr
FKOMlpr
MKOFlpr
CD38KO
Murine strains
B
RF
RF
OD450
2.5
2.0
1.5
1.0
0.5
0.0
Murine strains

**Figure 5**

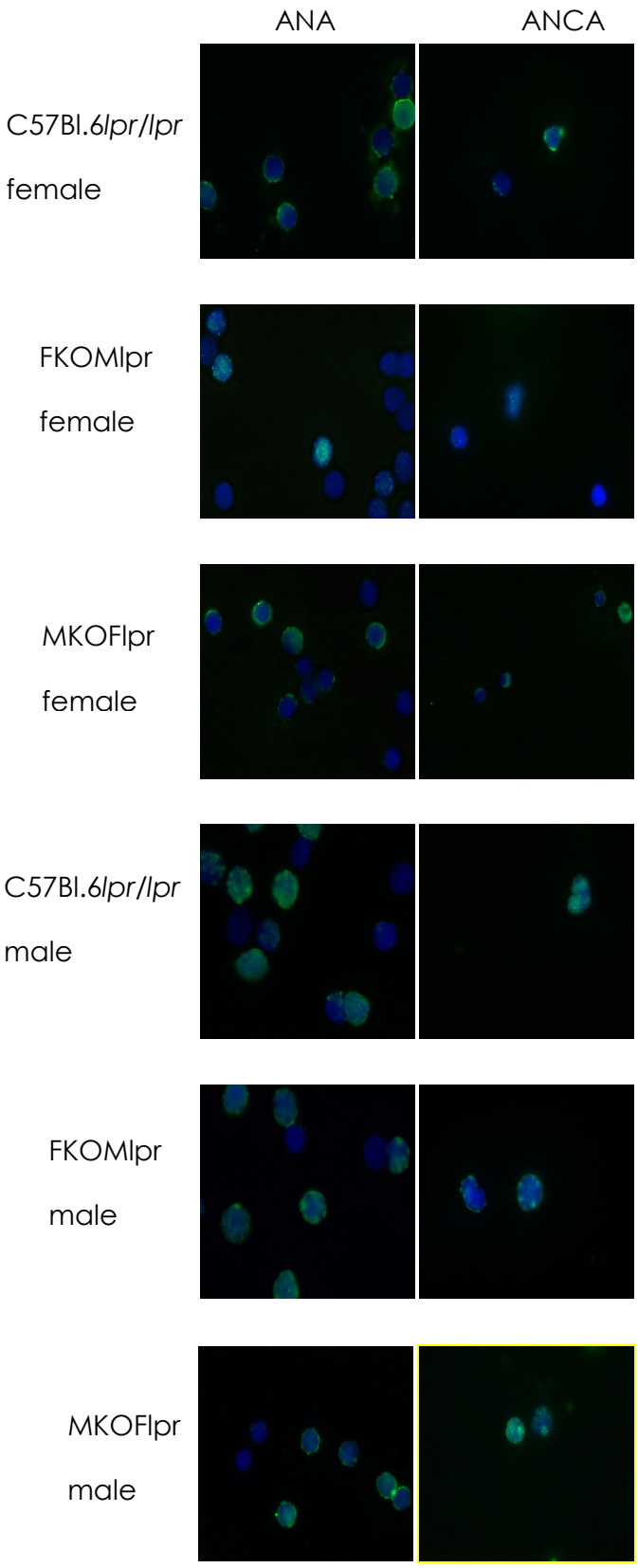
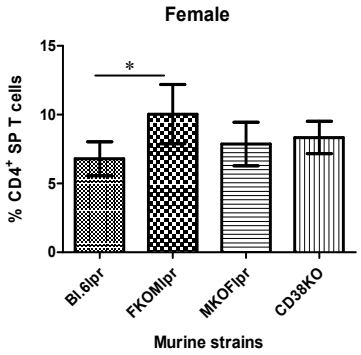
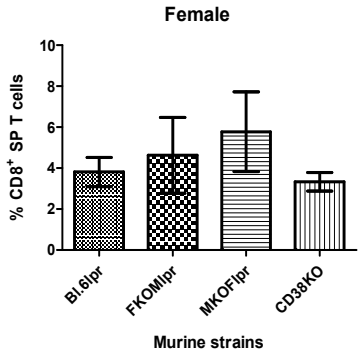

ANA
ANCA
C57Bl.6lpr/lpr
female
FKOMlpr
female
MKOFlpr
female
C57Bl.6lpr/lpr
male
FKOMlpr
male
MKOFlpr
male

**Figure 6**

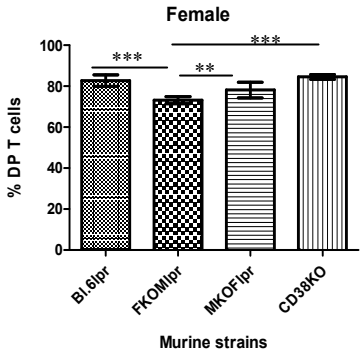
a)


Female
% CD4+ SP T cells
15
10
5
0
*
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO
Murine strains

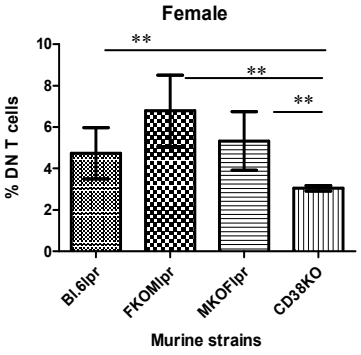
b)


Female
% CD8+ SP T cells
10
8
6
4
2
0
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO
Murine strains

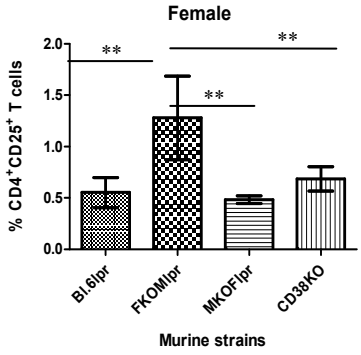
c)


Female
% DP T cells
100
80
60
40
20
0
***
**
***
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO
Murine strains

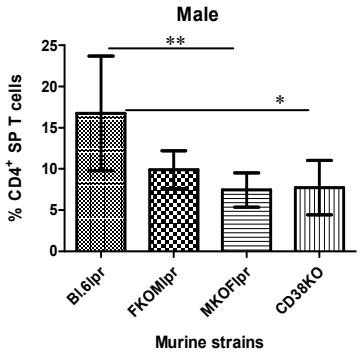
d)


Female
% DN T cells
10
8
6
4
2
0
**
**
**
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO
Murine strains

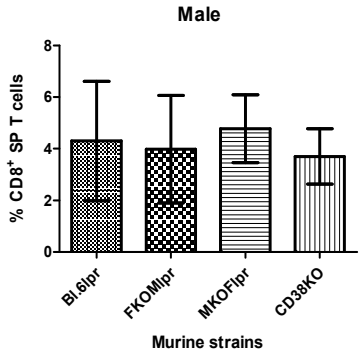
e)


Female
% CD4+CD25+ T cells
2.0
1.5
1.0
0.5
0.0
**
**
**
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO
Murine strains

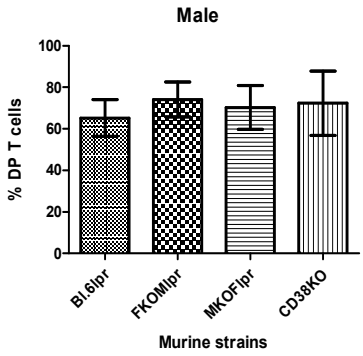
g)


Male
% CD4+ SP T cells
25
20
15
10
5
0
**
*
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO
Murine strains

h)


Male
% CD8+ SP T cells
8
6
4
2
0
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO
Murine strains

i)


Male
% DP T cells
100
80
60
40
20
0
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO
Murine strains

j) k)

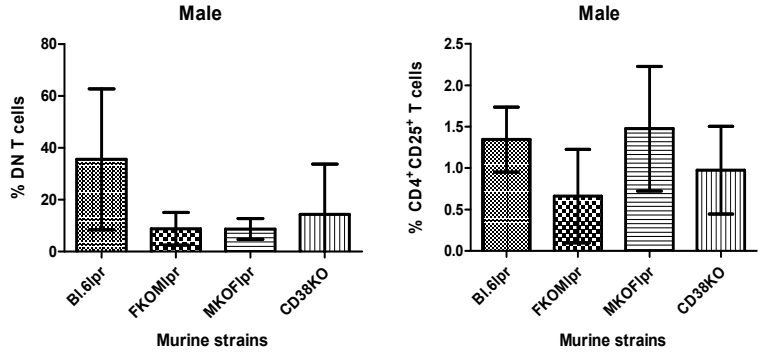
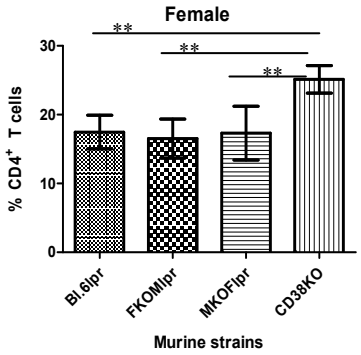
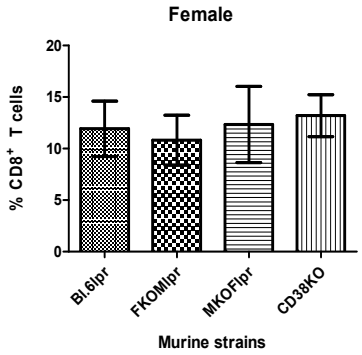

Male
% DN T cells
80
60
40
20
0
Bl.6lpr
FKOMIpr
MKOFIpr
CD38KO
Murine strains
Male
% CD4+CD25+ T cells
2.5
2.0
1.5
1.0
0.5
0.0
Bl.6lpr
FKOMIpr
MKOFIpr
CD38KO
Murine strains

**Figure 7**

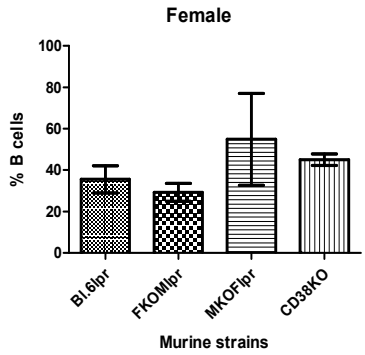
A)


Female
% CD4+ T cells
Murine strains
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO

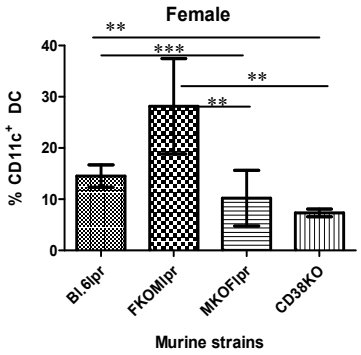
B)


Female
% CD8+ T cells
Murine strains
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO

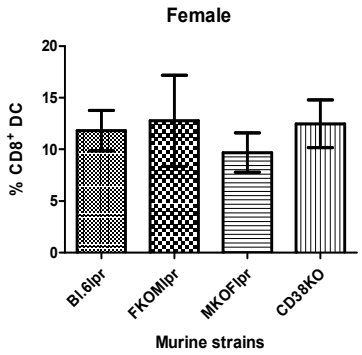
C)


Female
% B cells
Murine strains
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO

D)


Female
% CD11c+ DC
Murine strains
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO

E)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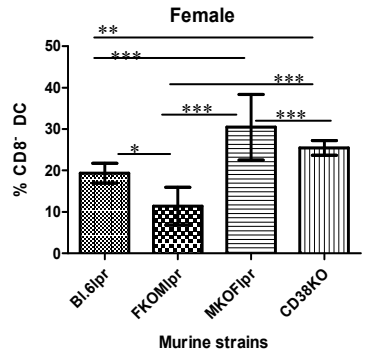
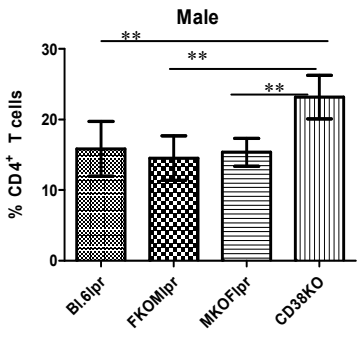
% CD8+ DC
Murine strains
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO

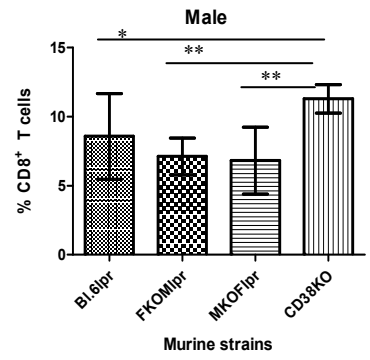
F)


Female
% CD8- DC
Murine strains
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO

G)


Male
% CD4+ T cells
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO

H)


Male
% CD8+ T cells
Murine strains
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO

I) J) K)

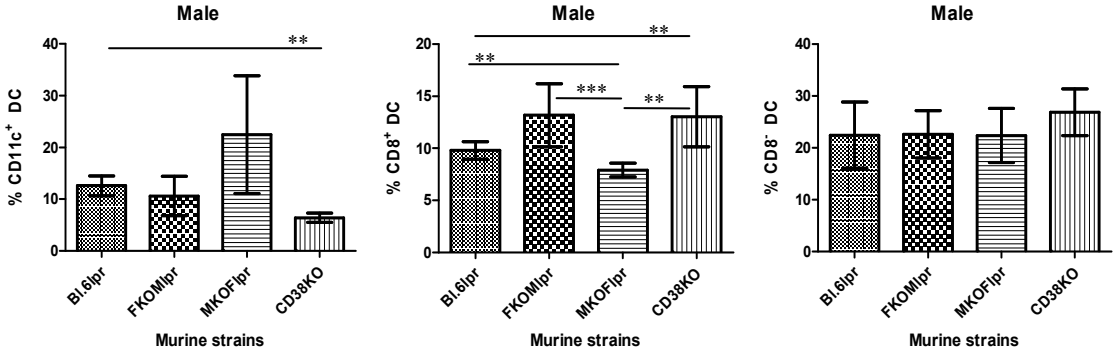
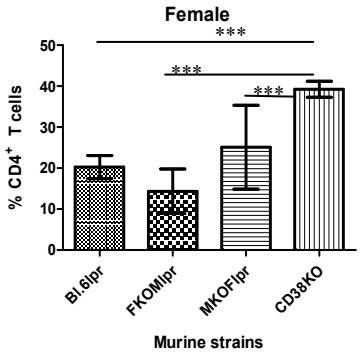
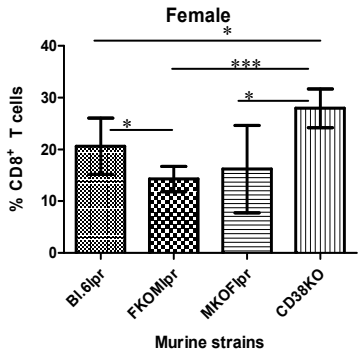

Male
% CD11c+ DC
40
30
20
10
0
**
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO
Murine strains
Male
% CD8+ DC
20
15
10
5
0
**
***
**
**
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO
Murine strains
Male
% CD8- DC
40
30
20
10
0
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO
Murine strains

**Figure 8**

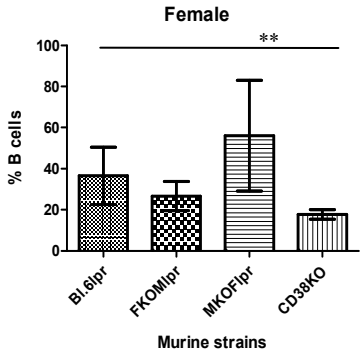
A)


Female
% CD4+ T cells
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO
Murine strains

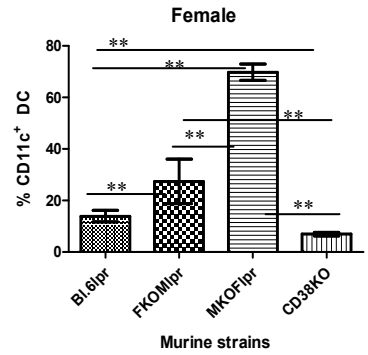
B)


Female
% CD8+ T cells
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO
Murine strains

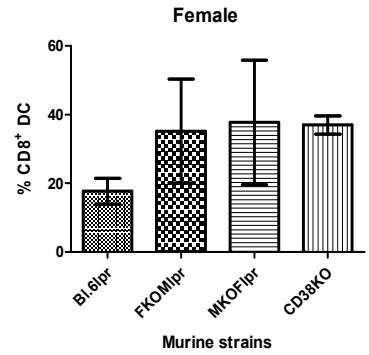
C)


Female
% B cells
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO
Murine strains

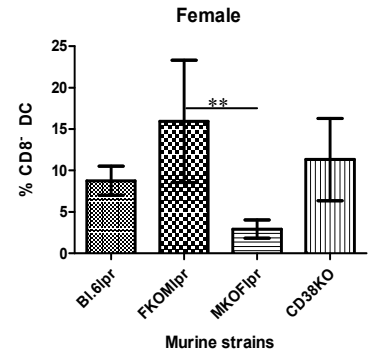
D)


Female
% CD11c+ DC
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO
Murine strains

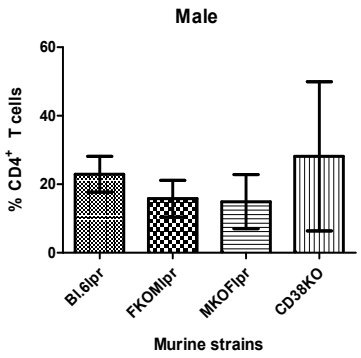
E)


Female
% CD8+ DC
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO
Murine strains

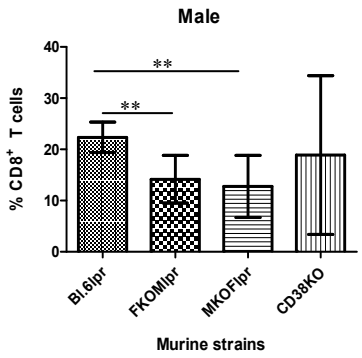
F)


Female
% CD8- DC
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO
Murine strains

G)


Male
% CD4+ T cells
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO
Murine strains

H)


Male
% CD8+ T cells
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO
Murine strains

I) J) K)

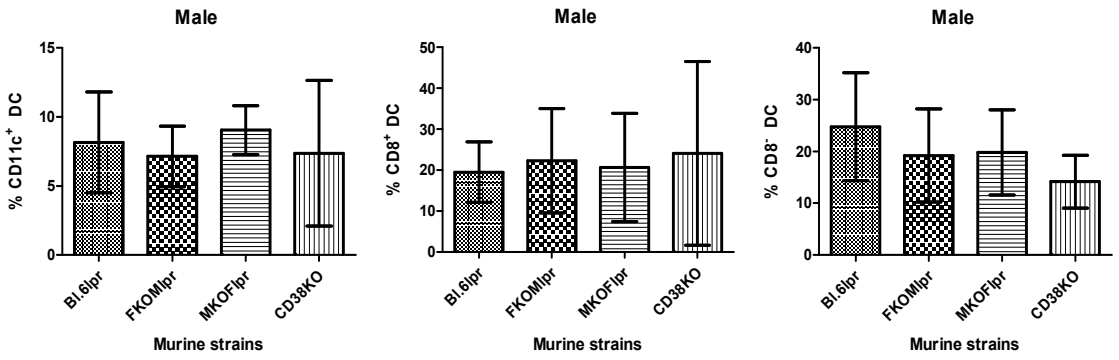

Male
% CD11c+ DC
15
10
5
0
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO
Murine strains
Male
% CD8+ DC
50
40
30
20
10
0
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO
Murine strains
Male
% CD8- DC
40
30
20
10
0
Bl.6lpr
FKOMlpr
MKOFlpr
CD38KO
Murine strains

**Figure 9**

# Chapter Four

# 4. Discussion

CD38 belongs to a family of multifunctional ectoenzymes, the ADP-ribosyl cyclases. It is able to transduce signaling cascades and has intracellular and extracellular enzymatic activity (Funaro *et al*., 1999).

As an enzyme, it is able to metabolize NAD to yield ADPR and cADPR, two calcium mobilizing metabolites (Howard *et al*., 1993; Schubber *et al*., 2004; Kuhn *et al*., 2006; Lee, 2006). The role of NAD in the immune system is now beginning to be unraveled (Haag *et al*., 2007; Di Virgilio *et al*., 2001; La Sala *et al*., 2003; Hanley *et al*., 2004; Di Virgilio *et al*., 2005). It is released upon cellular damage and is thought to serve as a "danger signal". In addition, the cADPR produced after NAD metabolism is required for cell chemotaxis (Partida-Sànchez *et al*., 2001). So, CD38, through its enzymatic activity, may act as a "danger sensor" or a "chemotaxis controller", playing a role in development of immune responses.

CD38 was shown to behave as a selectin and, through interaction with CD31, to promote cell adhesion (Deaglio *et al*., 1998; Deaglio *et al*., 2000; Dianzani *et al*., 1994). Cell adhesion has an important role in holding two interacting cells bound to each other long enough to allow proper communication. Thus, CD38 may mediate intercellular communication, playing a role in development of immune responses.

CD38 has been shown to be a promiscuous molecule broadly expressed within and outside the immune system. Expression of CD38 is not restricted to a cell lineage or to a functional cellular state. CD38 associates with known cell receptors that are able to transduce signals (Lund *et al*., 1996; Morra *et al*., 1998; Deaglio *et al*., 2002; DiRosa *et al*., 1997; Partida-Sànchez *et al*., 2001; Inove *et al*., 1997; Fedele *et al*., 2004; Zilber *et al*., 2000). Acting as a co-receptor, CD38 modulates the signaling cascade of each of these receptors and, as such, the end result of cell activation, playing a role in shaping of the immune response.

Nevertheless, the biological relevance of CD38 is still far from being understood. Our main goal was to extend the current knowledge on the role of CD38 in immune homeostasis. For this, we have focused our attention on two distinct situations in which the immune system is challenged: i) infection by intracellular pathogens, namely mycobacteria; and ii) systemic autoimmune diseases, such as SLE. In the next sections, we will revisit our data and some mechanisms in which CD38 may be involved will be presented. In the final section, a model for the physiological role of CD38 will be proposed.

## 4.1. CD38 is required for protection against mycobacterial infection.

As CD38 was shown to play a functional role in several immune cells, it was hypothesized that it could also be involved in development of immune responses against invading pathogens, such as viruses and bacteria. Indeed, in humans, CD38 has been used as a prognostic marker in HIV infection. Increased expression of CD38 was associated with a poor prognosis (Giorgi *et al*., 1993; Savarino *et al*., 1999; Savarino *et al*., 2003 Chun *et al*., 2004; Zaunders *et al*., 2005; Echaniz *et al*., 1993). However, the exact role of CD38 is still unclear. In mice, CD38 was shown to be required for immunity against *S. pneumoniae*, as its absence rendered mice more susceptible to infection. The increased susceptibility of CD38KO mice was related to absence of CD38 on both the lymphoid and the myeloid axis. In addition, in CD38KO mice neutrophil chemotaxis towards bacterial peptides, namely fMLP, was reduced, which suggested that CD38 was required for innate immune responses, helping to restraint the pathogen at the site of infection (Partida-Sànchez *et al*., 2001; Partida-Sànchez *et al*., 2003).

Being required for immune responses against extracellular bacteria and playing a role in viral infections, it was conceivable that CD38 could also play a role in immunity against intracellular bacteria, namely mycobacteria. Much of the information on the immune responses against mycobacterial infection came from studies using *M. tuberculosis*. Mtb is spread via aerosols and establishes infection in the lungs. The route of *M. avium* infection is still unclear. Nevertheless, in both situations, resident macrophages are the first cells to encounter mycobacteria and to ingest the bacilli. At this stage, the destruction of mycobacteria will depend on the intrinsic microbicidal capacity of the host phagocytes and virulence factors of the ingested mycobacteria. Mycobacteria that escape the initial intracellular destruction multiply, lead to disruption of infected macrophages, and attract blood monocytes and other inflammatory cells to the site of infection. The newly arrived monocytes then differentiate into macrophages or DC, depending on the microenvironment they find upon arrival (Chapuis *et al*., 1997; Mariotti *et al*., 2002). These cells readily ingest mycobacteria but are not able to destroy them. At this stage, mycobacteria will grow logarithmically and blood-derived macrophages will accumulate. However, little tissue damage occurs. At two to three weeks after infection, T-cell immunity develops (Houben *et al*., 2006; North *et al*., 2004). Antigen-specific T cells arrive to and proliferate within early granulomata, and activate macrophages, which are then able to kill the intracellular mycobacteria. Consequently, bacillary growth stops.

Central solid necrosis in these primary lesions inhibits extracellular growth of mycobacteria. As a result, infection may become stationary or dormant (Sneller, 2002; Ehlers *et al*., 2000; Volkman *et al*., 2004; Roach *et al*., 2002; Flórido *et al*., 2004). When mycobacterial infections develop, localization, disease severity and outcome are highly variable. The different manifestations of infection reflect the balance between the bacilli and the host defence mechanisms, the quality of the host response determining the outcome of infection (van Crevel *et al*., 2002; Kurbel *et al*., 2008; Martino, 2008).

CD38 may participate in anti-mycobacterial responses at several levels: CD38 may be involved in development of innate immune responses, namely at the level of macrophages, which are the main host cells of mycobacteria and are essential for the control of infection. CD38 was shown to be functionally important in macrophages, being capable of modulating their function. In fact, CD38 was shown to synergize with MHC II molecules, augmenting macrophage's responses to superantigens (Zilber *et al*., 2000). In addition, CD38 modulates the response of human monocytes to respiratory burst activators (Zilber *et al*., 2005). CD38 may also impact the immune response against mycobacteria through the modulation of leukocyte trafficking to and from the infection site. Indeed, CD38 may act as a selectin, through interaction with CD31 (Deaglio *et al*., 2000). It is also able to modulate chemokine signalling and has already been shown to affect DC and neutrophil chemotaxis (Partida-Sànchez *et al*., 2001; Partida-Sànchez *et al*., 2004). In addition, CD38 can impact triggering of acquired immune responses, which is mainly performed by DC. CD38 was shown to be required for human DC maturation, CD83 expression and IL-12 production (Fedele *et al*., 2004; Frasca *et al*., 2006), features that are necessary for development of protective anti-mycobacterial Th1 immune responses. Moreover, CD38 is able to modulate the response of DC to CCR7, the key chemokine that directs DC to T-cell areas of secondary lymphoid organs. CD38 may further impact development of T cell responses, through modulation of cytokine secretion, namely of Th1 cytokines (Morra *et al*., 1998; Cesano *et al*., 1998). CD38, either due to its ability to induce cytokine production and/or to interactions with hyaluronic acid (Nishina *et al*., 1994) may also play a role in development of granulomatous responses, which are protective against mycobacteria.

In the work presented herein, we have investigated whether CD38 participated in immune responses against intracellular pathogens. Two different models were used: i.p *M. avium* and aerosol *M. tuberculosis* infection. CD38KO and C57Bl.6 mice were infected with viable mycobacteria and their immune response compared. We have established that CD38KO mice were more susceptible to mycobacterial infection, as the CFU counts in

CD38KO mycobacterial target organs were significantly higher than those observed for C57Bl.6 mice.

As already mentioned, mycobacteria mainly infect macrophages. Thus, the initial phase of anti-mycobacterial responses depends on intrinsic macrophages' microbicidal mechanisms, including lowering of the phagolysosome pH and production of reactive species, such as NO (van Crevel *et al*., 2002). After ingestion, mycobacteria are directed to specialized vesicles inside macrophages, the phagosomes. Phagosomes are endocytic organelles that are specialized in the elimination of pathogens. The newly formed phagosome must undergo a step-by-step maturation process that culminates in the fusion with matured lysosomes forming phagolysosomes. Phagolysosomes have an acidic pH (pH 4-5) and contain the lysosomal enzymes required for denaturation and degradation of the ingested pathogen (Desjardiris, 1995; Huynh *et al*., 2007). After microbial challenge and cytokine exposure, iNOS isoform is expressed. iNOS assumes a relevant role in mycobacterial infections particularly during the later phases of infection (Cooper *et al*., 2000). *iNOS* is an IFNγ-inducible gene and induces production of large amounts of NO over a period of days (Bogdan, 2001). NO is formed upon cleavage of L-arginine (Barnes, 1993). Killing of mycobacteria can be achieved by several ways, including modification of mycobacterial DNA, proteins and lipids (Nathan *et al*., 2000; Gow *et al*., 1999).

We have found that absence of CD38 did not affect the initial course of infection. Our data have shown that CD38 was not required for mycobacterial entry into macrophages, nor did it participate in the initial control of intracellular mycobacterial growth. In fact, bacterial growth inside CD38KO and C57Bl.6 macrophages was similar. In addition, non-activated macrophages of the two strains were unable to acidify the phagolysosome and to produce NO. This inability to initiate microbicidal mechanisms is possibly related to mycobacterial survival strategies. Indeed, successful intracellular pathogens have evolved a number of strategies to avoid being destroyed inside phagolysosomes. For instance, mycobacteria can prevent or delay fusion of phagosomes with lysosomes and are able to modulate the phagosomal compartment by preventing the incorporation of the vesicular proton ATPase and, consequently, phagolysosome acidification (Sturgill-Koszycki *et al*., 1994; Gomes *et al*., 1999; Kelley *et al*., 2003; Mueller *et al*., 2006). In addition, phagocytosis via complement or scavenger receptors prevents phagosome maturation (Aderen *et al*., 1999; Hirsch *et al*., 1994; Schlesinger *et al*., 1993; Tailleux *et al*., 2003; Yadav *et al*., 2006; Bermudez *et al*., 1991; Bohlson *et al*., 2001). The reduced production of NO may relate to production of reactive species scavengers by mycobacteria, such as sulfatides and LAM (Martino, 2008; van Crevel *et al*., 2002;

Kusner, 2005; Sturgill-Koszycky *et al*., 1994; Xu *et al*., 1994; Sturgill-Koszycki *et al*., 1996; Clemens *et al*., 1990).

However, mycobacteria-mediated inhibition of these microbicidal mechanisms is not complete and relates to the state of activation of mycobacteria-infected macrophages. For successful killing of mycobacteria, macrophages need to be exogenously activated. Early in infection, this activation is performed by NK cells, which, in response to mycobacterial stimulation or IL-12 produced by infected macrophages, produce IFNγ. IFNγ then activates macrophages and potentiate their microbicidal mechanisms. As infection progresses, exogenous activation of mycobacteria-infected macrophages is mainly achieved by T-cell derived signals, such as CD40-CD40L interactions and IFNγ (Hostetter *et al*., 2002; Schaible *et al*., 1998). We have found that the ability of CD38KO macrophages to produce NO was dependent on the availability of IFNγ and that reduced NO production was not due to intrinsic defects of macrophages associated with absence of CD38. We have observed that exogenous activation of mycobacteria-infected macrophages of both strains by IFNγ increased the production of NO. Of note, the amounts of NO produced by CD38KO macrophages were higher than those produced by C57Bl.6 macrophages. In contrast, exogenous activation of mycobacteria-infected macrophages by IFNγ had no effect on phagolysosome acidification.

Even though innate immune responses are able to restrain mycobacterial growth for a limited time they are not sufficient for the efficient control of mycobacteria. Immunity to mycobacteria always requires development of acquired immune responses. The type of immune response developed determines the outcome of mycobacterial infection. It is well established that differentiation of Th1 cells is essential for protection (Winslow *et al*., 2008; Sohal *et al*., 2008; Flynn *et al*., 2001; Raupach *et al*., 2001). The main role of Th1 cells is the production of cytokines, such as IFNγ and TNFα, which help in bacterial elimination, killing of infected cells and containment of mycobacteria within granulomata, limiting bacterial expansion (Lande *et al*., 2003).

IFNγ is an essential player in immunity against mycobacteria (Cooper *et al*., 1993; Flynn *et al*., 1993; Pearl *et al*., 2001), although some evidence supporting a role for IFNγ-independent mechanisms have been published (Caruso *et al*., 1999; Cowley *et al*., 2003). In the absence of IFNγ, mycobacteria are allowed to grow uncontrolled, ultimately leading to death of infected mice (Cooper *et al*., 1993). IFNγ is required for full activation of mycobacteria-infected macrophages enabling them to kill intracellular mycobacteria (Flynn *et al*., 1993; Pearl *et al*., 2001; Dalton *et al*., 1993). It is important to note that, although $CD4^+$ T cells are believed to be the main source of IFNγ, other cell populations are also

capable of producing this cytokine. These include activated mycobacteria-specific $CD8^+$ T cells, NK T cells and, as mentioned above, NK cells (Serbina *et al*., 2000; Kameth *et al*., 2004; Winau *et al*., 2006). Our data have shown that CD38 is required for the production of high levels of IFNγ in response to mycobacterial infection, as its absence leads to reduced production of this cytokine. The reduced production of IFNγ may be a consequence of reduced numbers of IFNγ-producing cells or reduced IFNγ secretion by each cell. Our data have shown that in *M. avium* infections CD38 was necessary both for the differentiation of IFNγ-secreting cells, namely Th1 cells, and for efficient IFNγ production. In fact, besides producing reduced amounts of IFNγ, *M. avium*-infected CD38KO mice also presented reduced numbers of IFNγ-producing cells and of IFNγ-producing $CD4^+$ T cells with regard to C57Bl.6 mice. In contrast, in Mtb infections similar numbers of IFNγ-producing cells were found in both strains. In this model, CD38 was only required for efficient IFNγ secretion. Indeed, we have found that Mtb-infected CD38KO mice had reduced amounts of IFNγ mRNA with regard to C57Bl.6 mice. The difference between the *M. avium* and Mtb models of mycobacterial infection may relate to the route of infection, to the organ in which infection is first established or to the virulence of mycobacteria.

CD38 may directly impact IFNγ production by T cells, as activation of T cells via CD38 is associated with production of different Th1 cytokines, including IFNγ (Lund *et al*., 1998; Morra *et al*., 1998; Cesano *et al*., 1998). Alternatively, inability to produce IFNγ may be a consequence of altered DC functions, reduced production of IL-12 or to a tolerogenic microenvironment associated with absence of CD38.

DC are professional APC that are responsible for the initiation of acquired immune responses (Jiao *et al*., 2002; deJong *et al*., 2005; Proietto *et al*., 2004; Naik, 2008). Upon activation, DC upregulate MHC I and II molecules and increase the expression of costimulatory molecules. They acquire motility and are able to migrate to secondary lymphoid organs where they engage in antigen presentation and priming of T cells (Shortman *et al*., 2002; Shortman *et al*., 2007; Villadangos *et al*., 2007; Rescigno *et al*., 1997). In addition, DC also constitute an important *in vivo* reservoir for mycobacteria. Indeed, cumulating evidence suggest that mycobacteria are able to survive within DC in which they replicate very slowly. Due to decreased turnover rates, DC are unable to kill the mycobacteria, but mycobacterial antigens are continuously available for presentation to T cells, this way, boosting the acquired immune response against this pathogen (Hope *et al*., 2004; Bodnar *et al*., 2001; Tailleux *et al*., 2003). As already mentioned, control of mycobacterial infections requires development of Th1 immune responses. For this, T-cell

priming must be performed in an IL-12-rich microenvironment. Absence of IL-12 during T-cell priming leads to uncontrolled mycobacterial growth (Cooper *et al*., 1997; Cooper *et al*., 2002). CD38 was shown to be required for IL-12 production by human DC. However, we have found that the reduced production of IFNγ by CD38KO mice was not associated with decreased ability of APC to produce IL-12. In fact, we have observed that Mtb-infected CD38KO and C57Bl.6 mice had similar amounts of IL-12 mRNA and that *M. avium*-infected CD38KO and C57Bl.6 macrophages produced comparable amounts of IL-12.

Immunity/tolerance decisions involve several factors. For instance, it depends on the immunogenic state of the APC and the cytokine milieu. In mice, two main DC subsets are recognised: myeloid and lymphoid DC (Wilson *et al*., 2003). Myeloid $CD11c^+CD8^-$ DC are found in splenic marginal zone (De Smedt *et al*., 1996; Agger *et al*., 1993) and induce production of large quantities of IL-4 and IL-10 (and few IFNγ) by activated T cells (Proietto *et al*., 2004), thus promoting Th2 differentiation and suppressive microenvironments (Maldonado-López *et al*., 1999; Pulendran *et al*., 1999; Soares *et al*., 2007). Lymphoid $CD11c^+CD8^+$ DC are found in splenic T-cell areas (De Smedt *et al*., 1996; Steinman *et al*., 1997) and produce high levels of IL-12, leading to Th1 differentiation, $CD8^+$ T cell priming and IFNγ production (Maldonado-López *et al*., 1999; Pulendran *et al*., 1999; Soares *et al*., 2007; Curtsinger *et al*., 2003). We have found that CD38 played a role in murine DC differentiation, its absence favouring differentiation of $CD8^-$ DC. These data suggest that the increased susceptibility of CD38KO mice to mycobacterial infections may be due to a Th2 bias. Indeed, we have found that, in the absence of CD38, activated $CD4^+$ T cells secreted increased amounts of IL-4. With regard to cellular immune responses, $CD8^-$ DC are non-immunogenic, promoting IL-10-rich suppressive microenvironments. IL-10 inhibits development of protective immune responses increasing murine susceptibility to mycobacterial infection (Demangel *et al*., 2002; Turner *et al*., 2002; Bearner *et al*., 2008; van Crevel *et al*., 2002). It was shown to inhibit macrophage function, by suppressing cytokine production, antigen presentation and TNFα production (Bogdan *et al*., 1992; Koppelman *et al*., 1997; Murray *et al*., 1997; Oswald *et al*., 1992). We have found that the reduced Th1 responses of CD38KO mice were also associated with increased IL-10 production. It is important to note that increased production of IL-10 may also result from mycobacterial survival strategies (Sturgill-Koszycki *et al*., 1994). Indeed, mycobacterial LAM binds to DC-SIGN molecules on DC inducing the production of IL-10 by these cells. As a consequence, antigen presentation, upregulation of MHC molecules and co-stimulatory molecules is prevented (Appelmelk *et al*., 2003; van Kooyk *et al*., 2003).

One of the hallmarks of mycobacterial infections is the development of granulomata. Productive granulomata require: 1) development of Th1-mediated immune responses; and 2) sustained co-localization signals to hold macrophages and lymphocytes close within granulomata (Sauders *et al*., 2007). Reduced Th1 responses may lead to reduced TNFα production. TNFα, which is mainly produced by activated macrophages and, to a lesser extent, by activated Th1 cells, is required for the formation of productive granulomata and for the inclusion of these structures inside fibrous walls. These features are essential to trap mycobacteria (Bean *et al*., 1999; Flynn *et al*., 1995; Roach *et al*., 2002). CD38 may impact the formation of granulomata through its role in cytokine production. Activation of T cells via CD38 leads to secretion of Th1 cytokines, including TNFα (Lund *et al*., 1998; Morra *et al*., 1998; Cesano *et al*., 1998). Moreover, CD38, through interaction with hyaluronic acid (Nishina *et al*., 1994), may play a role in maintenance of granulomata structure. We have found that CD38 was not required for production of TNFα by splenocytes or non-activated macrophages, as *M. avium*-infected CD38KO and C57Bl.6 splenocytes and macrophages produced similar amounts of this cytokine. However, CD38 was necessary for upregulation of TNFα production by activated macrophages, as CD38KO macrophages co-cultured with splenocytes recovered from *M. avium*-infected CD38KO mice, that is in the presence of activated T cells, were unable to produce this cytokine. These data suggest that CD38, indirectly, through production of IFNγ by activated T cells, plays a role in the upregulation of TNFα production by macrophages. Our data also showed that CD38 was required for the formation of a fibrotic rim around granulomata this way contributing to containment of mycobacteria in a restricted area. This may be associated to the ability of CD38 to stimulate TNFα production by granulomata macrophages. Inability to close the granulomata may affect the proliferation rate of mycobacteria, as mycobacteria were found to be actively replicating in open lesions, whereas those in closed lesions were non-dividing (Young *et al*., 2002). As such, bacteria will disseminate to nearby areas leading to the formation of additional lesions at distinct tissue sites (Roach *et al*., 2002). Indeed, we have found that mycobacteria-infected CD38KO mice had a higher number of granulomata and that these structures occupied a larger area than that observed for C57Bl.6 mice.

One way to kill mycobacteria inside granulomata is to induce apoptosis of mycobacteria-infected macrophages. Apoptosis is a mechanism that only occurs in productive granulomata and the presence of apoptotic cells in these structures was associated with resistance to mycobacterial infection (Gutierrez *et al*., 2004; Kremer *et al*., 1997; Bocchino *et al*., 2005). Apoptosis prevents spreading of mycobacteria, limits tissue damage and provides mycobacterial antigens for presentation to primed T cells present in

the granuloma (Fayyazi *et al*., 2000). TNFα and NO production is required for the induction of apoptosis of mycobacteria-infected macrophages (Dalton *et al*., 2000). It is important to note that apoptosis, but not necrosis, prevents spreading and survival of mycobacteria (Frattazzi *et al*., 1997; Bocchino *et al*., 2005; Proskuryakov *et al*., 2003; Danelishvili *et al*., 2003). Not surprisingly, mycobacteria have evolved mechanisms that counteract apoptosis induction and instead promote necrosis, this way guaranteeing their survival (Gehring *et al*., 2003; Tobian *et al*., 2003; Budak *et al*., 2008; Gan *et al*., 2008; Mustafa *et al*., 2005; Porcelli *et al*., 2008; Chen *et al*., 2006). Our data showed that *M. avium*-infected CD38KO macrophages, in contrast to C57Bl.6 macrophages, were unable to produce high amounts of TNFα and NO but produced increased amounts of IL-10. This may be associated with reduced activation of infected macrophages, a consequence of decreased production of IFNγ by CD38KO T cells. It is conceivable that CD38, indirectly, through its ability to control IL-10, TNFα and NO production, may modulate macrophage apoptosis. Indeed, we have found that in Mtb-infected CD38KO mice there were less apoptotic cells than in C57Bl.6 mice and that these cells were restricted to the border of the granulomata, where activated $CD4^+$ T cells are found. This can have deleterious effects to the host, as *in situ* selective killing of activated $CD4^+$ T cells may lead to reduced *in situ* IFNγ production and to decreased activation of mycobacteria-infected macrophages.

Although our data on *M. avium* and Mtb infection clearly support a role for CD38 in resistance to mycobacterial infection; it also evidences some differences between the two models. For instance, at early time points of infection, CD38KO mice had a lower *M. avium* burden in the spleen than C57Bl.6 mice. The opposite was observed for Mtb-infected mice: CD38KO mice had a higher mycobacterial burden in the lungs than C57Bl.6 mice. These differences may relate to the route of infection and to the organ in which the bacilli are found. In our work, the route of infection used for *M. avium* was the intraperitoneal cavity whereas the aerogenic route of infection was used for Mtb. Mycobacteria grow more rapidly in some organs than others. For instance, mycobacteria grow faster in the lungs and establish infection earlier than in the spleen and liver. Another explanation for the differences between the two models may regard the virulence of the mycobacteria. Mtb is a much more virulent strain than *M. avium*, as it is capable of establishing infection even in immunocompetent individuals, whereas *M. avium* bacilli are rapidly controlled and hardly establish infection in immunocompetent individuals.

Development of effective immune responses requires that immune cells are in the right place at the right time. Orchestration of leukocyte movement and localization is performed by chemokines. Leukocytes are also attracted to the infection site by pathogen-

derived products. CD38 may impact the immune response against mycobacteria through modulation of leukocyte chemotaxis and trafficking. It is well established that CD38 participates in chemotaxis of DC and neutrophils. This can be due to its role as an adhesion molecule or the modulation of CCR7-, CXCR4- and CCR2-signaling pathways (Deaglio *et al*., 2000; Partida-Sànchez *et al*., 2001; Partida-Sànchez *et al*., 2004; Fedele *et al*., 2004). The increased susceptibility of CD38KO to *S. pneumoniae* infection was associated with reduced chemotaxis of neutrophils to bacterial peptides (Partida-Sànchez *et al*., 2001). We have found that CD38 was also required for chemotaxis of leukocytes, including mononuclear cells and granulocytes, to the site of *M. avium* infection. The reduced trafficking of leukocytes may compromise the initial ability to control mycobacterial growth. For instance, neutrophils enhance macrophage bacteriostatic activity and actively contribute to the containment of mycobacteria (Flórido *et al*., 1997; Appelberg, 1992; Appelberg *et al*., 1995). Reduced chemotaxis of neutrophils may then contribute to the increased susceptibility of CD38KO mice. The reduced trafficking of leukocytes to the site of inflammation may be due to disrupted CD38/CD31 interactions, compromised chemokine receptor signaling or to decreased production of chemokines. We have found that CD38 is required for chemokine production, namely that of MCP-3, MCP-1 and RANTES. MCP-1 and -3 are mainly produced by monocytes and macrophages and are responsible for the recruitment of monocytes, activated T cells and immature DC to the site of infection. RANTES is produced by a variety of cells, and is required for chemotaxis of monocytes and memory T cells. Absence or decreased production of these chemokines may thus be responsible for the reduced recruitment of mononuclear cells to the site of infection, this way compromising the ability of CD38KO mice to control initial mycobacterial growth.

It is well established that Mtb dissemination to MLN always precedes and plays a central role in development of T cell–mediated immune responses. Indeed, T-cell priming does not occur unless mycobacteria are present at these sites (Chackerian *et al*., 2002; Wolf *et al*., 2008). Antigen-loaded DC arrive at the MLN and engage in antigen presentation, leading to $CD4^+$ T cell activation, differentiation and proliferation. After activation, these cells leave the MLN and travel to the lungs where they help in mycobacteria containment (Lande *et al*., 2003). We have found that CD38 does not interfere with trafficking of $CD4^+$ T cells to mycobacterial target organs. We have found that Mtb-infected CD38KO mice had a higher number of $CD4^+$ T cells in MLN at early time points than C57Bl.6 mice. This may be associated with a rapid dissemination of mycobacteria to these organs, allowing a faster initiation of T cell responses. This was also reflected in an earlier exit of $CD4^+$ T cells from the MLN to the lungs. These data are

in agreement with Shi and coworkers (Shi *et al*., 2007) suggestion that CD38-independent chemotatic responses exist.

Taking all the available information into account, a model for the role of CD38 in mycobacterial infections may be proposed (Fig. 2).

Immune responses to mycobacteria require initial phagocytosis of mycobacteria by professional phagocytes, such as macrophages and neutrophils. Mycobacteria are first ingested by resident inflammatory cells. Chemokines and other inflammatory mediators are then produced, attracting blood leukocytes to the site of infection. CD38 is required for migration of these cells to the site of infection, namely through induction of MCP-1, MCP-3 and RANTES production. In addition, CD38 may also control leukocyte chemotaxis acting as an adhesion molecule. Indeed, CD38 may act as a selectin through interaction with CD31. As such, it may have an important role in leukocyte rolling, before diapedesis and extravasion. For effective killing of mycobacteria and restrain of mycobacterial growth, macrophages need to be exogenously activated. This activation is initially performed by NK cells, which, upon activation, produce IFN$\gamma$. IFN$\gamma$ induces full activation of microbicidal mechanisms of mycobacteria-infected macrophages endowing them with the capacity to kill intracellular bacteria. CD38 is required for efficient production of IFN$\gamma$.

The main source of IFN$\gamma$ during anti-mycobacterial immune responses are Th1 cells. Th1 responses are central in protection against mycobacteria. CD38 is required for Th1 differentiation and for IFN$\gamma$ production by these cells. DC, being professional APC, have a central role in guiding polarization of immune responses. Depending on the immunogenic state of the DC and DC subsets, different responses are triggered. Murine $CD8^-$ DC promote Th2 differentiation and production of IL-4 and IL-10, whereas $CD8^+$ DC produce IL-12 and induce Th1 differentiation. CD38 favors development of $CD8^+$ DC and Th1 differentiation. CD38, indirectly, through induction of IFN$\gamma$ secretion by activated T cells, stimulates NO production and hence contributes to killing of intracellular mycobacteria. In addition, it indirectly leads to activation of $CD8^+$ T cells and NK cells, this way contributing to the elimination of infected cells.

Continuous antigenic stimulation, as well as T cell and macrophage activation, results in the formation of granulomata. Successful formation of these structures is dependent on *in situ* TNF$\alpha$ production. CD38, through its ability to induce IFN$\gamma$ production, stimulates TNF$\alpha$ production by granulomata mycobacteria-infected macrophages. As such, it plays an active role in the restraint of mycobacteria at target organs.

In conclusion, our data clearly evidence a role for CD38 during the second phase of the anti-mycobacterial responses, when Th1 immune responses are triggered.

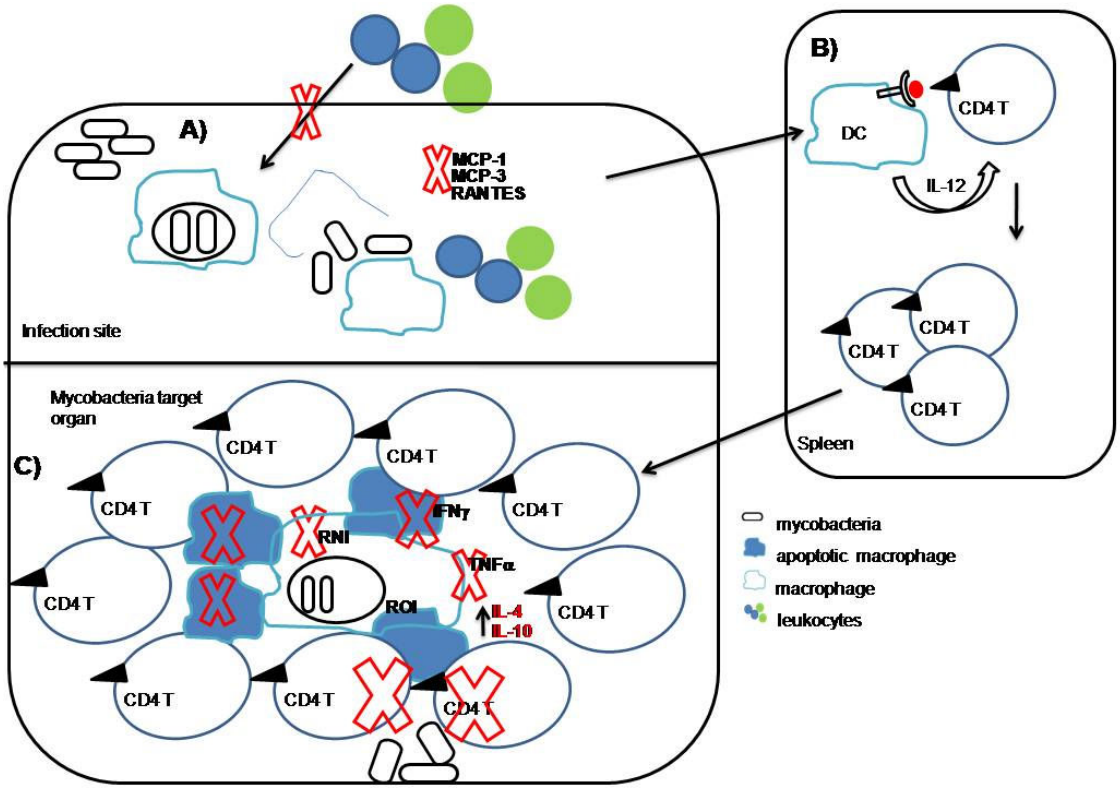



**Figure 2. Model for the role of CD38 in immune responses against mycobacteria.** CD38 may participate in the immune response against mycobacteria in several ways: A) CD38 is necessary for chemotaxis of blood leukocytes to the site of infection. This effect may be associated with the ability of CD38 to induce chemokine production, namely MCP-1 and -3 and RANTES. In addition, acting as a selectin, CD38 participates in the arrest of cells from the bloodstream, which is essential for their migration to the site of infection; B) CD38 is necessary for development of Th1 immune responses and IFNγ production. This feature may be associated with its capacity to direct DC differentiation towards a $CD8^+$ DC phenotype and to its modulation of the T-cell response by stimulating IFNγ secretion by T cells; C) CD38 is necessary for formation of a fibrous wall around granulomata and killing of mycobacteria by infected macrophages. This may be due to the ability of CD38 to induce IFNγ production by activated $CD4^+$ T cells. IFNγ-mediated activation of infected macrophages promotes TNFα and NO production. TNFα is required for the effective containment of mycobacteria inside granulomata; NO is a key molecule in the induction of apoptosis of infected macrophages, this way helping in the control of mycobacterial growth. Red cross, pathways compromised due to absence of CD38; RNI, reactive nitrogen intermediates; ROI, reactive oxygen intermediates; MCP, macrophages chemoattractant protein; RANTES, regulated upon activation normal T cell express sequence.

## 4.2. CD38 has a protective role in systemic autoimmunity.

CD38, due to its immunological functions, has long been suspected to play a role in autoimmune diseases. In fact, anti-CD38 antibodies have been established as an independent clinical marker for type 1 diabetes (Pupilli *et al*., 1999) and chronic autoimmune thyroiditis (Antonelli *et al*., 2001). Nevertheless, the exact role of CD38 is still unclear. Recently, absence of CD38 was shown to accelerate diabetes development in NOD mice, a feature that was associated with an effector: regulatory T-cell imbalance in the periphery, possibly due to selective NAD-induced killing of regulatory T cells (Chen *et al*., 2006a; Krebs *et al*., 2005). Even though the role of CD38 in organ-specific autoimmune diseases, such as diabetes, is starting to be understood, its involvement in systemic autoimmunity, such as SLE, is still unknown. Only indirect evidence suggesting the involvement of CD38 in SLE was published. CD38 was proposed as a candidate gene for human (Harley *et al*., 2002; Olson *et al*., 2002; Kelly *et al*., 2002) and mice (Vidal *et al*., 1998) lupus-susceptibility *loci*. Moreover, in SLE patients, a known CD38 polymorphism was shown to have a protective role in discoid rash development (Ferrero *et al*., 1999; González-Escribano *et al*., 2004). In addition, increased numbers of CD38 expressing T and B cells have been identified in SLE patients (Al-Janedi *et al*., 1993; Spronk *et al*., 1996; Lindquist *et al*., 1999; Pavón *et al*., 2006).

Murine lupus-like disease, similarly to SLE, is characterized by a female bias, development of IC-mediated GN, increased production of autoantibodies of multiple specificities, mainly against nuclear antigens and increased amounts of circulating immunoglobulins, IC and complement (Andrews *et al*., 1978). In murine lupus, the increased availability of apoptotic material, either due to defective clearance or excessive tissue damage, induces activation of DC (Marshak-Rothstein *et al*., 2007). Self-antigen loaded DC travel to secondary lymphoid organs where they present autoantigens to and activate autoreactive T cells. Chronic activation of autoreactive $CD4^+$ T cells provides excessive help to autoreactive B cells, leading to production of autoantibodies of multiple specificities (Andrews *et al*., 1978; Theofilopoulos *et al*., 1985; Waters *et al*., 2004), which will form IC. These can be trapped anywhere in the body initiating local inflammatory events. Local inflammation disrupts the normal functioning of the tissues, which become, themselves, a source of autoantigens, this way perpetuating the autoimmunity loop.

CD38 may be involved in development of systemic autoimmunity at several levels. It may play a role in lymphopoiesis, as its expression is tightly controlled during differentiation of T (Bean *et al*., 1995) and B cells (Malavasi *et al*., 1994; Kumagai *et al*., 1995). In the thymus, CD38 expression is upregulated during the DN2 and DN3 stages and the process of β-selection, suggesting that CD38 may be involved in TCR

rearrangements (Malavasi *et al*., 1994; Spits, 2002; Dik *et al*., 2005; Staal *et al*., 2001). CD38 may also be involved in the delivery of survival signals to developing thymocytes. Indeed, CD38 was shown to be able to modulate TCR signaling and, through its ability to recruit adaptor molecules and downstream effectors, the outcome of cell activation (Morra *et al*., 1998; Munoz *et al*., 2003; Zubiaur *et al*., 2002; Cho *et al*., 2000; Lund *et al*., 1996; Lund *et al*., 1998; Boffil *et al*., 2000). In addition, CD38 may also behave as an adhesion molecule (Deaglio *et al*., 2000). As such, it may be important for keeping two interacting cells together long enough to allow signal initiation. In the bone marrow, CD38 expression is also tightly controlled. CD38 is expressed by pro- and pre-B cells, during the rearrangements of the heavy and light chains of the BCR, which suggests a role for CD38 in this process (Dónis-Hernandez *et al*., 2001). In the periphery, CD38, through modulation of the BCR response, was proposed to shape the mature murine B cell repertoire (Rodriguez-Alba *et al*., 2008). CD38 may affect development of autoimmune responses by interfering with tolerance/immunity decisions, which involve DC. In fact, CD38 was shown to modulate DC maturation and function (Fedele *et al*., 2004; Frasca *et al*., 2006; Wykes *et al*., 2004; Partida-Sánchez *et al*., 2003; Partida-Sánchez *et al*., 2004). In human DC, it is required for CD83 expression and IL-12 secretion (Frasca *et al*., 2006; Fedele *et al*., 2004). Finally, CD38 may also participate in other peripheral tolerance mechanisms, such as survival of Treg cells. By competing with ART for NAD, CD38 may rescue Treg cells from apoptosis (Krebs *et al*., 2005; Han *et al*., 2000).

In the work presented herein, we have investigated whether CD38 was involved in lupus development. For that, we have used murine models of the disease. We have developed CD38KO-*lpr/lpr* mice, crossing CD38KO mice with C57Bl.6-*lpr/lpr* mice, a well established murine lupus model. The resulting CD38KO-*lpr/lpr* mice, of both sexes, and the autoimmunity-prone parental strain, C57Bl.6-*lpr/lpr* mice, were monitored for disease development through measurement of protein excretion in urine. We have found that, earlier in life, female and male CD38KO-*lpr/lpr* mice already presented pathological levels of proteinuria (≥100mg/dl), whereas C57Bl.6-*lpr/lpr* mice still had no signs of disease. These data suggest that absence of CD38 in *lpr* mice leads to kidney damage earlier in life. This was confirmed by histological analysis of the kidneys. We have observed that despite the fact that, at six months of age, all female and male *lpr* mice presented membranoproliferative GN, glomerulosclerosis was only seen in female CD38KO-*lpr/lpr* mice that reached 500mg/dl of proteinuria.

Lupus GN results from deposition of IC in the kidneys. The identification of IC deposits in these organs can be done in various ways, including direct immunofluorescence studies. However, immunofluorescence analysis by confocal laser

scanning microscopy (the methodology we have chosen) has a major difficulty: natural autofluorescence and autofluorescence induced by the use of tissue fixatives. Natural fluorescence is mainly due to the presence of endogenous flavins, reduced NAD(P)H, lipofuscins, reticulin fibres, collagen and elastin (Andersson *et al*., 1998; Baschong *et al*., 2001; Billinton *et al*., 2001). Tissues such as the kidneys can be very challenging as they have extremely high metabolic rates with constant NAD-NADPH turnover, high accumulation of flavins and lipofuscins and are highly vascularised. Tissue fixatives, such as neutral buffered formalin (the one used in our samples), form covalent bonds between adjacent amine-containing groups through Schiff acid-base reactions leading to the formation of fluorescent products that result in an intense fluorescent background (Beisker *et al*., 1987). Given the number of parameters that need to be taken into account when performing immunofluorescent studies, no single methodology has proven to be effective in the reduction of the fluorescent background. Thus, in order to be able to detect IC deposition, in the kidneys, by direct immunofluorescence, we had to improve the available methodologies so that we could achieve a drastic reduction in autofluorescence without compromising direct immunofluorescence labelling.

As mentioned before, natural autofluorescence may be due to intrinsic factors. These natural fluorophores are responsible for the emission of light ranging from 450nm to 650nm, wavelengths that overlap those of the fluorophores used in immunofluorescence studies (from 488nm to 568nm). One of the approaches used to quench natural autofluorescence is the use of dyes whose absorbance spectrum overlaps that of autofluorescence emission. A wide range of dyes is available and the choice for the most appropriate one should take into account tissue characteristics and the assays to be performed (Mosiman *et al*., 1997; Cowen *et al*., 1985). Sudan Black B, among other Sudan dyes, is the most appropriate dye to quench autofluorescence of lipofuscins, fats, triglycerides and lipoproteins (Baschong *et al*., 2001; Schnell *et al*., 1999). We have found that incubation of kidney tissue sections with Sudan Black B lead to a drastic reduction of the autofluorescent background. Nevertheless, this reduction was still not sufficient to allow detection of signals by direct immunofluorescence, a feature that could be associated with the autofluorescence induced by the use of fixatives. Treatment of tissue slides with either ammonia/ethanol or photobleaching was suggested to be a good approach to quench fixative-induced fluorescence (Baschong *et al*., 2001; Billinton *et al*., 2001; Neumann *et al*., 2002). However, application of either method in kidney tissue sections was not sufficient to quench autofluorescence to levels that would allow detection of direct immunofluorescence signals. We have found that treatment of tissue sections with a combination of photobleaching and Sudan Black B allowed the complete

elimination of the autofluorescent background in kidney tissue sections, thus allowing the detection of direct immunofluorescence signals. Application of this improved methodology to tissue sections of six-month old CD38KO-*lpr/lpr* and C57Bl.6-*lpr/lpr* mice allowed the detection $IgG_1$ and $IgG_3$-IC deposits in the kidneys of CD38KO-*lpr/lpr* and C57Bl.6-*lpr/lpr* mice, confirming that GN in CD38KO-lpr/lpr mice, similarly to that of the autoimmunity-prone parental strain, was IC-mediated.

Our next step was to characterize disease in this new murine model of lupus. Murine lupus, similarly to human SLE, is characterized by spontaneous production of autoAbs, mainly against nuclear antigens, and increased levels of circulating Ig and complement, all reflecting B cell hyperactivity (Andrews *et al*., 1978). We have found that female and male CD38KO-*lpr/lpr* mice share many of the serologic characteristics of murine models of lupus and human SLE, namely high titres of circulating IgG and production of ANCA and ANA and reduced amounts of anti-dsDNA autoantibodies and RF.

Altogether, our data suggest that, to some extent, CD38 plays a role in the control of systemic autoimmunity. We have observed that CD38KO-*lpr/lpr* mice developed a more severe and accelerated form of disease with regard to the parental strain, C57Bl.6-*lpr/lpr* mice. Although more severe, disease of CD38KO-*lpr/lpr* mice resembled that of C57Bl.6-*lpr/lpr* mice.

Development of autoimmune diseases requires breakage of tolerance. Central tolerance mechanisms mainly take place in the thymus during T-cell differentiation. The correct functioning of the thymus is essential to assure a competent and functional T-cell repertoire that is able to protect the host against foreign threats at the same time being unable to react with self-antigens. T cell ontogeny can be broadly divided into three major stages based on CD4 and CD8 expression on thymocytes: DN, DP and SP stages. At early stages of differentiation, pre-T cells are characterized by the absence of CD4, CD8 and TCR expression (DN T cells) (Ceredig *et al*., 2002). It is during the DN stage that cells begin to rearrange their TCR genes. Cells that successfully rearrange the TCRβ undergo a series of events known as β-selection, during which TCRβ, TCRγ and TCRδ rearrangements are arrested, CD4 and CD8 are upregulated and TCRα rearrangements initiated (DP T cells). If TCRα rearrangements are productive, DP T cells will receive survival signals and will undergo positive and negative selection. In contrast, if TCRα rearrangements are not productive, DP T cells do not receive survival signals and die by neglect (Klein *et al*., 2000; Sebzda *et al*., 1999; Sprent *et al*., 1995). The signaling cascade that provides survival signals and determines β-selection is similar to that

observed after activation of mature T cells through TCR (Hagenbeek *et al*., 2004). The TCRβ assembles with a surrogate a chain (*p*Ta) and CD3ζ forming a pre-TCR. The pre-TCR moves to lipid rafts on the cell membrane and spontaneously initiates the signaling cascade by inducing Lck phosphorylation and activation of the protein kinase C, Ras and MAPK cascades (Hagenbeek *et al*., 2004; Schmedt *et al*., 2001; von Boehmer, 2005; Gartner *et al*., 1999; Iritani *et al*., 1999). CD38 may be involved in the process of β-selection and in TCR rearrangements, through its function as a receptor. (Malavasi *et al*., 1994; Spits, 2002; Dik *et al*., 2005; Staal *et al*., 2001). On mature T cells, CD38 was shown to be constitutively associated with lipid rafts (Zubiaur *et al*., 2002) and to be able to modulate TCR signaling through its ability to interact with Lck and CD3ζ/ZAP-70 and activate the Raf1/MAPK signaling cascade (Munoz *et al*., 2003). In addition, CD38 may impact β-selection through its role as an adhesion molecule (Deaglio *et al*., 2000). Adhesion molecules are important in keeping two interacting cells together long enough to allow signal initiation. If the cellular adhesion is disrupted signals may not be tranduced. As a consequence, DP T cells will not receive survival signals and may die by neglect. We have found that CD38 impacts selection of DP T cells. We have observed that female FKOMlpr had decreased percentages of DP T cells with regard to C57Bl.6-*lpr/lpr* mice. These data suggest an increased thymocyte death prior to and/or during the DP stage.

After positive selection, developing DP thymocytes must commit to either the CD4 or CD8 lineage. This is determined by the duration and kinetics of TCR signalling (Germain, 2002; Singer, 2002; Yasutomo *et al*., 2000; Liu *et al*., 2004), as well as by cytokines of the common cytokine-receptorγ-chain family, such as IL-7 (Singer *et al*., 2008). The signaling cascade elicited by TCR/CD4-MHC and TCR/CD8-MHC interactions is similar, although Lck is differently recruited in the two situations. It is the amount of Lck recruited to the vicinity of the immunological synapse that dictates whether the developing thymocyte will become a $CD4^+$ SP or a $CD8^+$ SP T cell. The kinetics of the interaction also influence the process: thymocytes become a $CD4^+$ SP T cell, if the signal is sustained; and a $CD8^+$ SP T cell, if the signal is transient (Germain, 2002; Singer, 2002; Yasutomo *et al*., 2000; Liu *et al*., 2004). However, it is still controversial whether TCR/co-receptor interactions are, *per se*, sufficient to determine CD4/CD8 fate and cumulating evidence points to the involvement of other signaling pathways, such as Notch. Although still unclear, it was proposed that rather than being associated with quantitative changes in TCR signaling, Notch would influence the time-course of the TCR response or recruitment of downstream effectors in events prior to CD4/CD8 lineage choice (Laky *et al*., 2006; Ho *et al*., 2009; Collins *et al*., 2009). Thus, receptors or molecules that are similarly able to modulate TCR signaling may also participate in CD4/CD8 lineage decisions. This is the

case of CD38 due to its function as a receptor. For instance, CD38 may amplify the signal elicited by TCR ligation by augmenting recruitment of Lck and downstream effectors to the lipid raft (Zubiaur *et al*., 2002; Munoz *et al*., 2003). Strong TCR-mediated signals more rapidly elicit negative feedback mechanisms, leading to termination of TCR signaling and this way favoring differentiation of CD8 SP T cells. In the absence of CD38, the initiation of these negative feedback mechanisms would be delayed allowing continuous engagement of the TCR/CD4 and differentiation of CD4 SP T cells. Alternatively, CD38 may be able to "highjack" Lck molecules, competing with the TCR. As the amount of Lck available for TCR signaling is critical and limited, highjacking of Lck by CD38 may disrupt TCR signaling, and bias differentiation towards CD8 SP T cells. In this scenario, absence of CD38 would contribute to an increased availability of Lck, allowing for sustained TCR signaling and thus promoting commitment to the CD4 lineage. We have found that CD38 impacts CD4/CD8 linage choices. Supporting this idea is the observation that female FKOMlpr mice had increased percentages of $CD4^+$ SP T cells with regard to the autoimmunity-prone parental strain.

Before release to the periphery, SP thymocytes must undergo negative and positive selection. Negative selection is a more exigent process than positive selection and assures that cells presenting TCRs that strongly react with self-antigens are eliminated by apoptosis, this way guaranteeing that the majority of T cells that are exported to the periphery are self-tolerant. Negative selection depends on TCR reactivity and requires transient ERK, JNK and p38 activation. During this stage, there is also selection of SP T cells that bear highly autoreactive TCR and will exert a regulatory function in the periphery (Lio *et al*., 2008, Bassiri *et al*., 2001; Fontenot *et al*., 2003; Fontenot *et al*., 2005; Fontenot *et al*., 2005a). The TCR repertoire of Treg cells overlaps that of SP T cells undergoing negative selection (Hsieh *et al*., 2006). It was suggested that events that dictate whether cells will become Treg take place during β-selection, as alterations in this process lead to altered frequencies of $CD4^+CD25^+$ T cells (Bosco *et al*., 2006; Pennington *et al*., 2006). Cumulating evidence suggest that directing SP T cells to the Treg pathway or to negative selection requires additional signals (Salomon *et al*., 2000; Tai *et al*., 2005). However, these signals have not been identified yet. CD38 may play a role in directing developing thymocytes to the negative or Treg selection pathway. As already mentioned, CD38 is expressed at high levels during β-selection, when TCR reactivity is determined. Furthermore CD38 modulates TCR responses by recruiting adaptor molecules and downstream effectors (Zubiaur *et al*., 2002; Munoz *et al*., 2003). It is possible that CD38, through amplification of TCR-mediated signals, may direct SP T cells to negative selection. In the absence of such signals, differentiation of $CD4^+CD25^+$

Treg cells would be favored. Our data suggest that CD38 is required for directing SP T cells to the process of negative selection, as female FKOMlpr had a higher percentage of $CD4^+CD25^+$ T cells than the autoimmunity-prone parental strain.

Altogether, our data suggest that CD38 impacts T cell differentiation in the thymus and, as such, contributes to the establishment of a peripheral T-cell repertoire that is tolerant to self.

Besides the central mechanisms that operate in the thymus, maintenance of self tolerance is also dependent on peripheral mechanisms, which include activation-induced cell death (Nagata *et al*., 1995), anergy (Evavold *et al*., 1991; Sloan-Lancaster *et al*., 1993; Sloan-Lancaster *et al*., 1994; Bhandola *et al*., 1993; Lanfman *et al*., 1996; Banchereau *et al*., 1998; Banchereau *et al*., 2004), regulation by Treg cells (Izcue *et al*., 2006; Sakaguchi, 2004; Sakaguchi *et al*., 2007) and clonal ignorance (Ohashi *et al*., 1991). DC are key players in the decision between tolerance and immunity (Steinman *et al*., 2003; Gad *et al*., 2003). In the periphery, DC continuously present autoantigens to autoreactive T cells and, in the absence of co-stimulatory signals, induce anergy or deletion of these cells (Steinman *et al*., 2000; Moser, 2003). In addition, DC participate in Treg differentiation (Battaglia *et al*., 2004; Sakaguchi, 2005).

In mice, two main DC subsets have been identified. Myeloid DC ($CD11c^+CD8^-$) are mainly found in the splenic marginal zone (De Smedt *et al*., 1996; Agger *et al*., 1992). Upon activation, these cells migrate to T cell areas (Reis e Sousa *et al*., 1997), produce large amounts of inflammatory chemokines (Proietto *et al*., 2004) and induce Th2 differentiation with concomitant production of IL-4 (Maldonado-Lopez *et al*., 1999; Pulendra *et al*., 1999; Soares *et al*., 2007). They are also involved in promoting IL-10 rich tolerogenic environments. Lymphoid DC ($CD11c^+CD8^+$) are found in splenic T-cell areas (De Smedt *et al*., 1996; Steinman *et al*., 1997) and produce high levels of IL-12, promoting Th1 differentiation and $CD8^+$ T cell priming, with concomitant secretion of IFN$\gamma$ (Maldonado-López *et al*., 1999; Pulendran *et al*., 1999; Soares *et al*., 2007; Curtsinger *et al*., 2003). Altered $CD8^-/CD8^+$ DC ratios may then shift immune responses to Th1 or Th2 poles. Lupus is classically seen as a Th2-mediated autoimmune disease. Hyperactivated Th2 cells provide excessive help to autoreactive B cells leading to augmented production of autoantibodies and increased formation of IC. The newly formed IC will be deposited, for instance, in the kidneys, initiating local inflammatory events that, in the case of the kidneys, lead to development of IC-mediated GN. Increased $CD8^-/CD8^+$ DC ratios can be predicted to exacerbate murine lupus-like disease through promotion of Th2 differentiation. Our data have shown that CD38 modulates $CD8^-/CD8^+$ DC ratios in the

spleen. We have observed that CD38KO-*lpr/lpr* mice had increased frequencies of $CD8^-$ DC. Preferential activation of $CD4^+$ T cells of CD38KO-*lpr/lpr* mice by $CD8^-$ DC will bias their differentiation to Th2 cells and further promote B-cell hyperactivation. Supporting this hypothesis is the observation that CD38KO-*lpr/lpr* mice presented $IgG_1$- and $IgG_3$-mediated GN, which was more severe than that observed in C57Bl.6-*lpr/lpr* mice. These two IgG subclasses are associated with Th2 responses. Moreover, we have evidence that activated CD38KO T cells produce high amounts of IL-4 in response to antigenic stimuli.

Development of autoimmune diseases is usually associated with decreased frequencies and/or function of regulatory T cells. Treg cells are central for maintenance of peripheral tolerance and prevention of autoimmune diseases (Fontenot *et al*., 2005; Izcue *et al*., 2006; Sakaguchi, 2004; Sakaguchi *et al*., 2007; Ni Choileain *et al*., 2006; Shevach, 2000). There are several subsets of Treg cells, including natural (Sakaguchi *et al*., 1995; Groux *et al*., 1999) and induced $CD4^+CD25^+$ T cells (O'Garra *et al*., 2004) and NK T cells (Higuchi *et al*., 2002). Induced $CD4^+CD25^+$ T cells can be further divided into type 1 regulatory (Tr1) and type 3 helper (Th3) T cells (Bach, 2003; Zou, 2006; Yi *et al*., 2006). Differentiation and activation of Treg cells greatly depends on the microenvironment, namely on the cytokines present (Malek *et al*., 2004; La Cava, 2008; Fu *et al*., 2004). Treg cells exert their regulatory function through inhibition of *IL-2* transcription in target cells, cell-cell contact or TGFβ and IL-10 secretion (Wing *et al*., 2006; Taylor *et al*., 2006; Roncarolo *et al*., 2006).

Murine lupus has been associated with reduced numbers of Treg cells (NZM2410*sle*1 mice) and/or lack of suppressive function (MRL-*lpr/lpr*, NZB/WF1 and male BXSB) (Mudd *et al*., 2006; Chen *et al*., 2005; Monk *et al*, 2005). In humans, conflicting evidence has been published (Kuhn *et al*., 2009). Accelerated development of autoimmune diabetes in CD38KO NOD mice seems to be due to a peripheral imbalance between effector and regulatory T cells (Chen *et al*., 2006). It was proposed that this could be due to selective killing of Treg cells by NAD (Chen *et al*., 2006). Purine nucleotides are normally released under inflammatory or cellular stress. Released NAD accumulates in the extracellular microenvironment and may cause death of T cells. T cells are not equally sensitive to NAD-induced killing (Haag *et al*., 2002). Naïve T cells and Treg cells are highly susceptible to NAD-mediated killing (Seman *et al*., 2003; Adriouch *et al*., 2001; Aswad *et al*., 2005; Haag *et al*., 2002), whereas antigen-activated T cells are resistant to NAD (Seman *et al*., 2003; Kahl *et al*., 2000). Selective killing of Treg cells and naïve T cells limit the chance of activation of undesired T cells, allowing proliferation of antigen-specific T cells. When tissue damage becomes controlled, NAD levels decrease, allowing Treg cell survival and termination of the ongoing immune response. In the context of

autoimmunity, NAD-induced death may have a deleterious effect through the elimination of Treg cells.

NAD-induced cell death is primarily controlled by mono (ADP-ribosyl) transferases (ART) (Koch-Nolte *et al*., 1996; DiVirgilio *et al*., 2001; Seman *et al*., 2003). CD38 was shown to control ART functions by competing for NAD. It has been suggested that CD38 could this way control NAD-induced killing of Treg cells (Krebs *et al*., 2005). CD38 may act as a NAD sensor and control the duration and intensity of NAD/ART interactions, this way shaping immune responses (Han *et al*., 2000). Similarly to the observations made in CD38KO NOD mice, and although not addressed in this work, it is possible that absence of CD38 in *lpr* mice may lead to increased NAD availability and to ART-mediated selective killing of Treg cells. This would weaken peripheral tolerance and contribute to lupus development/exacerbation.

Interestingly, we have found that female mice descending from the two different original crosses were not equally susceptible to lupus development. FKOMlpr presented a more severe form of disease than MKOFlpr. We believe that this effect may stem from a maternal parent-of-origin effect that proposes that gene regulation may be controlled by the expression of a single allele in a parent-of-origin-dependent manner (Zeft *et al*., 2008, Ideraabdullah *et al*., 2008). Such an effect has already been observed in some autoimmune diseases, including juvenile idiopathic arthritis.

Based on the data presented herein and current knowledge on CD38, we propose the following model for the protective role of CD38 in lupus. CD38 may be involved in thymic differentiation of T cells (Fig. 3), namely at the level of β-selection, positive and negative selection and Treg differentiation. During β-selection, the TCR is assembled and the specificity and affinity of this receptor is determined. Those thymocytes harboring functional TCRs receive survival signals and are allowed to proceed the selection process. CD38 may be an active player in the assembly of productive α and β chains of the TCR and/or modulate the delivery of survival signals. After positive selection, developing thymocytes commit to either the CD4 or the CD8 lineage. This process depends on the duration and kinetics of the TCR signal, which can be influenced by other receptors. CD38, by modulating the TCR response through recruitment of adaptor molecules and/or downstream effectors, may play a role in CD4/CD8 lineage commitment. The final stage of thymic maturation involves negative selection and Treg differentiation. CD38 may help in directing developing thymocytes to either pathway. This effect can be associated with its ability to modulate TCR signalling.

CD38 may also participate in peripheral tolerance induction (Fig. 4). For instance, it may take part in decisions between tolerance and immunity, acting at the level of DC .It may also influence the kind of immune response developed. CD38 may promote $CD8^+$ DC differentiation, this way contributing to Th1 shifts. Th1 responses are protective in the context of lupus as they inhibit development of Th2-mediated immune responses. Finally, CD38 may promote Treg-cell survival, namely through competition with ART for NAD.

## Thymus

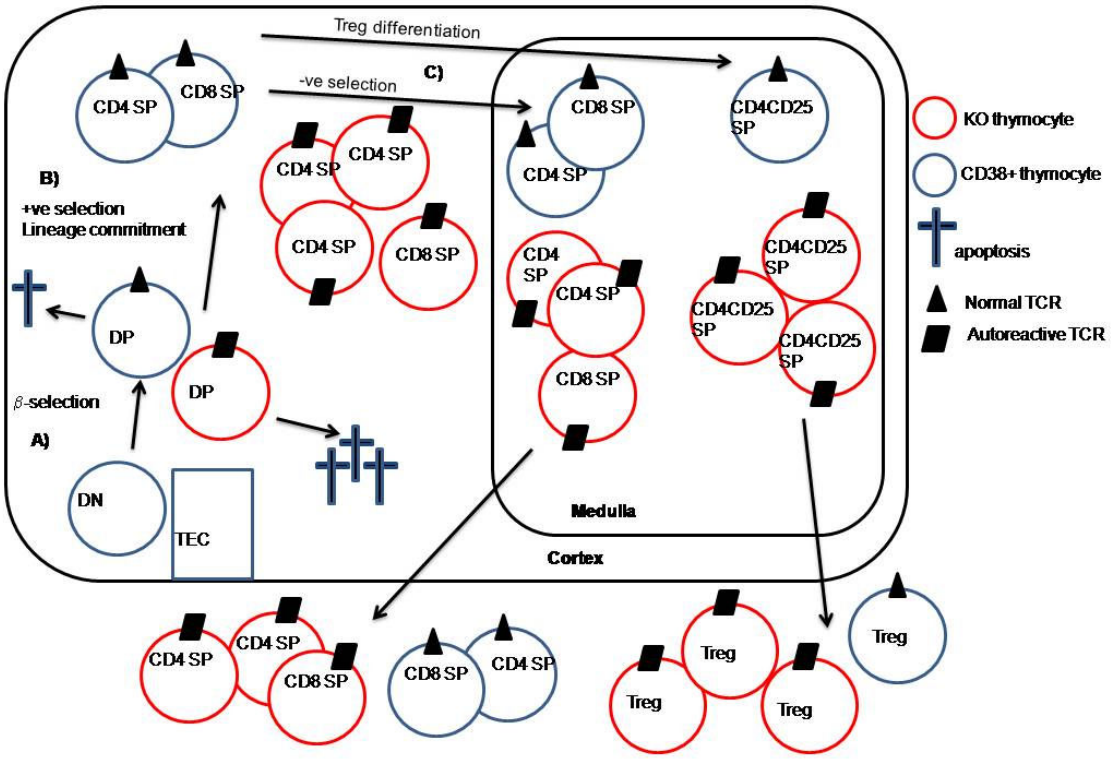



**Figure 3. Model for the role of CD38 in thymic differentiation of T cells.** CD38 may participate in thymic differentiation of T cells in several ways: A) CD38 is required for selection of DP thymocytes, namely at the level of TCR rearrangements and/or delivery of survival signals to these thymocytes, through modulation of TCR signalling; B) CD38 participates in CD4/CD8 lineage commitment, due to its ability to modulate the kinetics of the TCR response and the recruitment of downstream effectors; C) CD38 is responsible for directing SP thymocytes to negative selection or Treg differentiation; this effect is related to its ability to modulate TCR signalling. TEC, thymic epithelial cell; DN, double negative T cell; DP, double positive T cell; SP, single positive T cell; Treg, regulatory T cell.

## Periphery

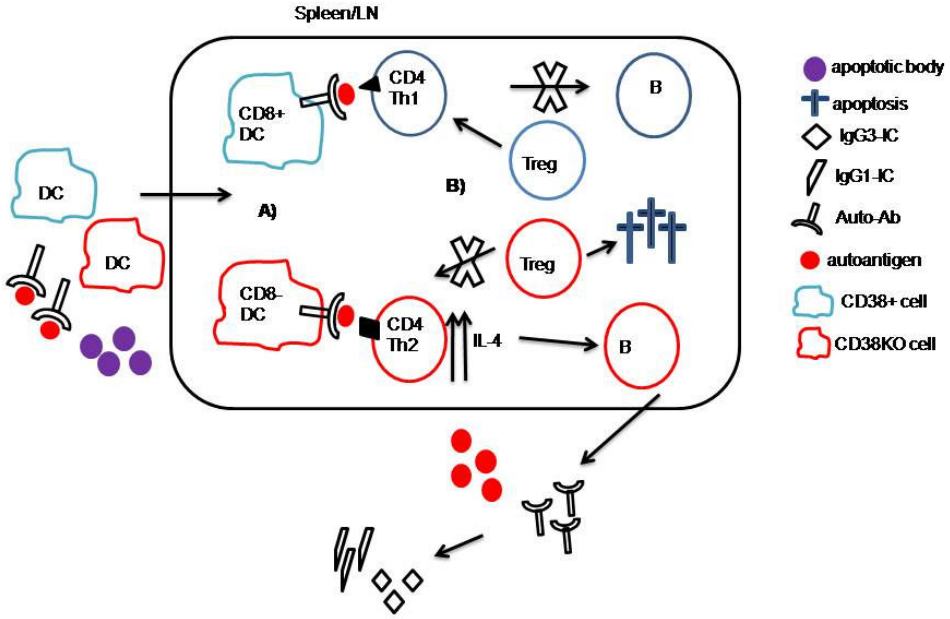



**Figure 4. Model for the role of CD38 in peripheral tolerance induction.** CD38 may participate in the tolerance induction in several ways: A) CD38 modulates DC differentiation into a $CD8^+$ phenotype. $CD8^+$ DC induce Th1 differentiation, which is protective in a lupus context; B) CD38 is required for survival of Treg cells. This feature is associated with competition of CD38 with ART for NAD.

## 4.3. References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# Chapter Five

# 5. Conclusions

From the data presented herein eight major conclusions can be drawn with regard to the role of CD38 in mycobacterial infections and murine lupus. With regard to the immune response against mycobacteria, CD38 seems to be required for:

1. full activation of mycobacteria-infected macrophages. This seems to depend on the role of CD38 in the induction of IFN$\gamma$ secretion by activated T cells.

2. recruitment of leukocytes to the site of infection. Reduced chemotaxis can result from decreased production of chemokines, such as MCP1/3 and RANTES, or to the action of CD38 as a selectin, through interaction with CD31.

3. development of Th1 immune responses and concomitant production of IFN$\gamma$. This may stem from a direct role of CD38 on T cells, stimulating IFN$\gamma$ production, or may constitute an indirect effect of CD38, which may act at the level of DC, promoting $CD8^+$ DC differentiation.

4. maintenance of the integrity of granulomata and the formation of a fibrotic rim around these structures. This may be related to the ability of CD38 to induce TNF$\alpha$ production by activated macrophages and T cells.

5. apoptosis of mycobacteria-infected macrophages within granulomata, which may be associated with TNF$\alpha$ and NO production.

Concerning murine lupus, our data suggest that CD38:

1. is associated with resistance to development of systemic autoimmunity.

2. may be involved in thymic differentiation of T cells, namely on $\beta$-selection, positive and negative selection and Treg differentiation.

3. may promote $CD8^+$ DC differentiation, which stimulate Th1 development. Th1 cells are then able to counteract differentiation and action of Th2 cells, which are the main mediators of lupus.

Altogether, our data suggest that CD38 plays a role in immune homeostasis. It may act as a regulator of immune responses, promoting Th1 responses, and may also monitor the extracellular microenvironment modulating tolerance/immunity decisions.

# Chapter Six

# 6. Abstracts

## 6.1 Abstract

CD38 belongs to a family of multifunctional ectoenzymes possessing extracellular and intracellular enzymatic. It metabolizes NAD to yield ADPR and cADPR, two calcium mobilizing metabolites. It may also behave as an adhesion molecule through interaction with CD31. CD38 seems to play a role in lymphocyte ontogeny, as its expression is successively switched on and off throughout lymphocyte development. CD38 has a pivotal role in the regulation of lymphocyte function. It physically associates with other cell surface receptors, such as TCR and BCR. CD38 ligation induces calcium mobilization, cell activation and proliferation, activation of transcription factors, such as NF-κB, cytokine secretion, namely Th1 cytokines, immunoglobulin class switching and modulation of apoptosis. In this work we aimed at studying the role of CD38 in immune function. We have focused our attention in two distinct situations: infections by intracellular mycobacteria and systemic lupus erythematosus.

The role of CD38 in infections by extracellular bacteria is already established. However, little is known with regard to infection by intracellular parasites. *Mycobacterium avium* and *M. tuberculosis* (Mtb) are facultative intracellular bacteria that mainly infect macrophages, guaranteeing their survival by circumventing macrophages microbicidal strategies. Effective immune responses to mycobacteria involve Th1 cells and production of high amounts of IFNγ, along with the development of granulomatous responses. Granulomata are highly organized structures, composed of macrophages and T lymphocytes, which have a central role in protection against mycobacteria. To investigate the role of CD38 in development of anti-mycobacterial immune responses, we have used two different models: intraperitoneal *M. avium* and aerosol Mtb infection. CD38KO and C57Bl.6 mice were infected and their immune responses compared. Our data suggest that CD38 is required for development of protective immunity against mycobacteria, as CD38KO mice were more susceptible to infection than C57Bl.6 mice. We have found that CD38 may impact the immune response against mycobacteria at several levels. We have observed that CD38 was necessary for leukocyte chemotaxis to the infection site. This effect was mediated through modulation of chemokine secretion, namely that of MCP1/3 and RANTES. In addition, it may involve the action of CD38 as a selectin. Our data have shown that CD38 was required for full activation of mycobacteria-infected macrophages and NO production. This effect appears to be associated with the ability of CD38 to induce IFNγ secretion by activated T cells. We provide evidence that CD38 promotes Th1 differentiation. This can result from modulation of DC differentiation, as we have observed that CD38 promotes development of $CD8^+$DC, which foster Th1 immune responses. We

have observed that CD38 is required for formation of productive, closed granulomata, possibly through its ability to induce Th1 cytokine secretion, namely that of TNF$\alpha$.

While the role of CD38 in organ-specific autoimmune diseases, such as type I diabetes, is starting to be unravelled, involvement of CD38 in systemic autoimmune diseases, such as SLE, is far from being understood. SLE is a disease of unknown ethiology that affects multiple organ systems. Lupus-like disease, similarly to SLE, is characterized by B-cell hyperactivity, production of autoantibodies, high levels of circulating immune complexes and complement and immune complex-mediated tissue damage. To investigate whether CD38 had any bearing in development of murine lupus, we have developed CD38KO-*lpr/lpr* mice. Disease progression was compared to that of C57Bl.6-*lpr/lpr* mice, a well established murine model of lupus. We have found that absence of CD38 accelerated and exacerbated disease development. Disease of CD38KO-*lpr/lpr* mice was similar to that of C57Bl.6-*lpr/lpr*, and characterised by a female bias, increased proteinuria, high titres of circulating immunoglobulins, anti-nuclear and anti-neutrophil cytoplasmic autoantibodies and development of immune complex-mediated glomerulonephritits. Our data suggested that the protective role of CD38 could be exerted at distinct levels. CD38 may impact thymic differentiation of T cells. Within the thymus, CD38 can play a role in $\beta$-selection, namely through its function as a receptor and/or an adhesion molecule. CD38 may also participate in CD4/CD8 lineage choice and at directing developing thymocytes to either negative selection or Treg differentiation. This effect may stem from the ability of CD38 to modulate TCR-mediated signals. Additionally, CD38 may impact the induction of peripheral tolerance. This feature may be associated with modulation of DC differentiation by CD38, namely through promotion of $CD8^+$DC differentiation. Finally, CD38 may be involved in survival of Treg cells, through competition with ART for NAD.

In the final section, a model for the role of CD38 in mycobacterial infections and murine lupus is proposed. In general, CD38 seems to act as a regulator of immune responses, directing development of Th1 responses. It may also monitor the extracellular microenvironment and influence tolerance/immunity decisions.

## 6.2 Resumo

O CD38 é uma ectoenzima multifuncional com actividade enzimática intra e extracelular, que resulta na conversão de NAD extracelular em dois mobilizadores de cálcio intracelular: o ADPR e o cADPR. Pode também actuar como molécula de adesão, por interacção com o CD31. Dado que a expressão de CD38 é estritamente controlada durante a linfopoiese, pensa-se que poderá estar envolvido neste processo. O CD38 encontra-se fisicamente associado a vários receptores membranares como é o caso do TCR e do BCR. A activação de linfócitos através do CD38 induz mobilização de cálcio intracelular, proliferação celular, activação de factores de transcrição, como o NF-κB, secreção de citoquinas, nomeadamente do tipo Th1, mudança de classe das imunoglobulinas e apoptose. Neste trabalho, fomos investigar o papel do CD38 na função imunológica. Focámos a nossa atenção em duas situações distintas: infecções por microrganismos intracelulares, como as micobactérias, e doenças de autoimunidade sistémica.

O papel do CD38 em infecções por parasitas extracelulares encontra-se já bem documentado. No entanto, ainda está por esclarecer o seu papel nas infecções por parasitas intracelulares. As micobactérias são parasitas intracelulares facultativos que infectam preferencialmente macrófagos e asseguram a sua sobrevivência evitando os mecanismos microbicidas destas células. A resposta imunológica anti-micobacteriana protectora é mediada por linfócitos Th1, com produção elevada de IFNγ, e envolve a formação de granulomas, estruturas organizadas compostas por macrófagos e linfócitos T. Para determinar se o CD38 teria alguma influência na resposta imunológica contra micobactérias, utilizámos dois modelos: infecção intraperitoneal com *M. avium* e por aerosol com *M. tuberculosis*. Os ratinhos CD38KO e C57Bl.6 foram infectados e a resposta imunológica desenvolvida foi comparada. Verificámos que os ratinhos CD38KO eram mais susceptíveis à infecção por micobactérias do que os ratinhos C57Bl.6, o que sugere que o CD38 é necessário para o desenvolvimento de uma resposta anti-micobacteriana. O CD38 pode participar nesta resposta a vários níveis. Observámos que o CD38 era necessário para a quimiotaxia de leucócitos para o local de infecção, o que estava relacionado com a produção de quimioquinas, como o MCP-1/3 e RANTES. Adicionalmente, este efeito poderá dever-se à acção do CD38 como selectina. Os nossos dados mostraram que o CD38 era necessário para a activação total dos macrófagos infectados e consequente produção de NO. Este facto parece estar associado à indução, pelo CD38, da produção de IFNγ por linfócitos T activados. De facto, demonstrámos que o CD38 promove o desenvolvimento de respostas Th1. Tal poderá resultar de o CD38

favorecer a diferenciação de DC $CD8^+$, que estimulam a polarização Th1. Observámos, ainda, que o CD38 era essencial para a formação de granulomas fechados, possivelmente através da sua capacidade para induzir a produção de citoquinas Th1, nomeadamente de TNF$\alpha$.

Embora o papel do CD38 em doenças de autoimunidade órgão-específicas, como a diabetes do tipo I, comece a ser elucidado; o mesmo não acontece com as doenças de autoimunidade sistémica, como o lúpus eritematoso sistémico (LES). A etiologia do lúpus murino, tal como do LES, ainda está por determinar. São doenças caracterizadas por hiperactividade dos linfócitos B, produção aumentada de autoanticorpos de múltiplas especificidades, níveis elevados de imunoglobulinas e complexos imunes em circulação e desenvolvimento de danos tecidulares mediado por complexos imunes. Neste trabalho fomos investigar o papel do CD38 no desenvolvimento de lúpus murino. Para tal, desenvolvemos os ratinhos CD38KO-*lpr/lpr*. Os ratinhos CD38KO-*lpr/lpr* foram monitorizados para o desenvolvimento de doença, tendo sido comparados com os ratinhos C57Bl.6-*lpr/lpr*, um modelo de lúpus murino já bem estudado. Verificámos que a ausência do CD38 acelerava e agravava o desenvolvimento de lúpus murino. A doença dos ratinhos CD38KO-*lpr/lpr* é semelhante à desenvolvida pelos murganhos C57Bl.6-*lpr/lpr*, sendo caracterizada por uma maior prevalência nas fêmeas, valores elevados de proteinúria, níveis elevados de imunoglobulinas e autoanticorpos em circulação e desenvolvimento de glomerulonefrite associado à deposição renal de complexos imunes. Os nossos dados sugerem que o CD38 poderá exercer um papel protector a vários níveis. O CD38 pode estar envolvido na diferenciação tímica de linfócitos T, nomeadamente no processo de selecção $\beta$, através da sua função de receptor e/ou de molécula de adesão. Pode também estar envolvido no compromisso dos linfócitos T com as linhagens CD4/CD8 e na orientação dos timócitos para o processo de selecção negativa ou para a diferenciação de linfócitos Treg. Este efeito pode estar relacionado com a capacidade de o CD38 controlar a cascata de transdução de sinal mediada pelo TCR. Adicionalmente, o CD38 poderá participar na indução da tolerância periférica, nomeadamente por modulação da diferenciação de $CD8^+$ DC. Por último, o CD38 poderá ser necessário para a sobrevivência de linfócitos Treg por competição com as ART pelo NAD.

Na secção final, é proposto um modelo para o papel do CD38 nas infecções por micobactérias e no lúpus murino. De um modo geral, o CD38 actuará como um regulador das respostas imunológicas, favorecendo o desenvolvimento de respostas do tipo Th1. Adicionalmente, o CD38 poderá monitorizar o microambiente extracelular e, deste modo, influenciar as decisões de tolerância/imunidade.

## 6.3 Résumé

CD38 est une ectoenzyme multifonctionnelle avec activité enzymatique intra et extracellulaire, qui réduit NAD à deux mobilisateurs de calcium intracellulaire: ADPR et cADPR. Elle peut aussi agir comme une molécule d'adhésion, par l'interaction avec CD31. L'expression de CD38 est strictement régulée pendant la différentiation des lymphocytes. Donc on a proposé qu'elle participe en ce procès. CD38 s'associe physiquement avec d'autres récepteurs membranaires, comme le TCR ou le BCR. L'activation de lymphocytes à travers la CD38 induit la mobilisation de calcium, la prolifération cellulaire, l'activation de facteurs de transcription (ex, NF-κB), la sécrétion de cytokines Th1, l'altération de classe de immunoglobulines et l'apoptosis. En ce travail nous avons cherché le rôle de CD38 dans la fonction immunologique. Nous avons consideré deux situations: infections par mycobactéries et maladies de autoimmunité systémique.

Le rôle de CD38 dans les infections par bactéries extracellulaires est déjà bien documenté. Cependant, son rôle dans les infections par parasites intracellulaires n'est pas encore clair. Les mycobactéries sont des parasites intracellulaires facultatifs qu'infectent plutôt les macrophages et assurent leur survie en s'esquivant aux mécanismes microbicides des macrophages. La réponse immunologique anti-mycobactérienne inclut les lymphocytes Th1 et la production de IFNγ, et la formation de granulomata, qui sont des structures composées par des macrophages et des lymphocytes T. Pour déterminer si CD38 était nécessaire pour la réponse immunologique contre les mycobactéries, nous avons utilisé deux modèles: infection intrapéritonéal avec *Mycobacterium avium* et infection par aérosol avec *M. tuberculosis*. Les souris CD38KO et C57Bl.6 ont été infectées et leur réponse immunologique comparée. On a vérifié que les souris CD38KO étaient plus susceptibles que les souris C57Bl.6 à l'infection par mycobactéries. Ces résultats ont suggéré que CD38 est nécessaire pour le développement d'une réponse anti-mycobactérienne. CD38 pourra participer en cette réponse à plusieurs niveaux. On a observé que CD38 était nécessaire pour la chemotaxis des leucocytes vers la place d'infection, ce que impliquait la modulation de la production de chemokines, comme MCP1/3 et RANTES. En addition, c'effet pourra être dû au rôle de CD38 comme selectin. Nos résultats ont démontré que CD38 était nécessaire pour l'activation total des macrophages et la production de NO. Ce fait peut être associé à l'induction, par CD38, de la production de IFNγ par des lymphocytes T activés. En effet, on a démontré que CD38 favorise le développement des réponses Th1. Cela peut résulter de la promotion de la différenciation de DC $CD8^+$ par CD38. Ces cellules favorisent la

polarisation Th1. On a aussi observé que CD38 était essentiel pour la formation des granulomata fermés, possiblement à travers sa capacité pour induire la production de cytokines Th1, en particulier TNF$\alpha$.

Même si l'étude du rôle de CD38 en maladies autoimmunes organe-spécifiques, comme la diabètes type I, à déjà commencé, les maladies autoimmunes systémiques, comme le lupus erytemathosus systémique (SLE), n'ont pas être étudiés. L'étiologie du lupus des souris, ainsi comme celle du SLE, est toujours inconnue. Ces maladies se caractérisent par l'hyperactivité des lymphocytes B, la production augmentée des autoanticorps, les niveaux sériques élevés d'immunoglobulines et des complexes immunes, et le développement des dommages tissulaires en résultat de la déposition des complexes immunes. Pour la recherche du rôle de CD38 dans le développement de lupus, nous avons développé les souris CD38KO-*lpr/lpr*. Ces souris ont été surveillées pour le développement de lupus et ont été comparées avec les souris C57Bl.6-*lpr/lpr*, un modèle bien connu de lupus des souris. Nous avons vérifié que l'absence de CD38 précipitait et exacerbait le développement de lupus. La maladie des souris CD38KO-*lpr/lpr* était pareille à celle des souris C57Bl.6-*lpr/lpr* et était caractérisée par une incidence plus élevée chez les femelles, des niveaux élevés de protéinurie et niveaux sériques augmentés d'immunoglobulines et d'autoanticorps et le développement de glomérulonéphrites en résultat de la déposition rénal des complexes immunes. Nos résultats suggèrent que CD38 peut avoir un rôle protecteur à des niveaux différents. CD38 peut être engagé en la différentiation thymique des lymphocytes T, notamment dans les procès de sélection $\beta$, par sa fonction de recepteur et/ou de molécule d'adhésion. CD38 peut aussi participer à l'engagement des lymphocytes T avec les lignages CD4/CD8 et à l'orientation des thymocytes vers le procès de sélection négative ou vers la différenciation des lymphocytes Treg. Cet effet peut être associé à la capacité de CD38 de contrôler la cascade de transduction de signal du TCR. En plus, CD38 pourra participer à la survie des lymphocytes Treg par compétition avec ART par NAD.

Dans la section finale, on propose un modèle pour le rôle de CD38 dans les infections mycobacteriénnes et dans le lupus des souris. En général, CD38 agit comme un régulateur des réponses immunologiques, en favorisant le développement des réponses Th1. En addition, CD38 peut surveiller la microambiance extracellulaire e, ainsi, influencer les décisions de tolérance/immunité.