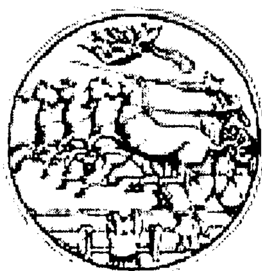
The Role of Regulatory CD4 T cells in Inflammation.

Thiago Lopes Carvalho



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Dissertação de candidatura ao grau de doutor
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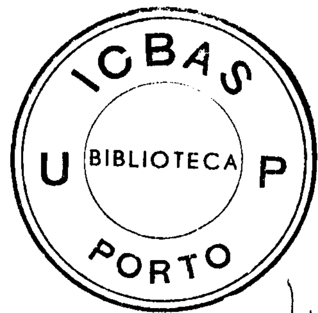

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To an absent friend,

Rodney E. Langman

Who probably would have disagreed with a lot of what follows if we had the chance to argue over a cold beer. This thesis is certainly much the poorer for it.

"Anybody can jump a motorcycle. The trouble begins when you try to land it."

Evel Knevel.

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Declaração

Os resultados do trabalho incluídos nesta Dissertação fazem parte dos artigos científicos indicados abaixo, alguns dos quais já publicados em revistas internacionais.
No cumprimento do disposto no n°2 do artigo 8° do Decreto Lei n°388/70, o autor desta Dissertação declara que participou na concepção e na execução do trabalho que esteve na base desses artigos, bem como na interpretação dos resultados e na redação dos respectivos artigos.

Trabalhos Publicados:

I. Hori, S., **T.L. Carvalho** and J. Demengeot. 2002. "CD25+ CD4+ regulatory T cells suppress CD4+ T cell-mediated pulmonary hyperinflammation driven by *Pneumocystis carinii* in immunodeficient mice." *European Journal of Immunology.* 32: 1282-1291.

II. Caramalho, I., **T. Lopes-Carvalho**, D. Ostler, S. Zelenay, M. Haury and J. Demengeot. 2003. "Regulatory T cells selectively express Toll-like receptors and are activated by lipopolysacharide." *Journal of Experimental Medicine.*

V. Coutinho, A., S. Hori, **T. Carvalho**, I. Caramalho and J. Demengeot. 2001."Regulatory T cells: the physiology of autoreactivity in dominant tolerance and 'quality control' of immune responses". *Immunological Reviews.* 182: 89-98.

Manuscritos Inéditos:

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IV. **Lopes-Carvalho, T.,** I. Caramalho and J. Demengeot. 2003. "CD4+CD25- T cells regulate the pool of CD4+CD25+ regulatory T cells". *Submitted.*

Abbreviation and Nomenclature

APC	Antigen Presenting Cell
BcR	B cell Receptor
CTLA4	Cytotoxic T Lymphocyte Antigen 4
DC	Dendritic Cell
DN	Double Negative thymocyte
DP	Double Positive thymocyte
EAE	Experimental Autoimmune Encephalomyelitis
HSP	Heat Shock Protein
IBD	Inflammatory Bowel Disease
IFN	Interferon
IL	Interleukin
IPD	Inflammatory Pulmonary Disease
LPS	Lipopolysacharide
MBP	Myelin Basic Protein
MHC	Major Histocompatibility Complex
NK	Natural Killer Cell
RAG	Recombination Activating Gene
SCID	Severe Combined Immunodeficiency
TCR	T cell receptor
TGFβ	Transforming Growth Factor β
TLR	Toll Like Receptor
Treg	Regulatory T cell (CD4+ CD25+)

Summary (English)

The Immune System efficiently protects the host from pathogens and parasites. These protective immune responses are however themselves destructive to the body and even in a properly functioning system, tissue damage always ensues. In addition, the Immune System may improperly target host antigens and lead to autoimmune diseases. It has long been known that a wide spectrum of these immune-pathological and autoimmune phenomena can be controlled or avoided by dominant regulatory mechanisms. A major recent advance has been the functional and phenotypic characterization of a population of central effectors of dominant tolerance, T cells that appear to be dedicated to the control of immune responses and avoidance of autoimmunity. These are CD4+ T cells that express the IL2Rα chain (CD25) constitutively, and are referred to as regulatory T cells (Tregs).

The present work seeks to understand how Tregs function in inflammatory conditions, and how inflammation and other lymphocytes affect the homeostasis of this population. We will argue that inflammation is the most important context for immune regulation, and that self/nonf-self discrimination must be particularly robust in this condition. We show that in inflammation induced by *Pneumocystis carinii* (an opportunist lung pathogen) or bacterial lipopolysacharide (LPS) injection in immunodeficient mice, Tregs control both the expansion of naive T cells and the extent of the inflammatory response itself. Furthermore, we show that inflammatory stimuli such as LPS promote Treg survival and proliferation, including a direct effect through innate immune system receptors expressed on Tregs. Finally, inflammation and naive cells are shown to contribute to the homeostasis of the Treg pool, indicating a regulatory role for the regulated population. The implications of these findings are discussed for induction and control of autoimmunity/immune pathology as well as in the evolution of the regulatory mechanisms of the adaptive immune system.

Summary (Portuguese)

O sistema imunológico protege eficientemente o organismo de patógenos e parasitas. No entanto, a própria resposta imunológica protectora causa danos aos tecidos do hospedeiro. Além destes danos colaterais fisiológicos, o sistema imune pode tomar como alvo da sua resposta antígenos do hospedeira, desecadeando as chamadas doenças autoimunes. Já é sabido há muito tempo que uma vasta gama destas patologias imunológicas podem ser controladas, ou evitadas, por mecanismos regulatórios dominantes. Um avanço recente importante foi a caracterização fenotípica e funcional de uma população de linfócitos T dedicada à regulação de respostas imunes e prevenção da autoimunidade. Estas células T são CD4+ e expressam constitutivamente a cadeia α do receptor da interleucina 2 na sua superfície, e foram batizadas como células T regulatórias (Tregs).

O presente trabalho busca compreender o funcionamento das Treg em condições inflamatórias, e como a inflamação afecta a homeostase desta população. Argumentaremos que a inflamação constitui o contexto mais importante para a imuno-regulação, e que os mecanismos que discriminan o próprio do não- próprio tem que ser particularmente robustos nesta condição. Demonstramos que na inflamação induzida pela infecção por *Pneumocystis carinii* (um patógeno oportunista do pulmão) ou pela injeção de lipopolisacarideo bacteriano em murganhos imunodeficientes as Tregs controlam a expansão das células T efectoras, bem como a resposta inflamatória em si. Além disto, as células T regulatórias respondem diretamente a compostos inflamatórios, expressando receptores específicos para tal, e podem interagir directamente com o sistema de imunidade inata.

Summary (French)

Le système immunitaire protège efficacement l'organisme des pathogènes et des parasites. Cependant la réponses immunitaires s'associe toujours d'un certain degrée d'endommagement des tissus. Ainsi, même lorsque le système est sain et fonctionnel, son activité est potentiellement destructive pour l'hôte. De plus, l'activité du système immunitaire peut cibler des antigènes de l'hôte ce qui conduit au développement de maladies autoimmunes. Il est établi depuis longtemps que la plus part de ces phénomènes immuno-pathologiques et auto-immuns peuvent être controlés ou évités par des mécanismes de régulation dominante. Une avancée majeure dans ce domaine est contenue dans la récente caractérisation phénotypique et functionnelle d'une population de lymphocytes T effecteurs de la tolérance dominante et responsable du contrôle des réponses immunes et auto-immunes. Ces cellules T CD4+ expriment de façon constitutive la chaîne alpha du récepteur à l'IL-2 (CD25) et sont dénommées cellules T regulatrices (Tregs).

Ce mémoire a pour ambition d'élucider le fonctionnement des Tregs en situation inflammatoires, et d'établir le rôle de l'inflammation et des autres lymphocytes sur l'homéostasie de cette population cellulaire. Nous défendons que l'inflammation crée le context e le plus important pour la régulation immunitaire, et que la discrimination soi/non-soi doit être praticulièrement robuste dans cette condition. Nous montrons qu'en situation d'inflammation induite par *Pneumocystis Carinii* (un pathogène opportuniste infectant les poumons) ou par injection de lipopolysaccharide (LPS) bactérien chez des animaux immuno-déficients, les Tregs limitent la réponse inflammatoire et contrôlent l'expansion des cellules T naives. Plus encore, nous révèlons qu'un stimulus inflammatoire tel que celui délivré par le LPS augmente la survie des Tregs et induit leur prolifération. Ces effets résultent d'un mécanisme incluant l'interaction directe entre le LPS et les Tregs par un « Toll like receptor », une molécules associée au système immunitaire inné, que nous trouvons exprimée de façon spécifique par les Tregs. Enfin, nous démontrons que les cellules T naives conditionnent aussi la survie et l'expansion des cellules Treg dans l'organisme, révèlant le rôle régulateur des cellules soumises à une régulation immune.

Nous discutons alors les conséquences de ces découvertes pour notre compréhension des mécanismes d'induction ou de contrôle des pathologies immunes et auto-immunes. Finalement nous intègrons ces nouvelles données

pour analyser l'évolution biologique des mécanismes de régulation au sein du système immunitaire adaptatif.

Introduction

1.1 The Immune System

Pathogens and parasites vastly outnumber free living organisms, and they exert an enormous pressure on hosts to defend themselves. All multi-cellular organisms possess some form of a defensive system (as do most single cell organisms), the overwhelming majority of which fall under the category of what is known as innate immunity. The innate immune system employs an efficient set of recognition and effector mechanisms to eliminate or control infections. This system utilizes conventional germ line encoded receptors to recognize either conserved pathogen related structures or markers of tissue damage/stress (see 1.2 below). Although most metazoan phyla display only innate immunity, jawed vertebrates have evolved a completely new recognition system, where specificities are somatically selected, differing in each individual, and even in different cellular clones within the same individual. By transferring genetic variation and selection from evolutionary to somatic time, the vertebrate immune system gives the host an extra edge when competing with pathogens evolving at much faster rates. This is referred to as the adaptive immune system and is embodied in a subset of white blood cells, the lymphocytes, their secreted products and the lymphoid organs.

Historically, adaptive immunity was first recognized in the light of the birth of clinical microbiology and the germ theory of disease, pioneered by the work of Louis Pasteur and Robert Koch. The idea that many diseases were caused by specific microbiological agents was soon linked to the phenomenon of immunity, i.e., the observation already present in ancient texts that individuals were often resistant to diseases they had previously suffered from. Pasteur made this link explicit when he showed that inoculating fowl with attenuated cholera pathogens led to specific resistance, in effect demonstrating the specific microbiological basis of immunity. This was soon confirmed by other observations that resistance to disease correlated well with elimination of associated microorganisms, and specific resistance to re-infection. The physiological means of acquired resistance however eluded Pasteur who proposed the interesting but erroneous nutrient theory of acquired immunity. A biochemical basis for this phenomenon was provided by the discovery of serum factors that were present in previously infected individuals that bound to the inoculated microorganism, but not to others. The protection conferred by

host soluble factors was dramatic in the early case studies of von Behring and Kitasato with diphteria and tetanus (where we now know that neutralization of potent toxins was crucial to survival of infection). Soon after this point, the diversity of specificities of the immune system began to be recognized and the experiments of Paul Ehrlich and others showed that the potential to specifically recognize foreign structures was large and not restricted to infectious agents. Even at this initial stage in the history of immunology, Ehrlich was aware that this diversity of specific recognition coupled to efficient elimination mechanisms posed a potential threat to the host – a concept he referred to as *horror autotoxicus*. The notion of avoiding forbidden specificities, and later forbidden clones, which he originated dominated thinking on immunological tolerance for a century. In parallel, sequestration or inavailability of self antigens were also proposed to avoid self reactivity, in particular during the years before the elucidation of the genetic basis of antibody diversity, when it was thought by many that antigen directed antibody specificity, by for example folding nascent polypeptide chains to fit itself. As knowledge on antibody specificity grew self-reactivity became an ever more pressing concern – particularly when Karl Landsteiner's experiments immunizing with entirely synthetic chemical compounds indicated that immune recognition seemed to be somehow unlimited. The second problem arising from this vast diversity was of, course, where did it come from (see 1.3 below).

These observations (among many others) were integrated to construct the dominant paradigm in immunology, the Clonal Selection Theory. In 1957, MacFarlane Burnet took Niels Jerne's proposal that antibodies were generated randomly with regard to specificity and that immunizations selected from a pre-existing pool and gave it a cellular basis by proposing that the immune system distributed these specifities clonally (leading Jerne to proclaim that "I hit the nail, but Burnet. hit it on the head"). This has been extensively borne out by the subsequent progress of cellular and molecular immunology. However, as a corollary of the Clonal Selection Theory, Burnett also proposed a mechanism for the avoidance of *horror autotoxicus*: that the cells of the immune system would be formed only in early ontogeny, with all cells that recognized self (axiomatically defined as what is present in the embryo) being eliminated. Although this model was initially supported by the experimental work of Owen, Medawar, Billingham and Brent showing that Tolerance does indeed have an ontogenic component, it was

ultimately shown to be untenable for the simple fact that lymphocytes develop throughout life. But the idea of a deletional Tolerance was not easily abandoned, and in 1959 Joshua Lederberg gave it the form it still bears today. Simply put, he transferred Burnet's tolerance mechanism from development of the organism to development of the cell, and proposed that when developing lymphocytes encountered antigen they would be deleted. Lederberg's solution imposes certain constraints in order to achieve effective tolerance. All relevant self antigens must be constantly expressed from the beginning of ontogeny- an antigen arising today will not delete cells that matured yesterday and developing immunocytes must be exposed to all antigens during their own development, as mature cells will be responsive and not deleted.

Ultimately, just as continuous lymphocyte development falsified Burnet's tolerance model, deletional mechanisms as a whole, although they clearly operate, can not be the only basis for tolerance simply because autoreactive T and B cells have been shown to exist in healthy hosts. As this became clearer, a series of peripheral recessive tolerance mechanisms were proposed, such as clonal anergy, exhaustion, ignorance or the old idea of antigen sequestration. The central fact remains however, that in the periphery of a normal, healthy individual there are lymphocytes capable of driving lethal autoimmune reactions. Recent popular theories on the functioning of the immune system such as Janeways's innate activation driven or Matzinger's "danger" model both assume that central (deletional) tolerance operated in the manner suggested by Lederberg, and thus add nothing to the solution of self/non-self discrimination problem. Other than dominant tolerance models, Bretscher and Cohn's associative recognition (two signal) model (later modified by Langman and Cohn) present a conceptual alternative to Lederberg's proposal. This thesis deals with one solution to this problem: the harnessing of self-reactivity to promote a dominant tolerance mechanism effected by regulatory T cells.

1.2 The Innate Immune System

While the adaptive immune system was first examined in the light of medical and biochemical schools of thought, the conceptual foundation of innate immunity was set down in a biological and evolutionary mold, primarily through the work of Elie Metchnikoff. In his studies of

phagocytosis, Metchnikoff linked this primordial defense strategy to fundamental physiological processes of digestion and ontogenesis. It is a tribute to his foresight that the renewed interest in innate immunity owes much of its impetus to studies that began with a focus on arthropod development.

The innate immune system is an evolutionarily ancient solution to defense and homeostasis of the host and employs essentially three basic strategies: (i) the recognition of damaged or altered self; (ii) recognition of the absence of self and (iii) recognition of conserved non-self. All of these strategies are present in various invertebrate and the vertebrate immune systems.

The recognition of damaged or altered self detects markers of tissue destruction or stress. The list can go from activation of plant defense mechanisms (oxidative bust or gall formation) by mechanical injury and cell wall damage (Yahraus et al., 1995) to the activation of inflammation in vertebrates by injury (Mannick et al., 2001) extra-cellular matrix degradation products (of which more below), for example. This strategy is wholly dependent on host molecules and thus has the advantage of being a difficult target for the evolution of pathogen evasion strategies. Among the disadvantages is its potentially self perpetuating nature, as inflammatory responses will inevitably produce more tissue damage, and an associated high potential for self directed responses in the absence of any real infectious threat to the host. The mechanistic basis of these processes is evolutionarily linked to tissue re-modeling and repair pathways.

The families of inhibitory natural killer cell receptors bearing ITIM motifs in vertebrates exemplify the recognition of the absence of self. Their discovery derived from hybrid resistance, the phenomenon whereby F1 mice reject parental cells, which is due to lack of recognition of MHC class I molecules by natural killer cells. This mechanism makes sense in the light of pathogen, especially viral, interference with antigen presentation as an immune evasion strategy (also commonly employed by tumors) (Karre, 2002). Of course, in this rationale for an innate strategy, adaptive immunity (and its breakdown) is implicit. A more likely origin for this system is suggested by the fact that homologs of NK receptors are found on colonial proto-chordates (Khalturin et al., 2003). In this context the receptors are used to maintain individuality, i.e., to avoid fusion of colonies with distinct genotypes through potent cytolytic

reactions at interface zones. Lack of self recognition is also a trigger for soluble factors, as in the case of spontaneous activation of complement C3 molecules on cell surfaces, unless turned off by host molecules such as CD46 and CD55 (Austen and Fearon, 1979). The obvious fault with this strategy is that pathogens may convergently evolve or even directly acquire from the host the "off-switch" molecules and neutralize host defense.

The third strategy, recognition of nonself, is currently the topic of intense research due to the characterization of a central pathway in its induction in both vertebrates and invertebrates. The principle of this strategy is to target invariant structures vital to pathogens and not produced by the host. Targets are often the product of complex metabolic pathways, such as lipopolysacharides or defining traits for certain groups, such as double stranded RNA molecules associated with retroviruses. That animals possessed receptors for these type of substances (mitogens) distinct from classical lymphocyte receptors (see below), and the central role of this mode of recognition in inducing an immune response was first proposed as the "one non-specific signal model" of lymphocyte activation by Coutinho and Moller (Coutinho and Moller, 1974a; Coutinho and Moller, 1974b; Coutinho and Moller, 1975). Janeway then expanded this model to include advances on co-stimulation and antigen presentation, among others. In his elegant and influential article(Janeway, 1989), he called the conserved foreign immune-activators Pathogen Associated Molecular Patterns (PAMPs), and their receptors Pattern Recognition Receptors (PRRs). It would seem a fundamental weakness of this system that PRRs would create an enormous selection pressure favoring the emergence of PAMP escape variants. That this does not occur although most metazoans seem to target many of the same pathogen molecules, frequently even using the same class of receptors clearly shows that modifying PAMPs has a cost that outweighs the benefit of evading host defenses. Of course the strategy is not bullet proof, and there are instances of either masking PAMPs, as in the case of bacteria that coat themselves with host glycans, or simply interfering with downstream signaling pathways and effector mechanisms. A fundamental limitation of this strategy is its reliance of a marker of "foreignness". While bacteria and RNA viruses, for example, may present a smorgasbord of attractive targets due to their highly divergent metabolic pathways, as we shift to pathogens phylogenetically closer to the host, such as eukaryotic protozoa and helminthes this becomes

progressively more difficult. Another problem with PAMPs pointed out by Medzhitov (Medzhitov, 2001) is that they do not allow for distinction between pathogenic and commensal or innocuous organisms in the same class (PAMP is thus a misnomer). PAMPs are not virulence factors. LPS from *Salmonella* and *E.coli* will both trigger the same PRR for example. This is a crucial point for the topic of this thesis, as uncontrolled responses to the resident intestinal and pulmonary flora are triggers for many immuno-pathological syndromes.

Our understanding of the nature of PRRs has progressed enormously over the last decade. This is mainly due to work on the Toll Receptor family of genes. The original Toll receptor was identified screening fly mutants as a master regulator of dorsal-ventral fate patterning in *Drosophila* (Hashimoto et al., 1988), where it responds to a maternally generated gradient of the *spatzle* gene product. This was revealed to be a cysteine knot molecule produced in an active form at the end of a proteolytic cascade . Analysis of the Toll receptor itself showed a leucine rich repeat (LRR) extracellular domain, and an intracellular domain homologous to that of the interleukin-1 receptor (TIR), a key player in vertebrate inflammatory responses. Furthermore, the signaling pathway used by the *Drosophila* Toll receptor was homologous to the NF-κB pathway used in vertebrates not just in IL1 receptor signaling, but by a wide variety of immune response and inflammatory associated receptor systems. LeMaitre, Hoffman and collaborators then showed that the Toll receptor in flies not only operated in development, it also controlled the induction of anti-fungal immune responses (Lemaitre et al., 1996). One intriguing aspect of their work that is often overlooked is that *Drosophila* Toll is, by definition, not a PRR, as its activating ligand in the anti-fungal response is not a PAMP but the same spatzle molecule that works in development – the true PRR must be somewhere upstream of the proteolytic cascade that generates active spatzle.

Subsequently to the discovery of the Toll receptor's role in *Drosophila* immunity, other Toll homologs were cloned and shown to activate the fly's immune response to different pathogens. While activation of Toll leads to the production of the anti-fungal peptide drosomycin, triggering of 18wheeler, a Toll Like Receptors (TLR), leads to the secretion of anti-microbial peptides (Williams et al., 1997). This suggests that the TLRs were either a family of PRRs themselves or were intermediates in PRR recognition. In addition, it points to role beyond

recognition, an instructive role in determining the appropriate class of response to distinct classes of pathogens.

TLR genes were identified in the genome of both mouse and man (and even in plants), and although no developmental role for them has been described yet, they are central mediators of PAMP recognition (Fig1).

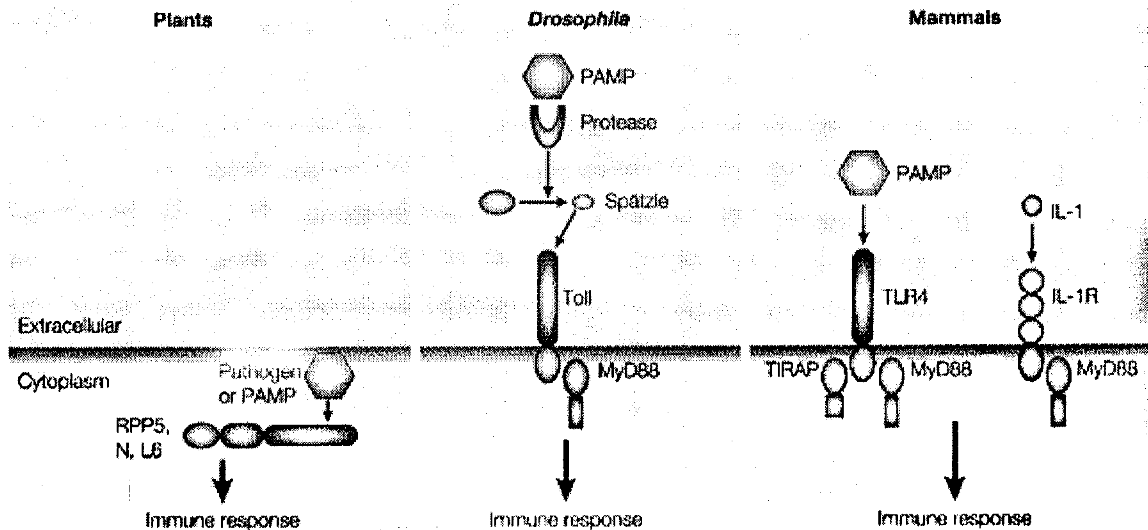

Figure 1. Toll receptor family molecules are mediators of innate immunity in many distinct phyla. (From Medzhitov, 2002)

The first characterized mammalian toll was human TLR4, which was shown to mediate expression of costimulatory molecules on APCs (Medzhitov et al., 1997), although its ligand was unknown. Subsequently it was shown in the mouse that mutations in the TLR4 gene were responsible for LPS unresponsiveness in two natural mutant strains (Poltorak et al., 1998; Qureshi et al., 1999). At present, 10 murine Toll genes have been identified. Ligands have been found for many of them, frequently (but not always) molecules that mark clear "foreigness", such as LPS, lipoteichic acid, double stranded RNA or umethylated CpG DNA motifs (reviewed in (Akira, 2003)). The picture is however turning out to be more complex than a simple TLR molecule per pathogen motif correlation, as at least some TLRs have been shown to interact in the formation of heterodimers with distinct recognition properties. Molecules which bind to stereotypical non-self and promote elimination

had long been known (e.g. CD14, LPS binding protein, Mannose-binding protein). The Toll receptor family's importance derives from its ability to not only mediate innate immunity, but as initially demonstrated by Medzhitov and Janeway, also to bridge innate and adaptive immune recognition by signaling the upregulation of costimulatory molecules. In line with this, the induction of inflammatory TH1 type responses is severely hampered in mice with a general deficiency for TLR signaling because of disruption of the MyD88 gene (Schnare et al., 2001) that encodes for and adaptor protein linking TLR engagement to the NF-κB pathway. Induction of TH2 responses was still efficient in these mice (indeed they seem biased towards them), but a role for other Toll signaling pathways (mainly the MAP kinase pathway) have not been discarded.

It should be noted however that if conceptually the above recognition strategies are distinct, in practice the situation is more complicated. Mammalian TLRs that have been shown to mediate recognition of foreign markers have also been implicated in the recognition of self-molecules (as for the prototypic Toll in *Drosophila)*. Furthermore, the self-molecules implicated are all markers of cellular damage or stress, such as Heat Shock Proteins (HSPs) or products of the degradation of the extracellular matrix (Asea et al., 2002; Ohashi et al., 2000; Vabulas et al., 2002a; Vabulas et al., 2002b; Vabulas et al., 2002c). The same is also true of several other molecules that double as receptors for foreign substances (PRRs) as well as those produced by stressed or damaged host cells ('altered' self molecules) (recently reviewed in (Gordon, 2002)).

1.3. Lymphocytes, antigen receptors and acquired immunity

The adaptive immune system is essentially embodied in the lymphocytes and in the clonal distribution of their receptors. It represents a new mode of recognition and is confined to this cellular subset, whereas innate responses are much more diffuse, and one can see not only lymphocytes or macrophage responding to LPS, for example, but also cells as distinct as fibroblasts- and tissue damage responses are necessarily even broader. When considering the selection pressures at the origin of adaptive immunity we should bear in mind that the host was not forced to choose between innate and adaptive immunity, but whether or not adaptive immunity conferred an advantage over innate immunity

alone. Second, the selection pressure(s) must relate essentially to recognition, as no truly novel effector mechanisms evolved with the appearance of adaptive immunity- rather what is seen is a modification of existing mechanisms to be activated by adaptive recognition, as in antibody complement fixation or opsonisation, for example. Finally, the fact that the somatic generation of receptors brings an emergent danger in each individual that there will arise potentially lethal self reactive clones (a danger not significant in innate immunity), coupled to the threat posed by the genetic mechanism for creating this diversity itself necessitate a high selective advantage for adaptive immunity to offset these costs. Qualitatively, the adaptive system allows for the new phenomenon of memory, i.e., it allows the host to respond faster, more efficiently and with higher specificity to previously encountered pathogens. At the population level the adaptive immune system in each individual will recognize different targets on the pathogen, which forces the pathogen to readapt to each host's immune system. Thus, adaptive immunity, even at a glance, presents clear advantages both for individuals and for populations.

50 years of immunochemistry in the first half of the 20th century exposed the diversity of antibody specificities. Once this diversity was evident, the problem how it originated became central to immunological research. At the cellular level, Burnet had stated that the lymphocyte "is the only possible candidate for the responsive cell of the clonal selection theory", i.e., the antibody producing cell. This was soon supported by the experimental work of Gowans and collaborators, showing that small lymphocytes did indeed carry immunological function. Remarkably, Murphy had come tantalizingly close to this discovery almost 40 years before (Silverstein, 2001). Work on antibody production in chickens had demonstrated that the bursa of Fabricius, an organ somewhat analogous to the bone marrow in birds, was responsible for this activity (or cells derived from it). Miller, however, described an essential role for the thymus in generating immunocompetent cells. Miller himself later integrated these seemingly conflicting results by showing that thymus and bone marrow derived cells cooperated to produce the classical antibody response. The resulting division of lymphocytes into T (thymus derived) and B (bone marrow or bursa derived) cells became a cornerstone of modern immunology (Miller, 1999).

While identifying conclusively the cell populations that carried adaptive immune function was vital, the core of Burnet's theory, the

clonal distribution of specificities had to be confirmed. Thus, Nossal, Lederberg and Jerne among others produced data validating the clonal distribution of antibody specificities, showing that each clone produced one antibody, and, crucially, that clones bearing antibodies against specific antigens -although rare- were present before immunization, and expanded in response to cognate antigen. Still the problem remained as to how to generate the diversity of recognition. Early instructive theories of antibody formation postulated a role for antigen in molding antibodies to fit itself, notably proposed by Linus Pauling. Biochemical research in the 1960s showed that diversity in specificity corresponded to amino acid sequence diversity at the N-terminal of immunoglobulins. The central dogma of molecular biology was by then established, and most people accepted that and that amino acid diversity in turn must correspond to genetic diversity. Thus the search switched to genetic mechanisms and various models of accelerated mutation, rearrangement or crossing-over in lymphocyte development were put forward in the 1960s and 70s. The matter was definitively put to rest by the elegant experiments of Susumu Tonegawa and collaborators, showing that antibodies were encoded by gene segments that were separate in the germline and rearranged in lymphocytes (initially shown in myeloma clones) to form a functional gene (Tonegawa, 1983). Subsequently it was demonstrated that in addition to the diversity generated by the combinatorial assortment of gene segments there was an additional, potentially larger level of diversity. This was provided by what is known as junctional diversity: nucleotides in the region where gene segments were joined were both lost through exonuclease activity and added (randomly by the enzyme TdT or from a template in P nucleotide addition). Importantly both combinatorial and junctional diversity are ontogenically regulated.

The diversity produced by somatic rearrangement of antigen receptor gene segments coupled to allelic exclusion provides the genetic basis for the Clonal Selection paradigm. It should be noted, however, that allelic exclusion is not absolute, and varies from high stringency at the T-cell Receptor (TcR) β and Immunoglobulin Heavy chain loci to apparently non-existent at the TCR α locus. The functional relevance of double expressors is currently the subject of debate – but it is clear that their frequency is low enough to allow them to be considered exceptions to the rule. Additionally, antigen receptors display degenerate recognition (which was in great part responsible for reports challenging the one lymphocyte clone, one antibody paradigm when it was being established,

specially when peritoneal cells were used), a property we shall return to soon.

These clonally distributed receptors come in to basic types, which in turn define the two distinct types of lymphocytes. Antibodies are easily detected and isolated from the serum, and were known long before B-lymphocytes were identified. They exist in both cell bound form, in which case they are referred to as the B-cell Receptor (BcR), or as secreted proteins (antibodies or immunoglobulins). Immunoglobulins recognize the three dimensional structures of native antigens. Conversely, the T cell receptor was only discovered more than 20 years after the thymus was shown to produce a specific set of immunocytes. The TCR exists only in membrane bound form (we now know) and its isolation and identification proved an intractable problem using cellular immunology and biochemical techniques. Only through the use of molecular biology in subtraction cloning experiments was it identified in Mark Davis' laboratory (Hedrick et al., 1984). Initially characterized as heterodimer of α and β chains, it was later shown to also exist as a distinct γδ heterodimer. Recognition by γδT cells, as well as their function, is still the subject of intense debate, and the rest of this Thesis will focus on αβ T cells.

The αβ T cell Receptor recognizes short linear peptides that are processed and presented on the surface of other cells by Major Histocompatibility Complex (MHC) molecules. As a general rule (to which there are exceptions), polypeptides captured from the extracellular medium are subjected to proteolysis and assorted peptides are then loaded onto MHC class II (MHCII) molecules for presentation to T cells expressing the CD4 co-receptor. Conversely, polypeptides produced intracellularly are processed and presented on MHC class I (MHCI) to CD8+ T cells. MHCI is present at the surface of most nucleated cells in the body, allowing CD8 cells to detect intracellular pathogens or abnormal self-proteins. Activated antigen specific CD8 cells will provoke lysis of the presenting cell- and thus CD8 T cells are referred to as cytotoxic or killer T cells. In addition, they may also secrete pro-inflammatory cytokines such as interferon γ(IFNγ) and IL2. MHCII is expressed mainly on specialized antigen presenting cells (e.g. macrophage and dentric cells) and present self and non-self peptides that are captured by pinocytosis or through phagocytosis of pathogens or apoptotic/necrotic host cells. Activation of CD4 T cells by cognate antigen can lead to, depending on the context, secretion of cytokines that

enhance inflammation, CD8 cell function antibody secretion or down-modulate immune responses. CD4 cells are thus termed Helper T cells, and also operate through cell contact dependent mechanisms, as exemplified by the CD40-CD40L system in the interaction with B lymphocytes.

1.4. Repertoire Selection

The generation of T and B cell receptors by random genetic mechanisms creates a need to organize this diversity, in particular to deal with the danger posed by anti-self specificities. T cells undergo development and selection in the thymus and once they have emigrated from this organ to the periphery, their specificities are fixed, so thymic development limits the peripheral repertoire. B cells on the other hand are subject to repertoire selection in bone marrow development (and here the rules are less clear than for T cell selection) but they may also alter their repertoire in the periphery, through somatic hypermutation. Generation of high affinity somatic mutants is apparently at the origin of many pathogenic auto-antibodies, and this process is strictly T cell dependent. Thus the T cell repertoire (the CD4 repertoire in particular) selection is a crucial step in natural tolerance, and a breakdown of Helper T cell repertoire selection may lead to high affinity pathogenic anti-self antibodies by disrupting the proper regulation of clonal selection during somatic hypermutation and proliferation of B cells in the germinal center.

T cells are produced from precursors that leave the bone marrow and colonize the thymus gland. Although sites of B cell differentiation and mechanisms of antibody formation vary widely between different species, even within mammals, T cell receptor genes have the same basic organization in all jawed vertebrates examined to date. Similarly, the gnathossome group phylogenetically most distant from mammals, cartilaginous fish, have a recognizable thymus gland (Litman, 1996). In evolutionary terms, T cell development seems to be a problem with only one solution. This is in great part due to the fact that, unlike B cells that recognize free or cell bound antigen directly, T cells must see small linear peptides processed and presented on the surface of other cells. The molecules that present peptides, major histocompatibility complex class (MHC) I and II proteins are among the most polymorphic genes known and the molecule that recognizes the MHC-peptide complex, the T cell

receptor (TCR) is made through somatic rearrangement. Thus, T cell development requires a selection step to ensure that nascent T cells are capable of productive interaction with the host MHC. This explanation is consistent with the fact that some T cell subsets that have limited diversity and interact with non-polymorphic MHC genes may have an extrathymic origin. Non-recognition of self MHC is not the only problem faced in T cell development: too high affinity for self MHC-peptide complexes could lead to deleterious responses against the host, as well as potentially making the induction of immune responses against non-self harder.

The cellular and molecular biology of T cell development have been the focus of intense research over the last thirty years and can be described in great (excruciating) detail. The earliest stage has been the most elusive, and the identification of T cell precursors in the bone marrow has only recently been related. These B220+CD19-c-Kit^{low} cells are a small population in the normal bone marrow that are, apparently the missing link between bone marrow common lymphoid precursor (CLP) cells and early thymocytes. At this stage, ligation of the Notch-1 receptor on these precursors either in the thymus or in the bone marrow provokes commitment to the T cell lineage (Pui et al., 1999). Several selection steps and at least 2 separate lineage commitment decisions must then take place (it should be noted however that by far the most common fate for a thymocyte is apoptosis, as most will fail at one of these steps). Thymic development of T cells is divided in three major phenotypic stages: cells that do not express either the CD4 or CD8 co-receptor molecules on their surface are termed double negatives and represent the earliest fraction; cells that express both are the next fraction, and are termed double positive and the last stage are cells expressing only one or the other (CD4 or CD8) associated to a functionally rearranged T cell Receptor, and are termed single positives.

Double negative (DN) T cells represent about 5% of murine thymocytes. Two key events will take place at this stage. DN cells rearrange TcR γ, $\delta$and βgenes. $\gamma\delta$T cell development remains mainly obscure and will not be dealt with further here. Suffice it to say that this is the first lineage commitment decision made, with a small fraction of T cells committing to the $\gamma\delta$lineage. What determines $\alpha\beta$ versus $\gamma\delta$ commitment is not known at present. For the cells committing to the $\alpha\beta$lineage, the DN stage comprises a selection step for functional βrearrangement. DN cells express an invariant α-like molecule, termed

the pre-T α chain. The recently formed β chain must be able to pair with this the pre-T α chain and assemble the pre-T cell Receptor, which is thought to signal spontaneously (*i.e* in a ligand independent fashion) leading to expansion and survival (termed β selection). DN cell will then initiate α rearrangement and mature to the subsequent, double positive (DP) stage. DP cells will then make the additional lineage decision, committing either to the CD4 or CD8 lineage. Here there is good evidence for an instructive role of the TcR in this decision, as TcR transgenes cloned from CD4 or CD8 cells overwhelmingly give rise to single positive cells with the same phenotype (Kisielow et al., 1988). Conversely, MHC molecules in the thymus will also play a key role, as MHCI-/- mice are essentially devoid of CD8 single positive thymocytes, and MHC-/-II have almost no CD4 single positive thymocytes (Von Boehmer et al., 2003). The two key events of repertoire selection take place at this stage: positive and negative selection. Although they are hard to dissociate temporally in terms of independent developmental stages within the DP subset, and may even happen more or less simultaneously, they are clearly separated in space. It has long been know that restriction to host MHC is dependent on the radio-resistant, epithelial, component of the thymus. This is now understood to be the result of positive selection, i.e., thymocytes that recognize self-peptides presented by host cortical epithelial cells with the appropriate avidity survive and proliferate. DP cells that fail to do so die (this is termed "death by neglect"). Hematopoetic (radio-sensitive) cells in the cortico-medullary junction region of the thymus are the main antigen presenting cells in negative selection (although this function may also be exerted by medullary epithelium cells), where high avidity interactions with developing T cells through their TcR leads to death by apoptosis (Starr et al., 2003). Thus as we follow the DP thymocyte in figure 2, it will undergo positive selection mainly in the cortex, and then undergo negative selection in the next zone it encounters. The net result of this is that cells that have functionally rearranged TcRs that can recognize self-MHC peptides and do not have an overly high avidity for these same complexes are prepared to leave the thymus as recent, single positive, thymic emigrants. Sadly, after all this effort, only a very small minority of them will be incorporated into the peripheral T cell pool- and the rules of recruitment here are far from clear.

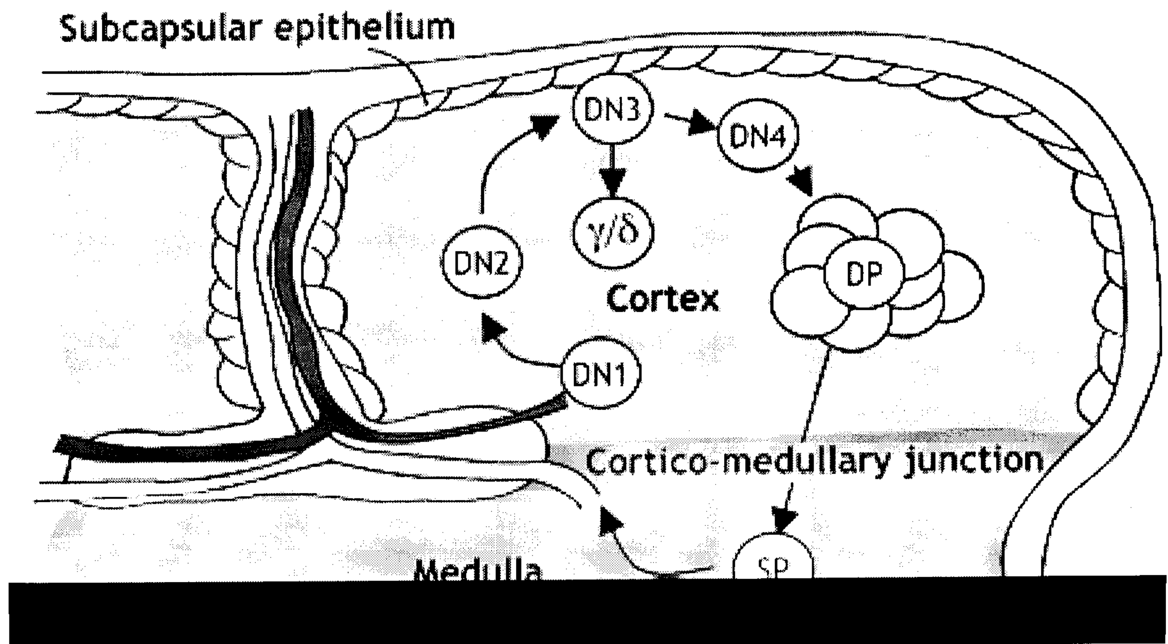

Figure2. The trajectory of thymocytes as they develop. DN cells enter the thymus after precursors leave the bone marrow and initially travel through the cortex, maturing into DP cells and finally single positive (SP) cells in the medulla where they leave the thymus and migrate to the periphery. (From Starr et al., 2003).

B cell development is also characterized by repertoire selection and in many ways parallels T cell development. Pro B cells must first rearrange their Heavy Chain (IgH) gene productively, and this is tested using a strategy similar to β chain selection in thymocytes. There is a pre-B cell receptor composed of two invariant molecules, the surrogate light chain (the λ5 protein) that must combine with the newly formed IgH chain. Thus, incidentally, the γδ T cell receptor appears to be the only antigen receptor that does not require an invariant partner to test its initial rearrangement. It is possible that γδ precursors are rearranging both loci simultaneously, and this is consistent with some descriptions in the literature. In any case, preB-cells that pass this step will then rearrange their light chain genes to form the B cell receptor. The rules of selection of the BcR repertoire are however much less clear than those of the TcR repertoire. There is evidence that strong BcR ligation in immature B cells can lead to cell death. Cross-linking of the BcR by polymeric antigens in particular is efficient in deleting immature B cells. However, there appears to be a second, salvage pathway, more recently described and termed receptor editing. In this process, strongly self-reactive immature B cells re-initiate light chain rearrangement, eventually testing both κ and

λ alleles for an appropriate specificity (Chen et al., 1994; Gay et al., 1993; Tiegs et al., 1993)(editing for TcRs in highly controversial and has not been satisfactorily demonstrated). Finally, whether or not positive selection by self ligands is important to B cell development is not clear, with the exception of a few subsets. As B cells recognize antigen directly, they do not require positive selection for the reasons that T cells do. Like with recent thymic emigrants, most B cells that leave the bone marrow will not be incorporated in to the peripheral lymphoid pool (Freitas and Rocha, 2000).

1.5. Tolerance and the Self Reactive Repertoire

Establishment of tolerance in each individual is a necessary cost of doing business for the adaptive immune system. The innate system faces this problem only in evolutionary time as it selects and fine-tunes its set of germline specificities. The random generation of diversity in the adaptive immune system means that any given specificity of a T or B cell receptor is (at the time it is generated) as likely to recognize self as non self antigens. Or, due to the degenerate nature of immune recognition, both (or even nothing at all). Although programmed gene rearrangement in embryonic and perinatal life offers a way around this and may reflect an initial strategy of a putative proto-immune system, this road is not open at later stages of the organism's development, with the exception of a few classes of so-called innate lymphocytes. As we saw above, immunological tolerance was recognized as a crucial problem as soon as the extent of diversity in antibody responses was first glimpsed, and was considering mainly the problem of avoiding or eliminating self-reactivity.

Self-reactivity is by now an established fact, but the approaches to it differ. While those working with natural antibodies and regulatory T cells, among others, seek a positive role for it in the physiology of the immune system, others are prepared to accept that it exists and write it off as irrelevant. Thus, Langman and Cohn state that "*While the immune system might recognize some self components, so long as these reactions are not debilitating enough to eliminate the individual there has been no violation of 'horror autotoxicus'*" (Langman and Cohn, SJI 1996). Another approach is to go further and postulate that while self-reactive lymphocytes are present, they are functionally unresponsive, or "anergic" (Schwartz et al., 1989). So a convincing case for self reactivity in the

lymphocyte repertoire will have to show that these cells are not only present, but functional- and in some cases, potentially dangerous to the host.

For T cells it has often been noted that positive selection introduces an obligatory degree of auto-reactivity in the emergent repertoire. This was highlighted by the discovery that not only the MHC molecules but also a diverse pool of self-peptides were involved in positive selection, and the subsequent demonstration that in the periphery these same self-peptide/MHC complexes could drive T cell proliferation (Ernst et al., 1999; Viret et al., 1999). Self-reactivity generated by positive selection was however postulated to be of too low affinity to generate T cell activation and autoimmunity. This explanation is not wholly satisfactory because TcR affinity is not the only relevant parameter for T cell activation and the avidity of the interaction with the APC is also crucial. While T cell affinity is an intrinsic and stable property for any given TcR/peptide-MHC interaction, avidity is a highly fluid quality. Changes in the expression level of the TcR, peptide-MHC or adhesion/costimulatory molecule will drastically alter avidity, and so a low/intermediate affinity interaction may also lead to T cell activation through increase in avidity- as in an inflammatory context, for example.

Throughout the 1970's and 80's a series of experimental models that had in common severe perturbation of lymphocyte homeostasis highlighted the existence of self reactive and potentially pathogenic T cells in normal individuals. Sakakura and collaborators found that neonatal thymectomy led to the development of a vast array of autoimmune syndromes. As the initial observation related to gonadal disfunction (Nishizuka and Sakakura, 1969) the authors sought a hormonal cause. As the list of target organs expanded and the autoimmune nature of the phenomenon became evident this was discarded in favor of the hypothesis of expansion of pathogenic lymphocytes (later shown to be primarily CD4+ cells). An exhaustive genetic study showed that in most isogenic strains of mice, neonatal thymectomy led to organ specific autoimmunity, with the target organ varying in a strain dependent manner. Interestingly, in his observations on the function of the thymus, Miller had already reported a wasting syndrome resulting from neonatal thymectomy (Miller, 1999). The original explanation was excessive pathogen load due to immune-deficiency, but this is not in agreement with the fact that completely immune-deficient SCID and Rag-/- mice do not normally display such

syndromes (although improvements in animal maintenance conditions can not be discarded). A similar pathology was observed when adult rats were thymectomized and irradiated, resulting in strain dependent autoimmunity (in the initial study, thyroiditis) (Penhale et al., 1976). In fact, several distinct protocols of inducing lymphopenia, including irradiation and cyclosporin-A treatment led to organ specific autoimmunity (Gleeson et al., 1996). Subsequently several groups succeeded in cloning T Cells with defined anti-self reactivities from normal mice, as was the case with myelin basic protein. Together these observations demonstrate that the immune system of normal, wild type, mice contain anti-self T cells with pathogenic potential that are somehow held in check by a then unknown mechanism (deletion is by definition excluded). Additionally, transferring small numbers of naive T cells into SCID mice leads to a lethal wasting disease, primarily targeting the gastrointestinal or respiratory mucosae. Here it is not clear whether or not the target antigens are self or non-self, or both, so it is not clear if this is a case of bona fide autoimmunity or immune pathology (or both). A similar procedure using athymic nude mice as recipients does, however, lead to multiple organ specific autoimmune disease, with the concomitant appearance of anti-self antibodies.

Finally, Mason has argued on theoretical grounds that it is unlikely that the level of cross-reactivity of TcRs needed to account for the observed diversity of peptide recogntion allows for deletional tolerance to be effective, as this would lead to a peripheral T cell pool incapable of mounting adequate immune responses (Mason, 1998).

Antibodies that bind to self components have long been known, and some were in fact detected by Karl Landsteiner. Initially these were thought to reflect aberrant, pathologic states (which in many of the early cases was true). Indeed, the first widely accepted presence of antibodies against the host in the 1950s were in acute cases of antibody mediated diseases, particularly anaphylaxis and thrombocytopenia purpura. We now know that certain subsets of B cells, in particular B1 and Marginal Zone cells are generated through positive selection and secrete high titers of anti-self antibodies. Many of these antibodies are important in house-keeping functions such as the clearance of dead cells and cellular debris. In general they are produced in a T independent manner. Most pathology caused by anti-self antibodies is derived from somatically mutated high affinity molecules. These are generated in a T dependent manner in the germinal center of peripheral lymphoid organs, where there is no doubt

that B cells undergo positive selection- this time ideally driven by non-self antigens. Failure in T cell regulation is usually necessary for the generation of these pathogenic antibodies, and in fact in many of the T cell lymphopenia models described above pathogenic antibodies can be easily detected.

1.6. Suppressor T cells

The idea that a population of T cells had as its function the suppression of immune responses was put forward more than 30 years ago. Gershon and Kondo showed that tolerance induced by injection of high number of sheep red blood cells into mice could be transferred by lymphocytes to naive hosts (Gershon and Kondo, 1970). Gershon coined the term "infectious tolerance" to describe the phenomenon and proposed the existence of a population of thymus dependent suppressor T cells (Gershon and Kondo, 1971). Later a specific genetic locus was linked to suppression, and was thought to represent a restriction element for suppressor T cells. This putative gene was christened the I-J, and was located between the I-A and I-E MHC loci. The I-J was studied not only through a genetic approach, but also by polyclonal antisera against it. Concomitantly, reports began to appear in the literature of T cell derived antigen specific soluble suppression factors. Phenotipically, suppressor T cells were described as CD8+ cells, but in a strange twist, they still required helper T (CD4) cells to achieve maximal efficiency. The latter were termed inducers of suppressor cell cells. The picture became even more baroque with the introduction of contra-suppressor cells, and soon a comprehensive model for suppressor T cell function became an ever more distant goal.

Although the phenomenology tied to suppressor T cells was enormous (far outstripping even the current rate of "regulatory T cell" papers), the field came crashing thunderously down. The causes were various: the I-J gene was shown to be a bizarre (still not properly explained) artifact, as there was no coding sequence in the intergenic region where it should be found; no one succeeded to clone an antigen specific soluble suppression factor, and with the clarification of the molecular nature of the T cell receptor it became difficult to accommodate their existence, even conceptually and the suppressor T cells themselves proved illusive- at some point thought to be a CD4 dependent CD8 T cell (a convoluted idea

that leaves the status of the CD4 cell in the system a bit mysterious). Suppressor T cells became anathema, and the fact that the *in vivo* dominant tolerance phenomenology still needed an explanation faded away from the immunological mainstream.

1.7. Regulatory T cells

The curse of suppressor cells, soluble specific suppression factors and the I/J locus haunted immunology for a decade before dominant T cell regulation became again acceptable again to most immunologists. However, a few groups continued to produce evidence of dominant regulation effected by T cells, and several observations discredited recessive mechanisms as the sole regulators of tolerance. Perhaps the strongest demonstration (apart from the above set of data showing self-reactivity) were the experiments of LaFaille and Tonegawa. They failed to induce encephalitis by cloning a TcR from a pathogenic anti-myelin basic protein T cell clone and constructing a transgenic mouse where the vast majority of T cells expressed the transgene (Lafaille et al., 1994). This was a limit case, with over 90% of the T cells expressing the transgene at the cell surface- T cells which they demonstrated to non-anergic. Crossing these mice to Rag-/- mice, however, led to 100% incidence of experimental autoimmune encephalomyelitis (EAE), indicating that a Rag dependent population (i.e. lymphocytes) was preventing disease. LaFaille, Tonegawa and collaborators used both knockout mouse models and cell transfer experiments to demonstrate that a population of CD4+ T cells was responsible for this effect. Unfortunately, while these experiments provided convincing evidence for dominant tolerance exerted by T cells, they failed to provide clues as to the phenotype of the regulatory CD4 population (other than, of course, the valuable clue that it was clearly a CD4 population).

A different approach to the problem was to purify cells according to cell surface phenotype and attempt to associate this to prevention of occurrence of immune pathology. Working on a mouse model of wasting disease two different groups demonstrated that fractioning CD4 T cells according to their expression level of the RB isoform of CD45 could lead to two distinct outcomes: SCID mice that received $CD45RB^{high}$ CD4 cells succumbed, usually within two months, to a wasting disease characterized by severe colonic inflammation, resembling human

Crohn's disease, a condition termed Inflammatory Bowel Disease (IBD). Transfer of the complementary CD45RB^{low} population not only did not lead to disease, it could prevent induction by the pathogenic subset (Morrissey et al., 1993; Powrie et al., 1993). The results were promising and strongly pointed to dominant regulation. However, CD45RB is a highly labile marker and about 33% of CD4 T cells have the low surface expression phenotype associated to regulation. Thus the study of CD45RB^{low} T cells in regulation was generally restricted to a small number of groups working on IBD and similar syndromes.

The major breakthrough came from Sakaguchi's group, which had been working for a decade to characterize the regulatory T cells in the neonatal thymectomy. As in the IBD system, they had found an association with cell surface phenotype, with CD5 expression levels for example, that was promising, but not practical, as the CD5 (Lyt-1) bright phenotype that was protective corresponded to about 80% of peripheral CD4 cells (Sakaguchi et al., 1985). Sakaguchi *et al.* surveyed CD4 cells for surface markers that correlated with the CD5^{bright} CD45RB^{low} phenotype. In this screen they found that CD25, an early activation marker, was expressed on roughly 10% of CD4 cells in unmanipulated mice (Sakaguchi et al., 1995). They demonstrated that depletion of CD25 cells before transfer of T cells into athymic nude mice led to the development of multiple organ specific autoimmune diseases, and that this could be prevented by restoring the CD25+CD4+ population. These observations were extended to the neonatal thymectomy model when they subsequently showed that CD25+ T cells were not found in the periphery at day 3 afterbirth, the preferred time point for thymectomy to lead to autoimmunity in the Balb/c strain studied (Asano et al., 1996), and consequently neonatal thymectomy resulted in adults that were deficient in this cell population. A semblance of unity came to the field when Powrie's group reported that although only a third of CD45RB^{low} CD4 cells are CD25+, this subpopulation was responsible for most of the protective effect of CD45RB^{low} cells in the IBD system (Read et al., 2000). To avoid association with the previous debacle, the new population of T cells that was proposed to perform the same basic function as suppressor T cells were re-christened regulatory T cells.

Another crucial difference between the CD45RB and CD25 markers is that while in thymic development recently formed single positive CD4 have a homogenous phenotype for CD45RB (intermediate), the same is not true for CD25 expression. Thymic single positive CD4 cells express

CD25 at a slightly lower frequency (around 5%) than peripheral cells (Papiernik et al., 1998). Thymic CD25+ cells are functional Tregs and are mainly not recirculating T cells (Itoh et al., 1999). Thus Tregs appear to belong to a separate developmental lineage, and the use of the CD25 marker allows the study of their development, which we shall return to below. This has recently been strengthened by the cloning of the first transcription factor shown to be important for their development. The gene, called Foxp3 was identified as an important player in severe X-linked immune dysfunction in both mice and humans (Bennett et al., 2001). The work of three independent groups now indicates that Foxp3 deficiency leads to autoimmunity because this molecule is important in the differentiation of Tregs (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003).

CD25 is a part of the high affinity IL2 receptor complex, together with the IL2Rβ chain and the common cytokine γ chain chaired with a large array of cytokine receptors, such as IL4, IL9, IL15 and IL21. Initially known as an activation inducible component of the IL2 receptor, the finding that the α chain is expressed constitutively on a population of CD4 cells with regulatory function cast a new light on a puzzling series of observations. The IL2-IL2R system was discovered as a potent promoter of T cell proliferation. However, mice made deficient by homologous recombination for IL2, IL2Rβ or IL2Rα (their severe lymphopenia and impaired signaling for multiple cytokines make the γc deficient mice uninformative in this regard) all showed severe lymphoproliferative or autoimmune disorders (Sadlack et al., 1993). This was interpreted as a cell autonomous defect of the responder/proliferating cells, based on Lenardo's demonstration that IL2 signaling could induce Activation Induced Cell Death (AICD) (Lenardo, 1991). However, consistent with Sakaguchi's proposal that CD25+ T cells were key regulators of the immune system, all of these systems were shown to be susceptible to dominant tolerance. Cell transfers from wild type mice or transient expression of missing components during thymic development led to healthy mice in all the above knockout animals (Almeida et al., 2002; Malek et al., 2002; Wolf et al., 2001). In addition, it was concomitantly shown that IL2 was necessary for the maintenance of CD25+ cells *in vivo* (presumably exogenous IL2, as CD25+ cells are very poor producers of this cytokine).

How regulatory T cells operate has become an intense area of research and produced a large set of apparently contradictory data. A simple

correlation with Th2 type regulation of inflammatory responses has been convincingly discarded in all systems tested to date, and IL4 for example, appears to play no role in their function. Interleukin-10 and TGFβ were shown to be indispensable mediators of $CD45RB^{low}$ immune regulation in the IBD system (Asseman et al., 1999; Powrie et al., 1996), and Tregs were shown to produce these cytokines. While *in vitro* suppression of T cell proliferation by Tregs is thought to be mediated by cell-cell contact and largely independent of secreted factors (for TGFβa role for a membrane bound form in *in vitro* suppression has been proposed, and disputed) a paracrine effect has not been ruled out. More importantly, cytokines, specially IL10 are important for *in vivo* function of Tregs. Thus Tregs from IL10-/- mice, while still able to control proliferation *in vitro* are unable to control homeostatic expansion of naive T cells and IBD induction (Annacker et al., 2001b). Conclusive experiments on the role of TGFβ *in vivo* have not yet been reported, in part due to the acute mortality of the knock out mice. A cell bound molecule that has been implicated in Treg function is cytotoxic T lymphocyte antigen 4 (CTLA4). CTLA4 expression is induced in T cells by activation, but Tregs express it constitutively. Blocking of CTLA4 with monoclonal antibodies (or F_{ab} fragments) abrogates *in vitro* suppression and control of IBD (Read et al., 2000; Takahashi et al., 2000)- but these experiments do not determine if the effect of treatment is on Tregs, responder T cells, or both. CTLA4 deficient mice also develop a fulminant lymphoproliferative syndrome, originally attributed to a cell autonomous defect in regulating the level of costimulation received by the T cells. Once again this was shown to be controlled by the presence of normal cells in bone marrow chimera experiments (Bachmann et al., 1999), lending further support to those that have long said that speaking of tolerant cells is nonsense, and one can only speak of the tolerant organism. Another level of complexity is added by the fact that these components need not be operating independently of each other. This is illustrated by that fact that in CD4 T cells, engagement of CTLA4 has been shown to stimulate TGFβ production (Chen et al., 1998).

Which cell types are the target of CD25 Tregs is also not clear. *In vitro* experiments have pointed to the importance of cell-cell contact, although it is not clear if the regulatory cell and its target must actually be on the same APC. Shevach's group has proposed and discarded the hypothesis that the primary mode of suppression might be through modulation of the APC itself, but the experiments done were indirect

(Thornton and Shevach, 2000). Others have claimed that an effect on the level of costimulatory molecules at the APC is significant (Cederbom et al., 2000). Certainly the important role of IL10 *in vivo* would point to direct or indirect (through lowering of inflammation) influence on APC function. Again *in vitro* CD25 Tregs can interact directly with and efficiently inhibit CD8 T cell activation and proliferation (including in APC free cultures) (Piccirillo and Shevach, 2001). Finally, two different groups reported an effect on B cell proliferation and antibody secretion (Bystry et al., 2001; Nakamura et al., 2001).

Other T cell subsets have been shown to have regulatory phenotype *in vivo*, in particular NKT and γδ cells. These cells however appear to play highly specialized roles in certain tissues, such as the mucosal surfaces and the liver. They shall not be dealt with further here, but are interesting in that they not only represent regulatory T cells, they also are part of the "innate" lymphocytes, with stereotypical antigen receptors and may be a clue as to the evolutionary origin of dominant regulation. CD8 T cells are still proposed by some to contain regulatory T cells, but it has been hard to demonstrate that they play a crucial role in Tolerance.

1.8 Regulatory T cells and thymic selection

While studies such as Medawar's with engraftment of hematopoetic cells induce strong deletional tolerance, this is not the whole story of T cell selection. After demonstrating that the thymus generated immunocompetent T cells, Miller also showed that thymus grafts induced tolerance to allo-antigens, and at the time also interpreted this in the frame of Burnet's model. Subsequently it was however shown that T cells are restricted to one thymic haplotype upon maturation, which argued strongly that they matured in one thymus (although it was clearly the case for positive selection, the possibility remained that they could be negatively selected on both haplotypes, either by recirculating or through reciprocal colonization of thymic lobes by bone marrow cells from the other background). The experiments of Singer and Matzinger argued against this and for a dominant mechanism. The most elegant demonstration of this principle was achieved by transferring only the epithelial component of the thymus and showing that this induced dominant tolerance. Initially in experiments done in *Xenopus* it was shown that grafting the portion of the embryo containing the thymic

epithelial rudiment would lead to lasting tolerance of allografts from the same background (Flajnik et al., 1985). Subsequently, the experiments done by Nicole LeDouarin and collaborators established the role of thymic epithelium in tolerance using much more meticulous transplantation, of only the third branchial pouch (which will originate the epithelial component of the thymus) in avian embryos prior to hematopoetic colonization. These experiments were not done with allografts, but in quail-chicken chimeras. The results were important from two different points of view. First, by showing that tissue grafts would be rejected even if present in the embryo *before* any hematopoesis is detectable they placed a very large nail in the coffin of recessive models were deletion or anergy are brought on by persistent self antigens and ensure tolerance. As grafts were spontaneously rejected in the birds as their immune system matured it would also be hard to argue for a danger based mechanism. Second, by showing that the thymic epithelium graft prevented rejection of other tissue grafts, *even though* the host thymus was still present and producing T cells, they argued strongly for a dominant regulatory mechanism dependent on T cells (reviewed in (Coutinho et al., 1993)

LeDouarin's group in collaboration with Antonio Coutinho's group then adapted this experimental model to mice and could repeat the same basic phenomena. Furthermore, in the murine model a more detailed analysis of the cells involved in this process was possible. Thus, it was shown that CD4 T cells selected on the donor thymic epithelium were the main effectors of tolerance in this model, and that they actively controlled other T cell populations. They proposed a model where regulatory T cells were selected by high affinity ligands on thymic epithelium (Modigliani et al., 1996). After the advent of CD25 this was confirmed by showing antigen specific selection of high-affinity anti-self cells into the regulatory compartment (CD25+) by the expression of cognate antigen on thymic epithelial cells (Bensinger et al., 2001; Jordan et al., 2001). Other predictions of the model, in particular a preferential generation of regulatory T cells in early ontogeny and peripheral "education" of self-reactive cells have proven more elusive (Annacker et al., 2001a; Coutinho et al., 2001)

Surprisingly, one of the seemingly more absurd aspects of fully deletional tolerance has been brought back in to play: the expression of a large set of peripheral peptides inside the thymus. However, the pattern of expression and localization of these peptides is entirely incompatible

with a role in efficient deletion. They are expressed on the thymic epithelium (Kyewski et al., 2002) in a mosaic fashion, in a poorly deleting cell type and in a pattern that would require a "pin-ball" mode of negative selection to be even minimally effective. The gene largely responsible for this mosaic expression of tissue specific antigens was identified following studies on patients suffering from a familial autoimmunity targeting glandular and other tissues (Autoimmune Polyendocrinopathy Candidiasis Ectodermal Dystrophy-APECED) (Bjorses et al., 1998). The mutated gene was identified as autoimmunity regulator (AIRE) and was shown to be expressed in the thymus. Subsequently, Mathis and collaborators showed that in mice AIRE controlled the expression of tissue specific peptides on thymic epithelium (Anderson et al., 2002). So far no evidence exists to directly link AIRE dependent expression of tissue peptides in the thymus to the development of regulatory T cells, but it is tempting to think that this may be a mechanism for the generation of tissue specific regulators. After all, deleting a fraction of self reactive cells may not be very efficient, but differentiating a fraction of tissue Tregs may.

Additional support for a role of positive selection in natural tolerance is also given by an unrelated study showing that drugs that interfere with positive, but not negative, selection lead to autoimmunity (Kretz-Rommel and Rubin, 2000).

Finally, the original model of thymic epithelium mediated positive selection incorporated an important new role for negative selection: allowing for immune responses in the periphery. By postulating that bone marrow derived thymic APCs would efficiently delete regulatory T cells, Coutinho and collaborators provided a way to avoid that dominant regulation cancel out immune responses. A key component in this model was that the colonization of the thymus by bone marrow APCs occurs after it has already exported T cells (negative selection is thus very inefficient in neonates) (Modigliani et al., 1996). This was an elegant solution that maintained an ontogenic component in natural tolerance. Unfortunately it has been very hard to demonstrate that neonates are particularly enriched for regulatory T cells- for those that express CD25 it is clearly not the case. The evidence for the expression of peripheral self antigens on thymic cortical epithelium cells, together with the demonstration that Tregs are positively selected by them, provides a slightly different view of how this may happen. In the terminology of Langman and Cohn, this would be establishing tolerance by allocating a

particular compartment to it, as opposed to a time window (Langman and Cohn, 1996). It also solves the apparent paradox of self reactive cells that undergo normal negative selection (Kawahata et al., 2002). It would be interesting to test the prediction of the necessity of deletion for responsiveness by using the K14 transgenic system described above, where there are not only potent syngeneic MLRs but also regulation of these by K14 Tregs, but not from wild type (Bensinger et al., 2001)

1.9. The immune response

So in effect what is our current understanding of how innate and adaptive immunity operate in practice? How does an immune response come about? Well the first answer is that the vast majority of daily contacts with microorganisms will not provoke any recognizable immune response at all. Most of these interactions will in fact occur with commensal bacteria and will not induce even innate immunity. The second largest set of contacts will come with bugs that are entirely controlled by the barrier surfaces of the body and innate immune mechanisms. Now let us consider the minority of cases where pathogens breach the body surfaces and activate innate and adaptive immunity.

The first event to take place will be activation of the innate immune system through the recognition of both tissue damage and PAMPS. This will lead to increased vascular permeability and release of chemotactic molecules at the infected site with the consequent efflux of circulating granulocytes and monocytes, provoking the easily perceived local swelling. At the same time, locally secreted inflammatory mediators will attract immature dendritic cell (DC) precursors that will pick up antigen at the infected site and then migrate to the draining lymph node where they will mature and present antigen to T cells together with co-stimulatory molecules. A key point here is that dendritic cells will be loaded with both self and non-self peptides, both presented in a highly stimulatory context for T cells (as we shall see later, depending on what induced the inflammation, DCs may be presenting only self). During the first few days, reactions against the pathogen will be carried out only by the cells of the innate immune system, and if they do not keep pathogen proliferation in check at this phase there is usually no hope that the adaptive immune response many days later will be able to do so. Naive T cells must be activated by antigen presented on the DCs in the lymph

node, and do not migrate into the target tissue. In addition to stimulation through their TcR by antigen-MHC, they require also signaling via a costimulatory receptor, the most important of which is the CD28 molecule. Activated, but not immature, DCs will present on their surface the CD28 stimulatory ligands, B7.1 and B7.2, which together with recognition of cognate antigen will activate the naive T cell. Memory/activated T cells will however penetrate tissues upon inflammatory stimulation, and if they encounter cognate antigen they can then be activated in the absence of co-stimulation. Thus, if the pathogen has been previously encountered, clonally expanded antigen specific cells will speed up the adaptive immune response, in what is known as immunological memory.

If however this is the first encounter, antigen specific lymphocytes will be rare. They must encounter cognate antigen on an activated DC and then expand. Reaching a clone size where they can mediate effective immune responses will take several days. When this happens, largely directed by cues from the innate immune system, lymphocytes can mount (in a overly simplified manner) two basic kinds of immune responses: inflammatory, cellular responses (termed Th1) and antibody (Th2) responses. Th1 responses are mainly directed against intracellular parasites and stimulate macrophage, neutrophil, natural killer and CD8 cell activity. Extracellular parasites and serum toxins are most efficiently countered by the secretion of antibody, which may also act to directly lyse target cells through complement fixation or tag them for phagocytosis (opsonization).

The end result of the adaptive immune response is that not only enhanced pathogen clearance, but also the persistence of clonally expanded antigen specific cells, which also belong to the appropriate (Th1, Th2 in the case of CD4 cells) effector class. These can then be activated independently of innate immunity, and provide the hallmark of adaptive immunity, immunological memory. Typically, the secondary response will be faster and more specific than the primary, and may also present vastly reduced collateral tissue damage.

1.10. Inflammation, autoimmunity and immune pathology

The association of inflammation to autoimmunity is much more complex than was originally thought. Cross reactivity and molecular mimicry have been proposed to link infections as triggers for autoimmunity. Thus, for a long time the idea has been around in the immunological community that autoimmune diseases represent an immune response that somehow misfired, and that infections are a root cause of autoimmunity. This is undoubtedly true in some cases, as in rheumatoid arthritis, but epidemiological data in humans and experimental work in animal models suggest that a more subtle correlation exists. The incidence of autoimmunity apparently grows with distance from the equator, while the inverse is true of the incidence of infectious disease. Exceptions to this rule may be found, but they usually reflect the genetic peculiarities of small isolated communities, such as the high incidence of autoimmunity in some regions of Sardinia. The general trend however seems to be independent of (tenuous) genetic differences, as third world migrant populations assume the profile of the host country, provided the individual in question immigrated before puberty (Bach, 2001).

Linking inflammation and autoimmunity is also not straightforward in mouse model systems. While some infections clearly precipitate autoimmune syndromes, this is by no means true across the board. Furthermore, inflammatory stimulation in the absence of infection, as with injection of LPS is in many models a potent inducer or re-enforcer of tolerance. Pre-treatment of mice with LPS has been shown to induce tolerance in models ranging from delayed type hypersensitivity responses to graft rejection and graft versus host disease. The NOD mouse model of type I diabetes provides many examples of this counter-intuitive role for inflammation in tolerance. The simplest observation in this system is that diabetes incidence shows an inverse correlation with the cleanliness of the animal facility. Conventional facilities have a much lower disease rate than specific pathogen free ones. This was later shown to be essentially a result of the inflammation, as injection of adjuvant alone (complete freund's adjuvant) mimics the effect. A similar finding shows the role of the inflammatory component in bacterial colonization dependent oral tolerance induction. In this model, germ-free mice are refractory to induction of systemic unresponsiveness to orally administered protein antigens, which can be induced in normal mice. Feeding LPS restores the ability to induce oral tolerance.

These diverse phenomena have all been shown to be strongly T cell dependent. In fact, many of them where studied in the context of suppressor T cells. Once again, the collapse of the suppressor cells left a fertile field of observations requiring a new cellular basis.

1.11 Aim of this thesis

The principal aim of this thesis is to examine how regulatory T cells behave under inflammatory conditions, because it is in this context that most, if not all, of the relevant decisions they must make *in vivo* will occur. Although it is a popular idea today that inflammation is an off switch for dominant regulation, we believe that this not tenable on conceptual grounds and not consistent with existent observations. We are constantly bathed in stimulation through damage or infection and in addition must live with an enormous load of commensal micro-organisms which are not the target of acute immune responses. The removal of regulatory T cells can reveal the frequently lethal auto-aggressive potential of these reactions, indicating that Tregs are present and functional during their occurrence.

Results

Manuscript I

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$CD25^+CD4^+$ regulatory T cells suppress $CD4^+$ T cell-mediated pulmonary hyperinflammation driven by *Pneumocystis carinii* in immunodeficient mice

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The $CD4^+$ T cell-mediated inflammatory response to *Pneumocystis carinii* (PC) critically contributes to the clinical severity of PC pneumonia. It has been suggested that lymphopenic conditions predispose individuals to this immunopathology, although the mechanisms remain poorly understood. Another set of evidence indicates that a subpopulation of $CD4^+$ T cells constitutively expressing the CD25 molecule prevent lymphopenia-induced autoimmunity and inflammatory bowel disease. We tested the ability of this $CD25^+CD4^+$ population to regulate $CD4^+$ T cell-mediated inflammatory response to PC. Adoptive transfer of $CD25^-CD4^+$ cells into PC-infected recombination-activating gene-2-deficient mice led to lethal pneumonia within 13 days post-transfer. PC infection appeared to trigger $CD25^-CD4^+$ cells, since recipients with reduced PC load survived up to 5 weeks after transfer. In contrast, transfer of $CD25^+CD4^+$ cells did not induce lethal pneumonia and prevented the development of the disease induced by $CD25^-CD4^+$ cells. Furthermore, $CD25^-CD4^+$ cells reduced the PC load in the lung, while $CD25^+CD4^+$ cells suppressed this immune response. Our results indicate an essential role for $CD25^+CD4^+$ T cells in the control of PC-driven immunopathology, and suggest that in immunocompromised hosts PC pneumonia may result from a deficiency in regulatory T cells.

Key words: Immunopathology / Immune regulation / Tolerance / *Pneumocystis carinii* pneumonia / T cell subset

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1 Introduction

Pneumocystis carinii (PC), an opportunistic pulmonary pathogen, causes life-threatening pneumonia (PC pneumonia: PCP) in immunocompromised individuals, including AIDS patients. Numerous studies have established the indispensable role of $CD4^+$ T cells for resistance to the microorganism [1]. Accumulating evidence indicates that, under some circumstances, $CD4^+$ T cell-mediated inflammatory responses to PC have deleterious consequences to the host and significantly contribute to PCP pathogenesis. PC-infected severe combined immunodeficient (SCID) mice develop fatal pulmonary hyperinflammation associated with pathogen clearance, when reconstituted with wild-type lymphocytes or sorted $CD4^+$ T cells, but not $CD8^+$ T cells [2]. Clinical studies also indicate that inflammatory responses to PC are more severe in non-AIDS patients receiving immunosuppressive therapies than in AIDS patients, specifically lacking $CD4^+$ T cells [3–5]. Furthermore, a recent study provided direct experimental evidence that the T cell-mediated inflammatory response to PC is the major contributor to the pulmonary dysfunction in PCP [6]. In contrast, $CD4^+$ T cells in normal individuals challenged with PC are able to mediate pathogen clearance through an immune response with minimum inflammation [7, 8]. Several findings suggest that a reduction in lymphocyte numbers facilitates the development of the $CD4^+$ T cell-mediated pulmonary hyperinflammatory reaction to PC; lymphocyte depletion by irradiation in mice results in interstitial pneumonia after PC inoculation [8] and induction of a fatal pulmonary hyperinflammation by adoptive transfer of lymphocytes in PC-infected SCID mice is observed only when small numbers of cells are injected [2].

Animals rendered lymphopenic, either by neonatal thymectomy, or adult thymectomy combined with split-dose γ-irradiation, develop organ-specific autoimmune diseases, prevented by reconstitution with $CD4^+$ T cells from normal animals. Thus, in normal animals, self-

[I 22135]

Abbreviations: IBD: Inflammatory bowel disease **PC:** *Pneumocystis carinii* **PCP:** PC Pneumonia **RAG:** Recombination-activating gene **SCID:** Severe combined immunodeficient T_{reg}: Regulatory T cells

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reactive T cells are under the control of a dominant protective mechanism mediated by $CD4^+$ regulatory T cells (T_{reg}), and a general reduction in lymphocyte numbers leads to deficient regulation by these cells and enhanced pathogenesis (reviewed in [9]). Recent studies have shown that T_{reg} capable of preventing autoimmunity are contained within a small subset of $CD4^+$ T cells that constitutively express the IL-2R α-chain (CD25) [10–15]. This $CD25^+CD4^+$ T cell population also inhibits enteric bacteria-driven inflammatory bowel disease (IBD) induced by transfer of $CD25^-CD45RB^{high}CD4^+$ T cells in SCID or recombination-activating gene (RAG)-deficient mice [16–20] and prevent hyper-IgE responses in immunized BCR/TCR double-transgenic system [21]. These analyses suggest that $CD25^+CD4^+$ T_{reg} play a fundamental role not only in preventing autoimmunity but also in controlling T cell-mediated inflammatory responses.

These findings prompted us to hypothesize that the PC-driven immunopathology in lymphopenic conditions is due to a deficiency in $CD25^+CD4^+$ T_{reg}. In this study, we analyzed the outcome of $CD25^-$ and $CD25^+CD4^+$ T cells transfer into PC-infected RAG-$2^{-/-}$ mice. We report that $CD25^+CD4^+$ cells prevent the development of the PC-driven fatal pulmonary inflammation induced by $CD25^-CD4^+$ lymphocytes. Furthermore, $CD25^+CD4^+$ cells are shown to suppress the elimination of PC mediated by $CD25^-CD4^+$ cells.

2 Results

2.1 Adoptive transfer of $CD25^+CD4^+$ T cells protects PC-infected RAG-$2^{-/-}$ mice from lethal pneumonia induced by $CD25^-CD4^+$ T cells

RAG-$2^{-/-}$ mice raised under specific pathogen-free conditions in our animal facilities were naturally infected with PC, as revealed by PCR detection of a PC-specific DNA sequence in the lung (Fig. 1A). As has been described for SCID mice heavily infected with PC [22, 23], histological examination of their lungs revealed thickened alveolar septa and exudation of amorphous foamy material into many of the alveoli (Fig. 1B). However, clinical signs of PCP, *i.e.* weight loss, rough hair coat, hunched posture, or reduced motor activity [23, 24] were absent.

C57BL/6 mouse splenocytes were fractionated into $CD25^+CD4^+$ and $CD25^-CD4^+$ populations by magnetic purification (hereafter denoted as $CD25^+$ and $CD25^-$ cells, respectively). The purity of the resulting $CD25^+$ and $CD25^-$ cells were routinely ~82% and ≥96%, respectively (Fig. 2A). The contaminating cells in the $CD25^+$ fraction were mainly $CD25^-CD4^+$ cells such that the

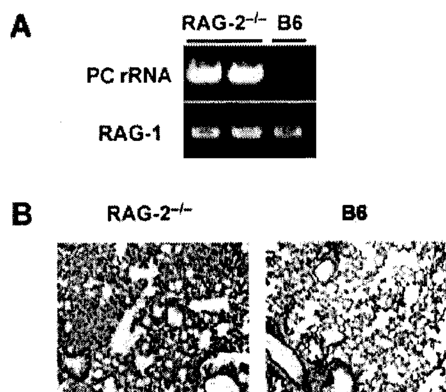

Fig. 1. PC infection in the lung of RAG-$2^{-/-}$ mice. (A) Detection of the PC mitochondrial large-subunit rRNA gene by PCR. Genomic DNA was isolated from the lung of RAG-$2^{-/-}$ or C57BL/6 (B6) mice, and subjected to PCR using primers specific for the rRNA gene, or mouse RAG-1 gene as a control. (B) PCP in RAG-$2^{-/-}$ mice. Representative histological sections of the lungs after hematoxylin and eosin staining are shown; ×100.

preparation contained a 6:1 ratio of $CD25^+$ versus $CD25^-$ cells. In each of the cell preparations, $CD8^+$ and $CD19^+$ cells were undetectable. Three-month-old PC-infected RAG-$2^{-/-}$ mice were injected i.v. with either 3×10^5 $CD25^-$, 3×10^5 $CD25^+$ cells, 3×10^5 of each, or none. Mice receiving $CD25^-$ cells alone exhibited acute clinical signs of PCP and died or became moribund 10–13 days after transfer (Fig. 2B, C). Unreconstituted RAG-$2^{-/-}$ mice gained weight slightly and showed no clinical sign of disease. Animals injected with $CD25^+$ cells alone never became moribund, although 33% exhibited a transient, moderate weight loss, which was recovered by the end of the 3rd week post-transfer. Only 25% of the mice receiving both $CD25^+$ and $CD25^-$ cells died of wasting disease. The majority (50%) of them showed moderate weight loss, which was also recovered by the end of the 3rd week. The remaining 25% did not show any sign of disease.

We killed the recipient mice 10–17 days after transfer, and examined the lungs and colons histologically (Fig. 3A, B). Moribund animals, showed extensive and widespread mononuclear infiltration in the alveolar spaces, perivascular, and peribronchiolar regions, while animals that did not develop lethal wasting disease displayed few infiltrates in the lung. In all groups of mice, the colon showed few mononuclear cells. Flow cytometric analyses of lung lymphocytes revealed large numbers of $CD4^+$ cells in moribund animals receiving $CD25^-$ cells alone, whereas infiltration of $CD4^+$ cells was minimal in healthy animals injected with both $CD25^-$ and $CD25^+$

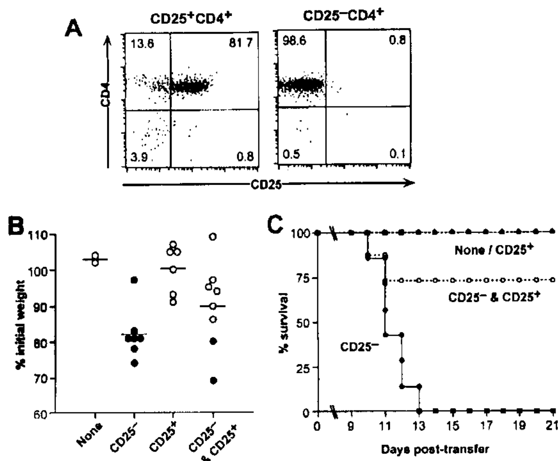

Fig. 2. Induction of acute lethal wasting disease in PC-infected RAG-$2^{-/-}$ mice by adoptive transfer of $CD25^-CD4^+$ T cells, and its prevention by the $CD25^+$ subset. PC-infected RAG-$2^{-/-}$ mice received either 3×10^5 $CD25^-CD4^+$ (n=7), 3×10^5 $CD25^+CD4^+$ (n=6), 3×10^5 of each ($CD25^-$ and $CD25^+$, n=8), or none (n=2). Results were pooled from two independent experiments. (A) Flow cytometric analyses of purified $CD25^+$ and $CD25^-CD4^+$ cells before adoptive transfer. (B) Relative body weight 10 days after transfer expressed as a percentage of the weight at the beginning of the experiment. The horizontal bars indicate the mean in each group. Group comparisons were statistically significant in the cases of: none *vs.* $CD25^-$ ($p=9\times10^{-5}$), none *vs.* $CD25^-$ and $CD25^+$ ($p=9\times10^{-5}$), none *vs.* total $CD4^+$ (p=0.02), and $CD25^-$ *vs.* $CD25^+$ ($p=4\times10^{-4}$), while $CD25^-$ *vs.* $CD25^-$ & $CD25^+$ was not. Open symbols, healthy mice; gray symbols, animals that recovered their initial weight by the end of the 3rd week post-transfer; black symbols, mice found dead or moribund. (C) Kaplan-Meier analysis of RAG-$2^{-/-}$ mice that received different $CD4^+$ T cell subsets. $CD25^-$ *vs.* $CD25^-$ and $CD25^+$, p=0.02 by the log rank test.

cells (Fig. 3C). TCR Vβ repertoire analysis of lung infiltrates from sick animals using multiple Vβ-specific mAb revealed restricted Vβ usage, suggesting Ag-driven clonal expansions in the lung (not shown). However, when comparing the profile of Vβ usage in different animals, no particular gene-segment was over-represented.

These results demonstrate that $CD25^-CD4^+$ cells induced an acute lethal pneumonia in PC-infected RAG-$2^{-/-}$ mice, while the $CD25^+$ population contained protective cells which inhibited the occurrence of the disease.

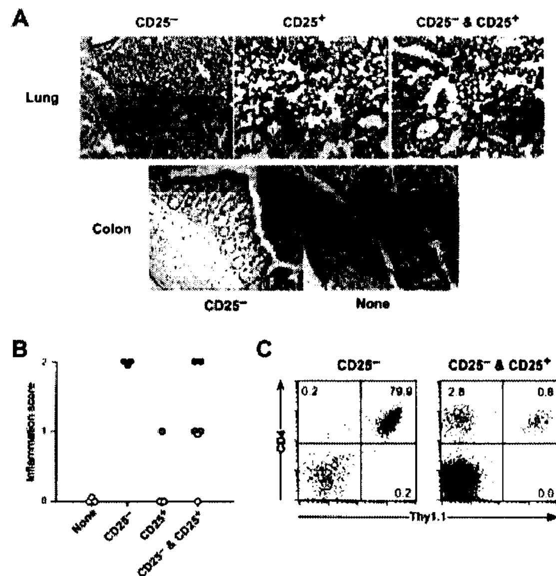

Fig. 3. Severe pneumonia and absence of colitis in PC-infected RAG-$2^{-/-}$ mice that received $CD25^-CD4^+$ cells. (A) Representative histology of the lung and colon sections 10–13 days after adoptive transfer of either 3×10^5 $CD25^-CD4^+$, 3×10^5 $CD25^+CD4^+$ cells, 3×10^5 of each, or none; ×100 for the lungs and ×200 for the colons. (B) Pulmonary inflammation scores determined 10–17 days after transfer (open symbols, healthy mice; gray symbols, animals that showed a transient and moderate weight loss; black symbols, moribund animals). For scoring procedure see Sect. 4.5. Results were pooled from two independent experiments. (C) Representative FACS analyses of lung infiltrates. RAG-$2^{-/-}$ mice received 3×10^5 $CD25^-CD4^+$ cells, or 3×10^5 $CD25^-CD4^+$ plus 3×10^5 $CD25^+CD4^+$ cells. $CD25^-CD4^+$ and $CD25^+CD4^+$ cells were purified from C57BL/6-Thy1.1 and C57BL/6-Thy1.2 mice, respectively. Note that Thy1.1^-CD4^+ cells seen in the co-transferred recipients are Thy1.2^+ cells originating from the injected $CD25^+CD4^+$ population.

2.2 The acuteness of the pneumonia induced by $CD25^-CD4^+$ T cells correlates with the degree of PC infection in the lung

In an attempt to clear the infection, our RAG-$2^{-/-}$ colony was rederived through embryo transfer. The resulting RAG-$2^{-/-}$ mice, born from immuno-competent foster mothers, were used as breeders, and the next generation analyzed when 2–3 months of age. The degree of PC infection in these animals was evaluated by quantitative PCR on lung DNA preparations using primers specific for the PC rRNA gene, a measure shown previously to directly correlate with the microorganism number in a given tissue sample [1]. The linear relationship between the fluorescence intensities of the PC DNA-specific PCR

products and the amounts of lung genomic DNA added in the PCR reactions is shown in Fig. 4A. Identical standard curves were also set for mouse RAG-1 sequences (not shown), and the relative quantities of the PC rRNA and mouse RAG-1 sequences in each sample were determined by reference to these standard curves. Embryo transfer resulted in a 34-fold decrease in the amount of PC DNA detected in the lung as compared with control RAG-$2^{-/-}$ mice of the previous experiment (Fig. 4B). Histological examination showed sparse foamy exudates in the alveoli and few thickened alveolar walls, indicating a less severe PCP (Fig. 4C). When these animals were used as recipients of adoptive transfer, all survived up to 5 weeks after transfer irrespectively of the cell populations they received. However, by this time, animals injected with $CD25^-$ cells alone when compared with those receiving both $CD25^-$ and $CD25^+$ cells showed significant weight loss (16.7 versus 19.4 g in the others; p=0.004) associated with clinical signs of PCP. Histological and cellular analyses were performed 5–6 weeks post transfer (Fig. 5). Similarly to the previous experiment, the lung of animals receiving $CD25^-$ cells alone revealed extensive and widespread infiltration of mononuclear cells. These features were identical, although more severe, in animals analyzed 6 weeks post transfer. Moderate focal infiltration of mononuclear cells was detected in the colons, although the intestinal pathology (hyperplasia of intestinal epithelial cells and loss of goblet cells) was less severe than previously reported [16–19] and animals displayed neither diarrhea nor rectal prolapse. The major cause of morbidity was, therefore, also pneumonia rather than colitis. Finally, animals receiving both $CD25^-$ and $CD25^+$ cells showed few infiltrates both in the lungs and in the colons, confirming that $CD25^+$ cells were capable of inhibiting both pneumonia and colitis.

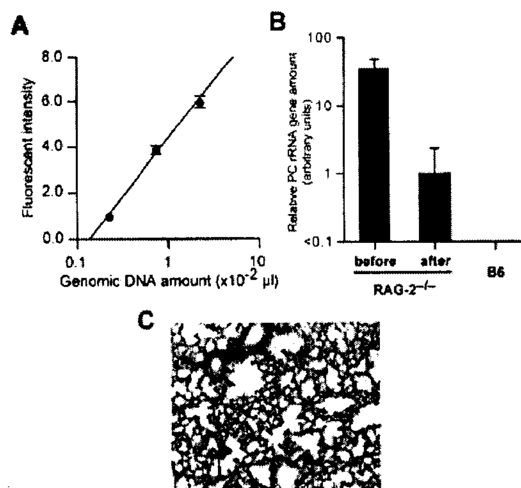

Fig. 4. Reduced PC infection in RAG-$2^{-/-}$ mice after rederivation of the colony. (A) Setup of a quantitative PCR assay for the PC rRNA gene sequences. A representative titration curve is shown where the fluorescent intensities (mean ± SD) of the specific PCR products were plotted as a function of the amounts of lung genomic DNA in the PCR reactions. (B) Relative amounts of the PC rRNA gene sequences in the lung of RAG-$2^{-/-}$ mice before or after rederivation, or of control C57BL/6 (B6) mice (geometric mean ± SD). Relative amounts of genomic DNA in the samples were first normalized according to the quantities of RAG-1 sequences. The difference between before and after rederivation was significant (p=0.01). (C) Representative histology of the lung from the rederived RAG-$2^{-/-}$ mice. Note sparse intra-alveolar exudates and few thickened septa as compared with Fig. 1B; ×100.

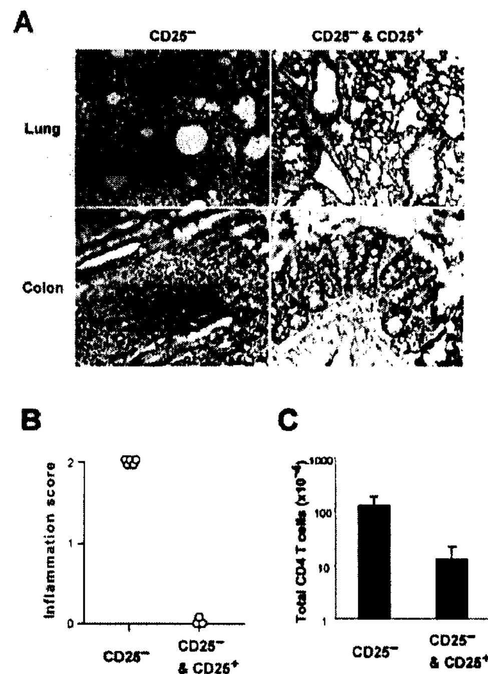

Fig. 5. $CD4^+$ T cell-mediated PCP in the rederived RAG-$2^{-/-}$ mice. (A) Representative histology of the lungs and the colons 5 or 6 weeks after adoptive transfer of $CD4^+$ T cell subsets. Mice received $3x10^5$ $CD25^-CD4^+$ cells, or $3x10^5$ $CD25^-CD4^+$ plus $3x10^5$ $CD25^+CD4^+$ cells. Original magnifications: x100 for the lungs; x200 for the colons. (B) Pulmonary inflammation scores determined 5 or 6 weeks after transfer. (C) Total number of $CD4^+$ T lymphocytes recovered from the lung.

2.3 CD25⁺CD4⁺ cells suppress the elimination of PC mediated by CD25⁻CD4⁺ cells

It was previously demonstrated that, in the absence of other T and B cells, $CD4^+$ T cells are able to reduce PC load in the lung of infected SCID mice [2]. To investigate whether $CD25^+$ cells suppress the elimination of PC, we determined the relative amount of PC DNA in the lungs using the quantitative PCR assay described above (Fig. 6). The RAG-$2^{-/-}$ mice receiving $CD25^-$ cells alone quantitatively carried on average 80-fold less of the rRNA sequences in their lungs than the unreconstituted mice or the recipients for both $CD25^-$ and $CD25^+$ cells. No significant difference was detected between the control animals and those receiving both subsets. Thus, $CD25^-CD4^+$ T cells mediated elimination of PC, and $CD25^+CD4^+$ T cells inhibited it.

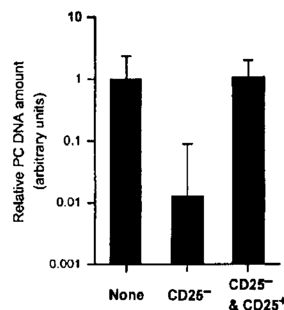

Fig. 6. Reduction of the PC load by $CD25^-CD4^+$ cells and its inhibition by $CD25^+CD4^+$ cells. Relative quantities of the PC rRNA gene sequences in the lungs were determined by the quantitative PCR method as in Fig. 4. RAG-$2^{-/-}$ mice received either 3×10^5 $CD25^-CD4^+$ (n=5), 3×10^5 $CD25^-CD4^+$ plus 3×10^5 $CD25^+CD4^+$ cells (n=3), or none (n=3) 5–6 weeks before. Geometric mean ± SD. None *vs.* $CD25^-$, p=0.006. $CD25^-$ *vs.* $CD25^-$ and $CD25^+$, p=0.005.

2.4 CD25⁺CD4⁺ cells limit the systemic expansion of CD25⁻CD4⁺ cells *in vivo*

Previous studies have shown that $CD25^+CD4^+$ T cells inhibit the expansion of normally responsive T cells both *in vitro* [25, 26] and *in vivo* [27]. We therefore assessed whether in our system $CD25^+$ cells control the expansion of $CD25^-$ cells equally in organs other than the lungs. To follow the expansion of each cell population, $CD25^-$ and $CD25^+$ cells were prepared from C57BL/6-Thy1.1 and -Thy1.2 mice, respectively. Animals receiving $CD25^-$ cells alone contained more $CD4^+$Thy1.1$^+$ cells than those receiving both subsets not only in the lung but also in the spleen and in the mesenteric LN (Table 1), indicating that $CD25^+$ cells have the capacity to limit the systemic expansion of $CD25^-$ cells *in vivo*. The number of $CD4^+$Thy1.2$^+$ cells recovered from the co-transferred recipients was ~3-fold higher than the number of the injected cells, confirming that, despite their inhibitory activity, $CD25^+CD4^+$ T cells can expand when transferred into RAG-$2^{-/-}$ recipients [27].

2.5 Infiltration of CD25⁺CD4⁺ cells in the lung correlates with the PC load and the ongoing inflammatory response

The significant representation of $CD25^+CD4^+$cells in the lung of PC infected mice (Fig. 3C and Table 1) together with the recent report that human $CD4^+CD25^+$ cells display a specific chemotactic response [28] prompted us to investigate whether their lung infiltration is dependent on the PC infection. For this purpose, Thy1.1 $CD25^-CD4^+$ and Thy1.2 $CD25^+CD4^+$ cells were prepared by cell sorting, such that the purity was routinely 99% and 98%, respectively. Monitoring the PC load in successive litters of RAG-$2^{-/-}$ mice revealed a progressive increment of the pathogen load, such that 1 year after rederivation animals showed similar amount of PC DNA in the lung to those used in the first set of experiments described above (Fig. 7A). In an attempt to also include non-infected animals in our analyses, we purchased RAG-$1^{-/-}$ mice from the Jackson laboratory. As shown in Fig. 7A, these animals were not free of PC, although the level of infection was much lower than in any RAG-$2^{-/-}$ mice bred in our facility. These two groups of animals received either population alone or together, and were analyzed 10 days later. Heavily infected mice that received $CD25^-$ cells alone were dead or moribund by this time (n=8). The number of Thy1.2 ($CD25^+$) cells recovered from the lung after single transfer or co-transfer with Thy1.1 ($CD25^-$) cells was one to two orders of magnitude higher in heavily than lightly infected animals (Fig. 7B), although constant in the spleen (not shown). Consistently, the later the experiment was done after rederivation of the RAG-$2^{-/-}$ animals, the higher the numbers. These results indicate that infection and ongoing inflammation targets the migration and/or expansion of both $CD25^-$ and $CD25^+CD4^+$ cells.

3 Discussion

The present work shows that RAG-$2^{-/-}$ mice heavily infected with PC rapidly die upon adoptive transfer of $CD25^-CD4^+$ T cells as a consequence of acute pneumonia. In contrast, adoptive transfer of $CD25^+CD4^+$ T cells does not affect survival of the recipients and prevents development of the disease induced by the $CD25^-CD4^+$ T cell transfer.

Table 1. Distribution of CD4⁺ T cell subsets in the rederived RAG-2$^{-/-}$ mice 5 or 6 weeks after adoptive transfer

Numbers and phenotype of the injected CD4⁺ T cells	n	Cell population	No. of CD4⁺ Thy1⁺ cells ($\times 10^{-5}$)[a) Lung	Spleen	Mesenteric LN
3×10^5 CD25⁻Thy1.1⁺	5	Thy1.1⁺	12.0 ± 6.7[b)] (24.9)[c)]	33.3 ± 1.1 (69.1)	2.9 ± 1.3 (6.0)
3×10^5 CD25⁻Thy1.1⁺ + 3×10^5 CD25⁺ Thy1.2⁺	3	Thy1.1⁺	0.85 ± 0.73 (14.6)	4.8 ± 1.7 (82.3)	0.18 ± 0.21 (3.1)
		Thy1.2⁺	0.20 ± 0.11 (2.2)	8.6 ± 1.3 (95.1)	0.24 ± 0.28 (2.7)
		Total	1.05 ± 0.84 (7.1)	13.4 ± 2.7 (90.1)	0.42 ± 0.49 (2.8)

a) Cells were counted and phenotyped by flow cytometric analysis using anti-Thy1.1, Thy1.2, and CD4 mAb.
b) Absolute numbers of CD4⁺Thy1⁺ cells were calculated by multiplying the percent positive cells by the total numbers of mononuclear cells. Mean ± SD.
c) Numbers in parentheses are the relative distribution of each T cell population expressed as percentage of the total cells recovered from the three organs.

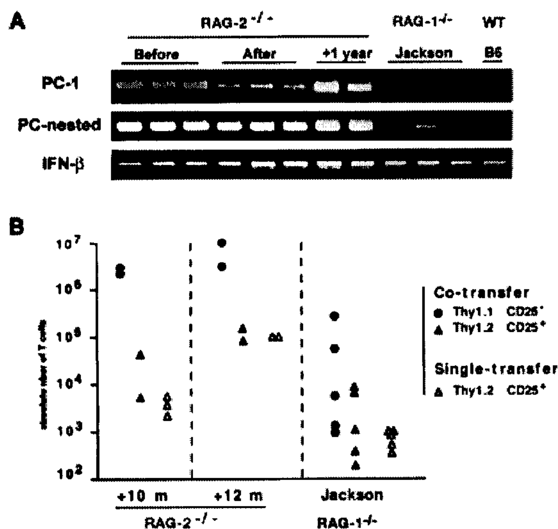

Fig. 7. PC infection targets infiltration of CD25⁺CD4⁺ cells to the lung. (A) Evolution of PC load in various unreconstituted mice. Lung DNA prepared from RAG-2$^{-/-}$ control animals used either in Fig. 2 (before rederivation), in Fig. 4 (second generation after rederivation), 12 months after rederivation (+1 year) or from recently purchased RAG-1$^{-/-}$ animals (Jackson) were submitted to PCR. PC-1, single PCR with primers pAZ102-H/pAZ102-E. PC-nested, nested PCR with primers pAZ102-X/pAZ102-Z. Total DNA content is shown to be homogenous after non-saturating PCR for IFN-β gene sequence. (B) Thy1.1 (CD25⁻) and Thy1.2 (CD25⁺) cell number recovered from the lung, 10 days after transfer. Mice received 3×10^5 Thy1.2 cells alone (empty symbols) or 3×10^5 of each population (filled symbols).

Our analyses reveal the relevance of testing for PC infection when following immunodeficient animals. PC is innocuous for immuno-competent animals and is, to this date, not included in the definition of the specific pathogen-free condition. The level of infection in SCID animals has been shown to worsen with age, and peak at about 60 days [23]. We now report that the PC load increases also with the age of the RAG-2$^{-/-}$ colony, and noticed that it associates with progressive inefficient breeding abilities. Our attempt to eradicate the infection with antibiotic treatments failed. However, our RAG-1$^{-/-}$ B10PL colony, maintained with breeding pairs that are systematically reconstituted with 3×10^7 wild-type splenocytes, consistently tested negative (not shown). Whether this protection is due to the breeding strategy or specific genetic background is currently being tested.

Several lines of evidence indicate that the cause of death in the experimental animals was an acute pneumonia induced by CD25⁻CD4⁺ T cells which were triggered by the pulmonary PC infection. It was previously reported that adoptive transfer of 1×10^5 CD4⁺ T cells induces a lethal pulmonary hyperinflammation in PC-infected SCID mice [23]. We show that the acuteness of the fatal disease in animals receiving CD25⁻CD4⁺ T cells correlated with the level of infection, and that moribund animals present extensive infiltration of inoculated CD25⁻CD4⁺ T cells into the lung. None of the control RAG-2$^{-/-}$ animals, although infected to various degrees by PC, developed PCP. Finally, RAG-1$^{-/-}$ B10.PL mice which tested negative for PC did not develop lung inflammation upon adoptive transfer of CD4⁺ cells (not shown).

We show that migration of the CD25⁺CD4⁺cells to the lung is also determined by the level of infection, and does not only depend on the presence of CD25⁻CD4⁺ cells. This result correlates with recent findings showing

that human $CD25^+CD4^+$ cells specifically expressed the chemokine receptors CCR4 and CCR8 and respond vigorously to macrophage and mature dendritic cells secreting their specific ligands [28]. Moreover, $CD45RB^{low}CD4^+$ cells, which contain all $CD25^+$ cells, isolated from germ-free mice prevent IBD [20], indicating that regulation does not require previous Ag encounter. As extensively discussed elsewhere [29], these analyses suggest that $CD25^+CD4^+$ migrate to the inflamed area, where they adjust immune responses to "appropriate" levels.

Our findings that transfer of $CD25^+CD4^+$ cells inhibits both the pulmonary inflammation and the elimination of the pathogen mediated by $CD25^-CD4^+$ cells provide the first evidence that the $CD25^+CD4^+$ population can control protective immunity to foreign pathogens. These results illustrate the nature of $CD25^+CD4^+$ T_{reg} as a "double-edged sword" capable of inhibiting both deleterious and advantageous aspects of an immune response. Considering the efficient innocuous immune response mounted by normal animals [7, 8], it is likely that immune suppression mediated by T_{reg} is not an all-or-nothing event but rather operates in a quantitative manner, which would depend on the balance between effector and T_{reg} numbers. The peripheral $CD4^+$ T cell compartment of a normal mouse contains 5–10% of cells expressing the CD25 marker, while in our experiments we transferred equal numbers of $CD4^+$ T cells of either population. In our assay, the quantitative balance between the two populations was therefore biased toward T_{reg} and may have induced "tolerance" to PC Ag. Further experiments involving adoptive transfer of various numbers and ratios of $CD25^+$ and $CD25^-CD4^+$ cells into RAG-$2^{-/-}$ mice will help clarify the quantitative aspects of the immune regulation by $CD25^+CD4^+$ T cells.

Our analyses may provide a rationale for a number of unexplained phenomena in the immunopathology of PC infection both in mice and humans. Adoptive transfer of 5×10^5 but not of 50×10^5 LN cells induces a fatal pulmonary inflammation in PC-infected SCID mice [2]. Similarly, inoculation of PC in irradiated but not in normal mice induces interstitial pneumonia [8]. In both cases, the inflammatory response is mediated by $CD4^+$ T cells, indicating that a reduction in total $CD4^+$ T cell numbers causes the development of pathological reactions to PC. It is therefore likely that the PC-driven immunopathology observed in lymphopenic animals results from a relative deficiency in the $CD25^+CD4^+$ T_{reg} population. In humans, several reports indicate that the association between PCP and the use of corticosteroids for cancer and bone marrow transplant patients is usually observed when the dosage is being reduced [3–5]. Corticosteroid-treatments are known to induce a state of lymphopenia, which may tilt the balance between effector cells and T_{reg} in favor of the former. Tapering of the immunosuppressive drug treatments will thus promote the activities of the effector cells now released from the control of T_{reg}. Recent studies showed the presence of a $CD25^+CD4^+$ T_{reg} population in humans [30, 31]. It is therefore likely that immunosuppressive therapies create a situation where the PC-reactive T cells escape from the regulatory activity of $CD25^+CD4^+$ T cells and participate in a pathological inflammatory response. Several reports have revealed an indispensable role for IL-10 [27], TGF-β [19, 32], and CTLA 4 [19, 33] in the immunoregulatory activities of $CD25^+CD4^+$ T cells. It will be critical to determine whether these molecules play an essential role in the control of PCP by T_{reg} to establish new therapeutic strategies for treatments of PCP patients.

4 Materials and methods

4.1 Mice

C57BL/6 and C57BL/6-*Thy1^a^,Igh^a^,Gpi1^a^*, and C57BL/6-RAG-$2^{-/-}$ mice were bred and maintained under specific pathogen-free conditions in our animal facility. Thy1.1 and RAG-$2^{-/-}$ mice were originally purchased from the Jackson Laboratory (Bar Harbor, ME) and CDTA (Orleans, France), respectively. C57BL/6-RAG-$1^{-/-}$ were purchased from the Jackson Laboratory at 6–8 weeks of age and utilized 2–3 weeks after their arrival. Rederivation of our RAG-$2^{-/-}$ colony was performed by embryo transfer in the oviduct [34]. For all experiment, donor and recipient mice were 2–3 months old.

4.2 Antibodies

The following mAb were purchased from PharMingen (San Diego, CA); PE- or allophycocyanin-conjugated anti-CD4 (RM4–5); FITC-labeled CD25 (7D4) and CD90.2 (Thy1.2) (53–2.1); biotinylated CD8α (53–6.7); CD11b (Mac1) (M1/70); CD19 (1D3); CD90.1 (Thy1.1) (OX-7); NK1.1 (PK136) and γδ TCR (GL3). Anti-CD25 mAb (PC61) was produced in the laboratory and coupled to Alexa Fluor™ 488 (Molecular Probes, Eugene, OR).

4.3 Purification of peripheral T cell subsets and T cell reconstitution

For magnetic purification, pooled LN cells and erythrocyte-depleted splenocytes were first enriched for $CD4^+$ lymphocytes. In brief, cell suspensions were stained with biotinylated mAb for CD8α, CD11b, CD19, γδ TCR, and NK1.1 and further incubated with Streptavidin-Dynabeads (Dynal, Oslo, Norway) before magnetic depletion. The $CD4^+$ cell-enriched fraction, was stained with FITC-anti-CD25 followed by anti-

FITC microbeads (Miltenyi Biotec, Bergisch-Gladbach, Germany) before separation on an LS⁺ column (Miltenyi Biotec). The positive fraction provided the CD25⁺CD4⁺ population. The negative fraction was further labeled with anti-CD4 (L3T4) microbeads (Miltenyi Biotec) and positively separated on a new LS⁺ column, providing the CD25⁻CD4⁺ population. In the last set of experiments (Fig. 7), pooled LN cells were stained with anti-CD4-PE and CD25-Alexa and cell sorting was performed on an MoFlo High Speed Cell-sorter (Cytomation Inc., Fort Collins, CO). RAG-$2^{-/-}$ or RAG-$1^{-/-}$ mice were injected i.v. with 200 μl of the purified CD4⁺ T cell subsets suspended in PBS.

4.4 Isolation of mononuclear cells from organs and flow cytometry

Single-cell suspension from spleen, mesenteric LN, and lung cells were obtained by forcing the organs through nylon mesh. Lung lymphocytes were isolated from the whole lung but the right superior lobe and separated using Lympholyte-M (Cedarlane Laboratories, Hornby, Canada). Cells were incubated for 20 min at 4°C in 50 μl of Ab solutions diluted in PBS/2% FBS containing 0.02% NaN_3. Biotinylated Ab were revealed with FITC- or PE-labeled streptavidin (PharMingen). Samples were suspended in 1 μg/ml propidium iodide to gate out dead cells, and analyzed on a FACSCalibur instrument (Becton Dickinson, Mountain View, CA).

4.5 Histological examination

The colon and half of the right superior lobes of the lungs were fixed in Zamboni's fixative, and 10-μm paraffin-embedded sections were stained with hematoxylin and eosin. Mononuclear cell infiltration in the lung was scored blindly as follows: 0, no infiltrates; 1, focal and mild infiltration restricted to the perivascular and peribronchiolar regions; 2, diffuse and intense infiltration in the alveolar spaces, perivascular, and peribronchiolar regions.

4.6 Quantitative PCR for determination of relative amounts of PC in the lung

Lung genomic DNA was prepared from the other half right superior lobe. Tissues were lysed in 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 100 mM NaCl, 0.5% SDS, 100 μg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) and DNA extracted by phenol-chloroform-isoamylalcohol. All PCR reactions (50 μl) contained ~20 ng genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 μM sense and antisense primer, and 1.25 U Taq DNA polymerase (Life Technologies, Gaithersburg, MD) in the supplier's buffer, and carried out in a PTC-100™ Programmable Thermal Controller (MJ Research Inc., Watertown, MA). For PC detection, the first amplifications consisted of 2.5 min at 94°C followed by 18 (unless otherwise mentioned) cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s, and terminated by 5 min at 72°C. In some experiments, a nested reaction was set, using 2 μl of the first PCR products as templates, and followed identical protocol. The outer (pAZ102-H/pAZ102-E) and internal primers (pAZ102-X/pAZ102-Z) were described previously [35, 36]. When testing RAG-$2^{-/-}$ animals, total DNA amount was assessed by PCR amplifications of the mouse RAG-1 gene with the specific primers 5'-CTTCGGAATGC-CGAGAAAGT-3' and 5'-TGTGAAGGGACCATTCAGGT-3'. The PCR condition was as above except for 30 cycles. The PCR products were visualized by ethidium bromide staining after separation on a 2% agarose gel, and the fluorescence intensities of the specific bands were quantified with an Eagle Eye®II Still Video System (Stratagene, La Jolla, CA). When analyzing RAG$2^{-/-}$ mice, samples of genomic DNA were first normalized to contain the same quantity of RAG-1 sequences by serial dilutions, and then relative quantities of the rRNA sequences were determined. For analyses of the evolution of the PC load in various strains of mice (Fig. 7A), amount of lung DNA in each sample was normalized after non-saturating PCR reaction specific for the mouse Interferon-β gene sequence. Primers were sense 5'-TATCTTCAGGGCTGTCTCCTTTCT-3' and antisense 5'-GGCATAGTTACTAGTTGTAACAGC-3'. The reaction was similar to others except for 25 cycles.

4.7 Statistical analysis

Analysis was performed using Student's *t*-test, unless otherwise indicated. Differences were considered statistically significant with $p<0.05$.

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<u>Manuscript II.</u>

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Regulatory T Cells Selectively Express Toll-like Receptors and Are Activated by Lipopolysaccharide

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Abstract

Regulatory CD4 T cells (Treg) control inflammatory reactions to commensal bacteria and opportunist pathogens. Activation of Treg functions during these processes might be mediated by host-derived proinflammatory molecules or directly by bacterial products. We tested the hypothesis that engagement of germline-encoded receptors expressed by Treg participate in the triggering of their function. We report that the subset of CD4 cells known to exert regulatory functions in vivo ($CD45RB^{low}$ $CD25^+$) selectively express Toll-like receptors (TLR)-4, -5, -7, and -8. Exposure of $CD4^+$ $CD25^+$ cells to the TLR-4 ligand lipopolysaccharide (LPS) induces up-regulation of several activation markers and enhances their survival/proliferation. This proliferative response does not require antigen-presenting cells and is augmented by T cell receptor triggering and interleukin 2 stimulation. Most importantly, LPS treatment increases $CD4^+$ $CD25^+$ cell suppressor efficiency by 10-fold and reveals suppressive activity in the $CD4^+$ $CD45RB^{low}$ $CD25^-$ subset that when tested ex-vivo, scores negative. Moreover, LPS-activated Treg efficiently control naive CD4 T cell–dependent wasting disease. These findings provide the first evidence that Treg respond directly to proinflammatory bacterial products, a mechanism that likely contributes to the control of inflammatory responses.

Key words: inflammation • tolerance • lymphocytes • regulation • innate immunity

Introduction

It is established that a subpopulation of CD4-αβ T cells controls inflammatory responses to commensal bacteria and pathogens (1–4). These cells are encompassed in the naturally activated lymphocytes ($CD45RB^{low}$) and enriched in the CD25-expressing subset. In the absence of regulatory T cells (Treg),* alymphoid animals reconstituted with CD4 cells isolated from normal mice develop severe inflammatory bowel disease if colonized by enteric bacteria (1), or lethal pneumonia if infected by *Pneumocystis carinii* (2). Cotransfers of Treg protect from disease by inhibiting both the protective (2) and inflammatory responses (1, 2), leading to the notion of "quality control" of the immune response (for review see reference 5).

These activities of Treg suggest their engagement at early stages of infection/inflammation. Accordingly, evidence exists for rapid Treg migration to inflammatory sites, likely as a result of the constitutive expression of chemokine receptors (6) and high sensitivity to inflammatory chemokines (7). Once at the site of infection, however, it remains unclear whether activation of Treg function is triggered by components of the host inflammatory response or by direct recognition of microbial products. In either case, engagement of germline-encoded receptors is an attractive possibility. Toll-like receptors (TLRs) ensure vertebrates with the means to recognize a vast range of microbial products and produce immediate, "innate" responses. Thus, TLRs expressed in B lymphocytes trigger effector functions such as antibody production, providing a direct link between innate and adaptive immunity (8, 9). Furthermore, endogenous molecules such as heat shock proteins (HSP; references 10–13) or oligosaccharides of hyaluronan (14) have recently been shown to functionally ligate TLR-4 in macrophages or dendritic cells, and may participate in inflammatory reactions. On the other hand, although TLR-4 expression in murine $CD3^+$ lymphocytes (15) and more specifically in particular subsets of γδ T cells (16) has been reported, expression of TLR genes in CD4-αβ T cell subpopulations has not been directly assessed.

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**Abbreviations used in this paper:* HSP, heat shock proteins; $I_{50\%}$, 50% inhibition; TLR, Toll-like receptor; Treg, regulatory T cells.

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In these experiments, we directly tested whether proinflammatory microbial products activate CD4 cells involved in the control of inflammatory reactions. We first monitored the expression profile of all nine murine TLR genes thus far identified in various subsets of CD4 T cells. The results show that TLR-4, -5, -7, and -8 are selectively expressed in CD4 T cell subsets that contain Treg. We then established that $CD4^+$ $CD25^+$ cells, unresponsive to TCR triggering, are activated and proliferate when treated with the TLR-4 ligand LPS. This proliferative response does not require APC, is augmented by TCR triggering, and synergizes with IL-2 stimulation. Finally, we show that exposure to LPS markedly increases Treg activity as measured in suppression assays in vitro and maintains their regulatory function in vivo.

Materials and Methods

Mice

All animals were bred and maintained under specific pathogen-free conditions in our animal facilities. C57BL/6 and C57BL/6-*Thy1.1* mice were originally purchased from The Jackson Laboratory. C57BL/10ScCr mice (B10ScCr) were obtained from CDTA. C3H/HeJ and C3H/HeN were purchased from The Jackson Laboratory. C57BL/6-*H-2*^{u} and RAG-$1^{-/-}$ animals were provided by S. Tonegawa, MIT, Boston, MA. C57Bl-*H-2*^{u} Thy1^a mice have been previously described (17). All animals used in this study were 6–8 wk old.

Cell Purification

Erythrocyte-depleted splenocytes and LN cells were prepared as previously described (17). For flow cytometry purification of $CD4^+$ cell subsets, pooled LN cells were stained with Cy-Chrome-conjugated CD4 mAb (clone RMA-5; BD Biosciences), Alexa Fluor™ 488-CD25 (PC61; produced in the laboratory), and CD45RB-PE (clone 16A; BD Biosciences). In some experiments, the following mAbs were pooled and used in addition to exclude nonconventional CD4 cells: B220-PE (RA3-6B2), CD11c-PE (clone HL3), PanNK-PE (DX-5), Mac I biotin (clone M1/70), and MHC-II biotin (clone M5-114; all from BD Biosciences). Biotinylated Abs were revealed with PE-labeled Streptavidin (BD Biosciences). The reference B cell population was sort-purified after staining with CD19-FITC (clone 1D3; BD Biosciences). Cell sorting was performed on a MoFlo® high speed cellsorter (DakoCytomation). The purity of each cell preparation was >97%. $CD4^-$ contaminants in the $CD4^+$ $CD25^+$ preparations represented routinely 0.5%. $CD4^+$ $CD25^-$ cells used in the suppression assays were prepared by magnetic purification. Total LN and erythrocyte-lysed splenocytes were first depleted of $CD25^+$ cells by treatment with 7D4 mAb (produced in the laboratory) and complement (low-tox rabbit complement; Cedarlane). Cells were then stained with anti-CD4 (L3T4) microbeads and positively separated on LS^+ columns (both from Miltenyi Biotec). Erythrocyte-depleted splenocytes, treated with J1J anti-Thy1 mAb (produced in the laboratory) and complement, were irradiated at 30 Gy and used as a source of APC. Additional antibodies used for FACS® analyses were: class I (H2Kb AF6-88.5) and B.7 (16-10A1), CD69 (H1.2F3), CD44 (IM7), CD38 (90), TCRβ (H57-597), Thy1.1 (OX-7), and Thy1.2 (53-2.1; all from BD Biosciences). FACS® analyses were performed on a FACSCalibur® instrument run with the CellQuest™ program (both from Becton Dickinson). Dead cells were gated out after propidium iodide staining.

RT-PCR Reactions

Total RNA was extracted from 10^6 cells using Trizol reagent, treated with DNase I and reverse transcribed using Superscript II RT and oligo(dT)$_{12-18}$ primer (all four reagents from Life Technologies). The amount of cDNA in each sample was first normalized after nonsaturating PCR for HPRT transcripts. 25 μl reaction mixture contained 1.5 mM $MgCl_2$, 0.2 mM dNTP, 25 pmol sense and antisense primer (5'-GTAATGATCGTCAACGGGGGAC and 5'-CCAGCAAGCTTGCAACCTTAACCA), and 1 U Taq DNA polymerase (Life Technologies) in the manufacturer's buffer. PCR consisted of 5 min at 94°C followed by 26 cycles of 30 s at 94°C, 55°C, and 72°C, terminated by 10 min at 72°C. The specific TLR primer sequences and their respective annealing temperatures are shown in Table I. Other conditions were as described above except for 35 cycles. Reactions were not saturated as controlled by increasing the amount of template (not depicted). All PCRs were performed on a PTC-100™ programmable thermal controller (MJ Research Inc.). After separation on 2% agarose gels containing ethidium bromide (Sigma-Aldrich), the specific bands were quantified with an Eagle Eye® II still video system (Stratagene).

Cell Cultures and Proliferation Assays

All cultures were set in RPMI-1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml

Table I. *Primer Sequences for Mouse TLR RT-PCR Used in This Study*

	Nucleic sequences[a]	Tp[b]
TLR-1	TCTCTGAAGGCTTTGTCGATACA GACAGAGCCTGTAAGCATATTCG	56
TLR-2	TCTAAAGTCGATCCGCGACAT TACCCAGCTCGCTCACTACGT	58
TLR-3	TTGTCTTCTGCACGAACCTG CGCAACGCAAGGATTTTATT	58
TLR-4	CAAGAACATAGATCTGAGCTTCAACCC GCTGTCCAATAGGGAAGCTTTCTAGAG	62
TLR-5	ACTGAATTCCTTAAGCGACGTA AGAAGATAAAGCCGTGCGAAA	56
TLR-6	AACAGGATACGGAGCCTTGA CCAGGAAAGTCAGCTTCGTC	58
TLR-7	TTCCGATACGATGAATATGCACG TGAGTTTGTCCAGAAGCCGTAAT	56
TLR-8	GGCACAACTCCCTTGTGATT CATTTGGGTGCTGTTGTTTG	58
TLR-9[c]	CCGCAAGACTCTATTTGTGCTGG TGTCCCTAGTCAGGGCTGTACTCAG	62

[a]Sequences are written 5' to 3', sense and antisense primers successively.
[b]Annealing temperature in °C.
[c]Reference 48.

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gentamycin, 50 μM 2β-ME, 10 mM Hepes, and 1 mM sodium pyruvate (all from Life Technologies).

For PCR controls, sort-purified CD4$^+$ CD25$^-$ CD45RB^{high} (10^6/well in 24-well plates) were stimulated for 3 d with 1 μg/ml plate-bound anti-CD3 mAb (145.2C11; produced in the laboratory) and 1 μg/ml soluble anti-CD28 mAb (clone 37.51; BD Biosciences).

LPS-induced Proliferation. Cultures were set in triplicates in 96-well plates maintained for 3 d at 37°C, 5% CO2. Each well contained 2.5×10^4 purified CD4$^+$ cells with or without 5×10^4 APC, 0.5 ug/ml anti-CD3 mAb, IL-2 from X63-IL2 cell supernatant (~10 U/ml) diluted at 1/500, and 10 μg/ml LPS from *Salmonella typhymurium* (Sigma-Aldrich). In experiments comparing cells purified from WT, B10ScCr, and C3H/HeJ animals, LPS from *Escherichia coli* EH100 was HPLC purified (provided by C. Galanos, Max-Planck Institute for Immunobiology, Freiburg, Germany).

IL-2 Production. Primary cultures were set in triplicate in 96-well plates containing 100 μl medium. After 48 (CD4$^+$ CD25$^-$ cells) or 72 (CD4$^+$ CD25$^+$ cells) h, 50 μl of the supernatant was transferred to a new well containing 1,000 CTLL-2 cells and 50 μl fresh medium. Amplification of the response was achieved 48 h later by adding saturating IL-2 (as described above) for 24 h.

LPS Pretreatments. Sort-purified CD4$^+$ subpopulations ($1.5–2 \times 10^6$/well) were seeded in 24-well plates for 3 d in the presence of 10 μg/ml LPS (Sigma-Aldrich) with or without 1 μg/ml soluble or plate-bound anti-CD3 mAb. In some experiments, after washing in media, cells were cultured for an additional 3 d in the presence of IL-2 and 1 μg/ml soluble anti-CD3 mAb. To assess the induction of surface molecules, erythrocyte-lysed splenocytes were plated at 1.5×10^6/ml in medium alone or supplemented with 10 μg/ml LPS (Sigma-Aldrich) for 18 h.

Suppression Assay. CD4$^+$ CD25$^-$ cells (target cells) were plated at 2.5×10^4/well in U-shape 96-well plates together with 10^5 APC and 0.5 μg/ml anti-CD3 mAb and variable numbers of the suppressor populations under test. Each dilution was set in triplicate and culture was maintained for 3 d. All proliferations were monitored by addition of [^{3}H]thymidine (1 μCi/well; Amersham Biosciences) for the last 6 h of culture.

Adoptive Transfer

Pooled LN from C57BL-*H-2^u Thy1.2* and C57BL-*H-2^u Thy1.1* mice were used to sort purify CD4$^+$ CD25$^-$ and CD4$^+$ CD25$^+$ cells, respectively. C57BL-*H-2^u* RAG-1$^{-/-}$ recipients were injected intravenously at 8 wk of age. Weight and general health status was monitored every 3 d for 21 d.

Results

Selective Expression of TLR-4, -5, -7, and -8 by Treg. LN lymphocytes were FACS® purified under highly stringent gate definitions according to the CD4 and CD45RB surface markers and TLR-4 expression was assessed in the various subsets by RT-PCR in nonsaturating conditions. Naive CD4$^+$ cells (CD45RB^{high}) scored negative whereas the samples of activated/memory CD45RB^{low} cells prepared in parallel displayed a clear signal (Fig. 1 a). Additional fractionation of the CD4$^+$ CD45RB^{low} subset according to the expression of the CD25 molecule revealed the heterogeneity of this population. The RT-PCR signal was reproducibly three- to fourfold higher in the CD25$^+$ compared with the CD25$^-$ subset (Fig. 1 b). The PCR product obtained in either CD45RB^{low} population was cloned and sequenced to confirm its identity with the published TLR-4 sequence (not depicted). Expression of TLR-4 by CD4 cells is not merely the result of activation because naive CD4$^+$ cells (CD45RB^{high} CD25$^-$) activated in culture for 3 d using plate-bound anti-CD3 together with anti-CD28 acquire a CD45RB^{low} CD25$^+$ phenotype (not depicted), but continue to score negative for TLR-4 expression in the RT-PCR assay (Fig. 1 b). Finally, analyses of CD4 cells in various lymphoid tissues (thymus, mesenteric LN, axillary LN, and spleen) revealed preferential expression of TLR-4 in CD4$^+$ CD25$^+$ cells independently of their location, although more marked in cells purified from the spleen (Fig. 1 c). Therefore, TLR-4 expression in CD4 T cell subsets is restricted to subpopulations with known in vivo regulatory functions and may represent a specific marker for Treg differentiation.

These results prompted us to monitor the expression of all identified murine TLR genes in the same CD4 T cell subsets. As shown in Fig. 1 c, three additional TLRs (TLR-5, -7, and -8) were found preferentially expressed by CD4$^+$ CD45RB^{low} cells. TLR-5 expression was undetectable in B and naive CD4 T cells and, similarly to TLR-4, markedly increased in the CD25$^+$ subset when compared with CD45RB^{low} CD25$^-$ cells (fourfold). TLR-7 transcripts were detectable in naive CD4 cells albeit to a lower level than in the naturally activated subset of CD4$^+$ cells (fourfold and twofold, respectively, when compared with CD45RB^{low} CD25$^+$ and CD25$^-$). Finally, TLR-8 ap-

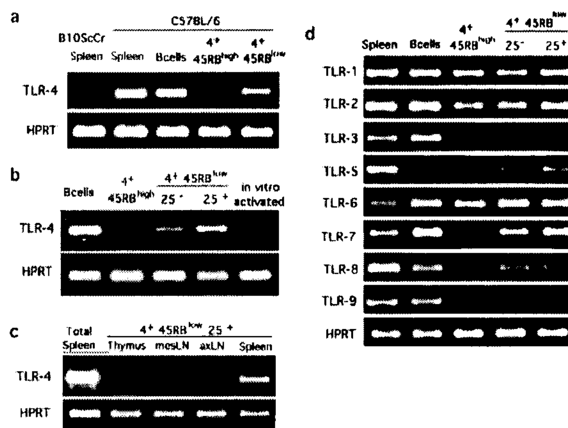

Figure 1. Specific expression of TLR genes by Treg. RNA from sort-purified CD19$^+$ (B cells) and various subpopulations of CD4 cells (4$^+$) from B6 mice, either pooled LNs (a, b, and d) or specific lymphoid organs (c) were submitted to RT-PCR. CD4$^+$ CD45RB^{low} or CD4$^+$ CD45RB^{high} (4$^+$ 45RB^{low} or 4$^+$ 45RB^{high}) were sorted as negative for CD11c, CD11b, B220, pan-NK, and MHC II in addition to positive for CD4 and CD45RB (a) or simply according to CD4, CD45RB, and CD25 expression (b, c, and d). Erythrocyte-lysed splenocytes from B6 or TLR-4–deficient B10ScCr animals and in vitro–activated B6 CD4$^+$ CD45RB^{high} served as controls. All RT-PCR were performed at least twice on independent samples.

peared specifically expressed in the CD4⁺ CD45RB^{low} compartment independently of its CD25 phenotype. Similarly to TLR-4, expression of these genes was not induced by in vitro activation of purified naive CD4⁺ T cells (not depicted). Less relevant for this study but noteworthy in the interplay of innate and adaptive immunity, expression of TLR-1, -2, and -6 was readily detectable in all CD4⁺ cell populations analyzed. Finally, expression of both TLR-3 and TLR-9 was clearly detectable in total splenocytes but not significantly in any of the CD4 T cell subsets.

LPS Treatment Induces the Expression of Several Activation Markers on CD4⁺ CD25⁺ T Cells. Next, we investigated whether the expression of TLR-4 by CD4⁺ CD45RB^{low} CD25⁺ cells was functionally relevant, i.e., if such cells responded to LPS, the classical TLR-4 ligand for B cells and macrophages (18). In a first step, we performed FACS® analyses of splenocytes treated for 18 h with LPS in vitro. As shown in Fig. 2, CD4⁺ CD25⁺ cells, similarly to macrophages and B lymphocytes, up-regulate the expression of Class I, CD69, and B7.1 surface molecules. In addition, expression of other T cell–specific activation markers (CD44 and CD38) were also significantly enhanced whereas the levels of TCR expression were not affected. In contrast, expression of CTLA-4, cytoplasmic IL-10, and TNF-α remained unaltered upon exposure to LPS (not depicted). Finally, CD4 ⁺CD25⁻ cells failed to up-regulate any of these molecules when exposed to LPS in the same conditions. Up-regulation of CD69 expression by T cells upon exposure to LPS in vitro has been previously described (19) and is a response we attribute to CD4⁺ CD25⁺ cells. A previous report also described the induction of B7 expression on CD4⁺ CD25⁺ cells upon TCR triggering (20), a finding that we now extend to LPS activation.

LPS Directly Induces CD4⁺ CD25⁺ Cell Survival/Proliferation. Previous studies indicate that LPS stimulation of B cells in vitro enhances their survival (21, 22). Similarly, survival of purified CD4⁺ CD25⁺ cells maintained in culture was greatly enhanced by the addition of LPS. Thus, although the number of cells recovered after a 3-d culture in medium supplemented or not with anti-CD3 antibody represented routinely 1–2% of the initial number of seeded cells, these values reached ~15% in cultures containing anti-CD3 antibody and LPS or IL-2 and ~30% when media was supplemented with both LPS and IL-2. In 6-d cultures containing anti-CD3 and IL-2, the addition of LPS for the first 3 d enhanced the cell recoveries from 32 to 62% (not depicted). These observations confirm that exocrine IL-2 acts as a survival/growth factor for CD4⁺ CD25⁺ cells (23) and demonstrates similar effects for LPS.

Because LPS is a potent B lymphocyte mitogen, we also tested its effect on CD4⁺ CD25⁺ proliferation. As reported (24, 25), highly purified CD4⁺ CD25⁺ cells do not proliferate in APC-supplemented cultures in response to TCR ligation whereas they do expand in the presence of IL-2. The addition of LPS to otherwise unresponsive cultures containing anti-CD3 antibodies and APC induced a readily detectable incorporation of [^{3}H]thymidine that was consistently 20-fold higher than in controls, although 5-fold lower than IL-2–induced responses (Fig. 3 a). The removal of APC from these cultures resulted in higher [^{3}H]thymidine incorporation in response to LPS while reducing the IL-2–mediated response. The responses of CD4⁺ CD25⁺ cells to LPS are also detectable in the absence of TCR triggering, albeit to a much lower level (Fig. 3 b, note the different scale). Interestingly, in the absence of both APC and anti-CD3 antibodies, responses to LPS scored higher than those to IL-2. Assessing the dose response of CD4⁺ CD25⁺ to LPS treatment (Fig. 3 c) reveals a peak of proliferation at 10 μg/ml, a result similar to what is routinely obtained when testing B lymphocytes (not depicted). This dose is much higher than that required to induce maximal responses in components of the innate immune system like dendritic cells and macrophages (in the order of nanograms). Contrary with B cell responses to LPS and anti-BCR (26), proliferation of CD4⁺ CD25⁺ is increased by the addition of anti-CD3 independently of the dose of LPS. These results indicate that LPS treatment induces TCR triggering sensitivity whereas TCR engagement does not affect the sensitivity to LPS.

To directly assess whether LPS induces proliferation of CD4⁺ CD25⁺ cells through engagement of the TLR-4 receptor, we tested cells from LPS nonresponder and responder animals (27). In similar cultures to those described above, CD4⁺ CD25⁺ cells purified from TLR-4–deficient B10ScCr or C3H/HeJ (28, 29) mice did not respond to LPS (Fig. 3, d and e) regardless of the presence of APC or anti-CD3 (not depicted), but they did respond to IL-2. Furthermore, the LPS and IL-2 effects are additive in B6 animals but not in the B10ScCr T cells. Finally, using either B6 or B10ScCr APC altered neither the LPS response of B6 CD4⁺ CD25⁺ cells nor the unresponsiveness of TLR-4–deficient CD4⁺ CD25⁺ cells (not depicted), confirming that such T cell responses are not mediated by LPS activation of APC.

Taken together, these results demonstrate that LPS directly activates survival/proliferation of CD4⁺ CD25⁺ T cells through the TLR-4 receptor molecule.

TCR Triggering of LPS-activated CD4⁺ CD25⁺ Cells Induces IL-2 Production. The inability of CD4⁺ CD25⁺ cells to engage in cycle upon TCR triggering has been attributed

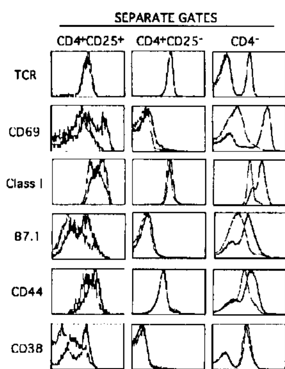

Figure 2. Activation of CD4⁺ CD25⁺ cells upon LPS treatment of splenocytes in vitro. Erythrocyte-depleted splenocytes were cultured for 18 h in the presence of LPS (bold) or medium alone (plain) and stained for CD4, CD25, and various other surface molecules. Histograms correspond to FACS® analyses for the indicated molecules inside the three independent gates. Representative analysis out of four independent assessments is shown. A minimum of 5,000 events was acquired in the CD4⁺ CD25⁺ gate.

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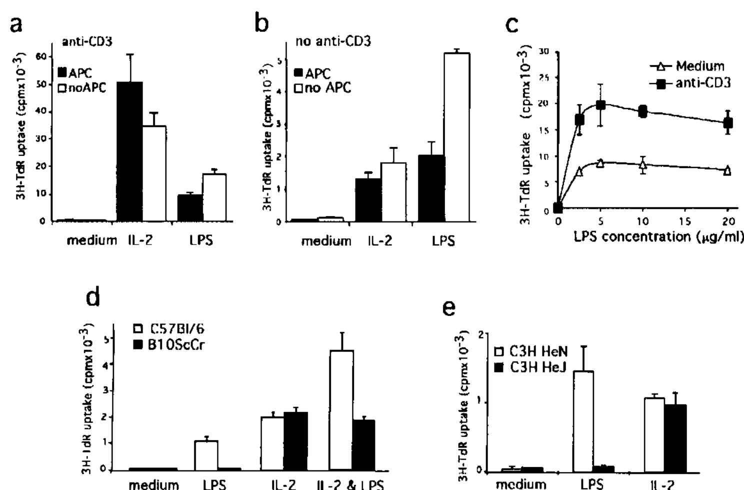

Figure 3. LPS directly induces CD4+ CD25+ proliferation through TLR-4. (a and b) Proliferative responses of CD4+ CD25+ cells to LPS are APC independent. Sort-purified CD4+ CD25+ from B6 animals were maintained in culture for 3 d in the presence of anti-CD3 (a) or not (b) and supplemented or not with APC (solid or open bars). For each culture condition, proliferative responses induced by either LPS or IL-2 are compared. (c) Purified CD4+ CD25+ cells were cultured for 3 d in the absence of APC in medium supplemented with various doses of LPS. (d and e) TLR-4–deficient CD4+ CD25+ cells do not proliferate in response to LPS. CD4+ CD25+ were sort purified from TLR-4–competent animals B6 (d) and C3H/HeN (e; open bars) or TLR-4–deficient B10ScCr (d) and C3H/HeJ (e) mice (solid bars) and maintained for 3 d in culture without APC nor anti-CD3. The medium was supplemented with LPS, IL-2, or both, as indicated. Each assay has been performed at least twice on independent cell samples and with similar results.

to their incapacity to produce IL-2 (24, 25). We described above that upon LPS stimulation, CD4+ CD25+ cells respond to TCR triggering by increased proliferation. This result may indicate that LPS induces endogenous IL-2 production. To directly address this point, we developed a highly sensitive assay to detect IL-2 produced by cells maintained in culture. As shown in Fig. 4 a, supernatants from 1,000 naive CD4 cells stimulated for 48 h in presence of APC and anti-CD3 induce readily detectable proliferation of the IL-2–dependent CTLL-2 cell line. This proliferation is proportional to the number of CD4 cells seeded. When testing the supernatants from 2.10^5 CD4+ CD25+ cells maintained in culture for 3 d in the presence of LPS, IL-2 production was undetectable (Fig. 4 b). However, similar cultures supplemented with anti-CD3 provided enough IL-2 to induce detectable proliferation of the CTLL-2 cells. Moreover, production of IL-2 was markedly enhanced when anti-CD3 was provided plate bound instead of soluble.

These results indicate that LPS per se does not induce endogenous IL-2 production. However, it seems to confer

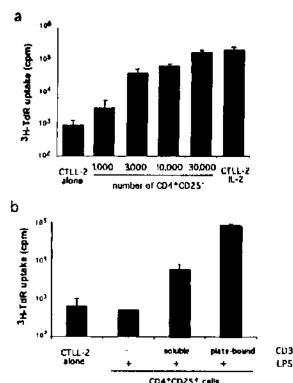

Figure 4. IL-2 production by CD4+ CD25+ cells upon LPS stimulation. (a) Sensitivity of the assay. Supernatant of various numbers of CD4+ CD25− cells cultured for 48 h in the presence of APC and anti-CD3 were used as a source of IL-2 to support CTLL-2 cell proliferation. Proliferation of CTLL-2 cells in the absence of supernatant (alone) or when they were provided with saturating amount of IL-2 (IL-2) are shown. (b) CTLL-2 proliferation induced by supernatants of 2.10^5 CD4+ CD25+ cells maintained in culture for 3 d in the presence of LPS and anti-CD3 as indicated. Background proliferation is shown on the left. Each assay has been performed twice, each in triplicates.

sensitivity to TCR triggering, a signal that in turn leads to a low level of IL-2 synthesis.

Exposure of CD45RB^{low} CD25+ or CD45RB^{low} CD25− T Cells to LPS Markedly Enhances Their Suppressive Functions. Next, we investigated whether LPS affects the effector functions of CD4+ CD25+ cells. Currently, the ability of CD4+ CD25+ cells to suppress the proliferative response of naive CD4 T cells to TCR triggering in the presence of APC is used as an in vitro correlate of their regulatory function in vivo (24, 25). According to the observations described above on LPS-induced survival/proliferation, we tested cells that were first differentially exposed to LPS for 3 d and then provided with IL-2 for an additional 3-d period. The inhibition of CD4+ CD25− proliferation is plotted in Fig. 5 as a function of the ratio of "regulatory" to target cells. The ratio corresponding to a 50% inhibition ($I_{50\%}$) can serve as an index of suppression efficiency. The $I_{50\%}$ of freshly isolated CD4+ CD25+ cells was ~0.25 (i.e., one regulatory to four target cells) in several independent experiments. As shown in Fig. 5 a, culturing this cell population for 6 d with IL-2 (and anti-CD3) improved the $I_{50\%}$ to 0.07 (1 to 14), a result consistent with previous findings (30). In the same conditions, however, exposure to LPS for the first 3 d resulted in even higher levels of suppressive activity, as indicated by an $I_{50\%}$ lower than 0.025, the last point in our titrations (1 to 40). We also tested CD4+ CD25+ cells that were pretreated with LPS in the absence or presence of plate-bound anti-CD3 antibodies. Independently of the presence of anti-CD3, the $I_{50\%}$ was consistently fourfold higher when cells had been exposed successively to LPS and IL-2, as compared with IL-2 alone (not depicted). These results demonstrate LPS-dependent activation of effector functions in CD4+ CD25+ cells.

CD4+ CD45RB^{low} CD25− cells exert regulatory functions in vivo (31), but do not show suppressor activity in vitro when freshly isolated (Fig. 5 b and reference 24). Strikingly, however, CD4+ CD45RB^{low} CD25− cells manifested a remarkable $I_{50\%}$ of ~0.1 (1 to 10) after in vitro ex-

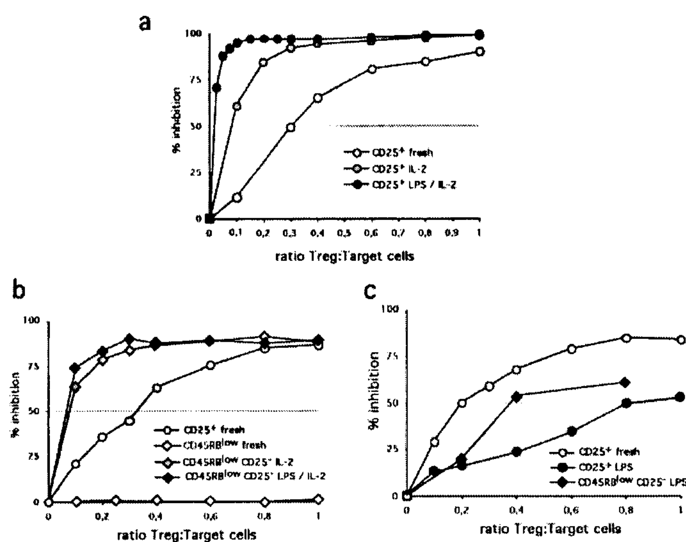

Figure 5. LPS treatment enhances the suppressor function of Treg. (a) Suppressor efficiency of CD4$^+$ CD25$^+$ is greatly enhanced upon exposure to LPS. Sort-purified CD4$^+$ CD25$^+$ T cells were maintained in culture in medium supplemented with anti-CD3 and either LPS for 3 d followed by 3 d with IL-2 (LPS/IL-2) or with IL-2 for 6 d (IL-2). The same cell population not submitted to culture was used as reference control (fresh). Proliferation of CD4$^+$ CD25$^-$ stimulated with anti-CD3 and APC in the presence of increasing numbers of these CD4$^+$ CD25$^+$ cells was monitored on day 3. The percent of inhibition ([cpm in control − cpm in experiment]/cpm in control) is plotted versus the ratio of CD4$^+$ CD25$^+$/CD4$^+$CD25$^-$ cell number at the origin of the culture. (b and c) LPS treatment of CD4$^+$ CD45RB^{low} CD25$^-$ cells reveals their suppressor functions. Sort-purified CD4$^+$ CD45RB^{low} CD25$^-$ cells were tested in suppression assays after 6 d culture (b) or after only 3 d exposure to LPS and anti-CD3. Suppression by CD4$^+$ CD25$^+$ treated similarly is also shown. (c) Freshly isolated CD4$^+$ CD25$^+$ and CD4$^+$ CD45RB^{low} CD25$^-$ served as control. Nomenclatures are as described in a. Each measurement has been performed at least twice on independent cell samples and resulted in similar curves.

posure to LPS followed by IL-2 (Fig. 5 b). As titration curves for cells exposed sequentially to LPS and IL-2 or to IL-2 alone were similar, we also tested CD4$^+$ CD45RB^{low} CD25$^-$ cells immediately after 3 d of LPS activation. As can be seen in Fig. 5 c, LPS clearly promotes suppressive activity in this cell population. CD4$^+$ CD25$^+$ similarly exposed for 3 d to LPS without subsequent exposure to IL-2 showed lower suppressor efficiency than untreated cells. This result is best explained by the poor survival of CD4$^+$ CD25$^+$ cells in absence of exogenous IL-2 (as described above), preventing a proper evaluation of suppressor efficiency on a per cell basis. This argument is supported by our results above, which show that when subsequently provided with IL-2, the same cell population displays maximal suppressor efficiency. Finally, as expected, CD4$^+$ CD45RB^{high} cells stimulated for 6 d in cultures supplemented with IL-2 and soluble anti-CD3 did not show any suppressor activity, regardless of their differential exposure to LPS for the first 3 d of culture (not depicted).

We conclude that LPS markedly enhances the suppressive activity of naturally activated CD45RB^{low} T cells, whether these are CD25$^+$ or CD25$^-$.

LPS-activated CD4$^+$ CD25$^+$ T Cells Prevent Wasting Disease Induced by Naive CD4 T Cells in Alymphoid Recipients. To assess whether the suppressive function we monitored in vitro correlates with in vivo regulatory functions, we tested the ability of LPS-treated CD4$^+$ CD25$^+$ cells to control the wasting disease that is induced in RAG-deficient animals upon transfer of naive CD4 cells (1). C57BL/6-*H-2*^{u} RAG-1$^{-/-}$ recipients of CD4$^+$ CD45RB^{high} (Thy1.2$^+$) cells developed a lethal wasting disease in less than 3 wk after adoptive transfer. This extremely fast development associated neither with pathological intestinal inflammation nor pneumonia, contrarily to what is observed in other genetic backgrounds (1, 2). Animals that received CD4$^+$ CD25$^+$ (Thy1.1), either freshly isolated or exposed to LPS followed by IL-2, remained healthy (Fig. 6 a). In addition, cotransfer with either population of CD4$^+$ CD25$^+$ cells reduced the number of transferred naive Thy1.2$^+$ lymphocytes recovered from mesenteric LNs (Fig. 6 b), spleen and pooled axillary and cervical LNs (not depicted) to a similar extent. Finally, the numbers of transferred CD4$^+$ CD25$^+$ cells recovered in recipients of the cotransfers were similar in the two groups of animals as were their respective levels of CD25 expression (exemplified in Fig. 6 c).

These results demonstrate efficient control of inflammation and homeostatic expansion by LPS activated CD4$^+$ CD25$^+$ cells in vivo.

Discussion

Here we provide the first evidence for the selective expression of pathogen-associated "pattern recognition receptors" by Treg and the functional relevance of one of them, TLR-4, in the physiology of this cell subpopulation. Our findings that LPS promotes Treg survival/proliferation and enhances their suppressive functions also demonstrate that T cells involved in the control of inflammation directly respond to proinflammatory microbial products. Thus, this analysis provides another link between innate and adaptive immunity and reveals a novel mechanism for the control of immune responses.

The rather high proportion of TLR genes, classical components of the innate immune system, selectively expressed in a subset of CD4 cells endowed with the function of regulating adaptive immune responses, may help us delineate the evolutionary scheme that led to the establishment of an adaptive immune system. Among the nine known murine TLR genes analyzed here, four (TLR-4, -5, -7, and -8) are selectively expressed in this particular CD4$^+$ subset. TLR-5 expression appeared the most selective for Treg. In line

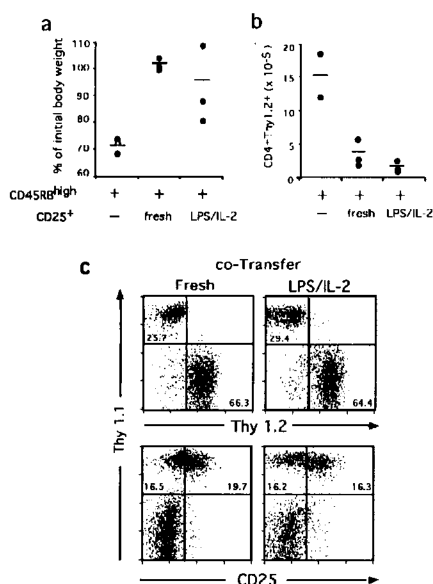

Figure 6. LPS-treated CD4$^+$ CD25$^+$ are efficient regulatory cells in vivo. RAG-1–deficient animals (three per experimental group) received 10^5 CD4$^+$ CD45RB^{high} Thy1.2 cells alone or together with the same number of CD4$^+$ CD25$^+$ Thy1.1 cells, either freshly isolated or maintained in culture sequentially with LPS and IL-2 for a total of 6 d (as described in Fig. 4). Mice were analyzed at 21 d after transfer. (a) Control of wasting disease. The white dot represents an animal found dead on day 17 after transfer. Its weight monitored on day 16 was used. (b) Control of lymphocyte expansion. Numbers of CD4$^+$ Thy1.2$^+$ cells (originally CD45RB^{high}) recovered in the mesenteric LNs. (c) Viability and phenotype of the transferred CD4$^+$ CD25$^+$ cells. Representative staining of axillary LNs from recipients of cotransfers.

with the present observations on LPS activation of Treg through TLR-4, it is striking that the TLR-5 receptor binds flagellin (32), another bacterial product. Furthermore, all endogenous ligands identified to date, which in addition to LPS, bind TLR-4 and induce signaling in various cell types, are molecules involved in inflammatory responses. Thus, this is the case for HSP, HSP-60 (10), HSP-70 (12), and GP96 (11), heparan sulfate (33), surfactant protein A (34), and the product of degradation of the extracellular matrix hyaluronic acid (14). Natural ligands for TLR-7 and TLR-8 are not yet identified, but the finding that seven out of nine TLR molecules are expressed by Treg suggests that a rather large universe of inflammation-related endogenous and pathogen-associated molecules might directly modulate their activities.

LPS effects on murine T cells have been generally interpreted as indirectly mediated through activation of accessory cells (19, 35, 36), although sporadic evidence of direct triggering has also been provided (37–39). The frequency of CD4 T cells expressing TLR-4 at the cell surface could not be assessed because the available antibodies that specifically recognize the murine TLR-4–MD2 complex on peritoneal macrophages fails to interact with both B and T cells (40 and unpublished data). Assuming homogeneous expression levels by positive cells, a quantitative interpretation of the present RT-PCR analyses would indicate TLR-4 expression in, at most, some 15% of the total CD4 compartment (10% CD25$^+$ and 1/4 of the 20% CD45RB^{low} CD25$^-$). In addition, the results of LPS-stimulated cell cultures suggest that only a fraction of CD4$^+$ CD45RB^{low} CD25$^{+/-}$ cells respond to TLR-4 ligation, thus being compatible with a previous report indicating that 3% total T splenocytes are LPS responders (37). The essential finding here, namely that all CD4 T cells that respond to LPS are encompassed in the Treg subpopulation, provides additional evidence for the evolutionary and functional relationship between innate and adaptive immunity. In turn, our results may suggest that Treg belong to a particular class of "nonclassical" $\alpha\beta$ T lymphocytes, along with NK and $\gamma\delta$ T cells. Whether the selective TLR expression by Treg described here defines a unique lineage or a particular differentiation stage will be a crucial issue to address in future experiments.

Several models of inflammation induced by pathogens in immunocompromised animals have shown that Treg are necessary to prevent deleterious immune responses (1, 2). The process of Treg activation in these systems had not been addressed until now. Our findings that a bacterial product can directly activate Treg and enhance their effector functions suggest polyclonal activation of these cells during infection. In turn, they provide the first evidence that cells involved in the control of inflammation respond to proinflammatory microbial molecules and add to previous reports demonstrating that Treg are particularly sensitive to inflammatory chemokines (6, 7). The survival/proliferative responses to LPS, TCR ligation, and IL-2 may suggest, however, that during bacterial infection unspecific activation of Treg through TLR is amplified by specific recognition of antigens. Moreover, specific delayed-type hypersensitivity and graft-versus-host reactions, as well as graft rejection and classical immunization, have all been shown to be down modulated or abolished by pretreatment of mice with various doses of LPS (41–43). Activation of Treg by LPS may well be the basis for these observations. In the course of an infection, however, Treg activation does not fully impair the protective response even though it may limit its magnitude (2) and, certainly, its deleterious pathogenic consequences for the host (1, 2). The specific immune response to nonself antigens may take place as the consequence of a natural ratio of regulatory to effector cells heavily biased toward the latter and a Treg TCR repertoire seemingly biased toward the recognition of self-antigens (17, 44–46). Thus, presentation of nonself peptides during acute infection is predominant, resulting in the initial preferential activation of naive responder cells. As infection regresses and pathogens are cleared, presentation of self-ligands to Treg predominates, leading to the control of inflammation and preventing the activation of naive, autoreactive T cells by self-peptides presented in an inflammatory context. Importantly, inflammatory stimuli may lead to detrimental systemic effects and the stimulation of Treg through TLRs, either by microbial or endogenous ligands

released during stress or tissue damage, might be required to prevent generalized immunopathologies.

Finally, these findings may contribute novel insights to the cellular basis of chronic infections and to the beneficial effect of various infections on the onset of autoimmune diseases (47, 48).

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$CD4^{+}CD25^{+}$ Regulatory T cells Control LPS Induced Inflammatory Reactions

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Abstract

Regulatory $CD4^+$ T cells constitutively expressing the IL-2 receptor α chain (CD25) control immune responses to pathogens and commensal bacteria and prevent occurrence of autoimmune disease. As inflammatory reactions associate with both infection and pathological autoimmunity, it is tempting to postulate a common mechanism of regulation targeted at limiting inflammation. In the present work, we investigated $CD4^+CD25^+$ regulatory activity in vivo during inflammation induced in absence of foreign protein antigens. Alymphoid RAG knockout mice were selectively reconstituted with $CD4^+CD25^+$, $CD4^+CD25^-$, or both cell populations, and peritoneal inflammation induced by injection of low dose purified LPS. Monitoring the number and phenotype of lymphocytes and components of the innate immune system recovered in various tissues, we evidenced that $CD4^+CD25^+$ inhibit $CD4^+CD25^-$, NK and neutrophil accumulation at the site of inflammation by a mechanism dependent on interleukin 10. Our analyses further indicate that regulation of CD4 cell number *in vivo* results from reduced migration capacity rather than limited proliferation. Furthermore, we demonstrate that regulatory T cells interact directly with cells of the innate immune system, and limit their inflammatory response to LPS. These results suggest a common regulatory mechanism independent of the nature of the presented antigen to control inflammatory reactions.

Introduction

Control of inflammatory responses to commensal bacteria and pathogens by a subset of CD4 $\alpha\beta$ T cells has been clearly demonstrated (1-4). T cells controlling immune pathology in these models are enriched in the naturally activated, CD45RB^{low}, population. In most, but not all of these systems, CD4+CD45RB^{low} cells which constitutively express the IL-2 receptor α chain (CD25) (5), show, to date, the best correlation with regulatory function. CD4+CD25+ were initially shown to avoid autoimmunity in neonatal thymectomy and adoptive transfer models (5), and have since been shown to play a central role in controlling immune responses in a large number of models. Thus, in non infectious models, type 1 diabetes (6), experimental autoimmune encephalomyelitis (7), gastritis (8), allergic IgE responses (9) and also lymphocyte homeostasic proliferation (10) have all been shown to be limited if not prevented by Treg. Protective immune responses are also limited by Treg as exemplified during *Pneumocystis carinii* (2) or *Leishmania major* infections (11). The activation of Treg function in dampening immune responses led to the concept that Tregs may have a role in "quality control" of immune responses be they against self or non self (12-14).

To dissociate the effects of TCR stimulation by foreign antigens from inflammatory signals on the function of Tregs, we developed a model of sterile inflammation, in absence of non-self protein antigens. The model we report here uses immune-deficient RAG knockout mice reconstituted with CD4+CD25-, CD4+CD25+, or both populations Recipients were then treated with either highly purified LPS or PBS by intra-peritoneal route. Our results show that regulatory T inhibit CD4 cell migration to the site of inflammation. We further evidence that Tregs are able to modulate the responses of components of the innate immune system, both in vivo and in vitro. Finally we establish that Il-10 is necessary for Treg control of either arms of the immune system.

Materials and Methods

Mice

C57BL/6, C57BL6/-*Thy1*^{a}, *Igh*^{a}, *Gpi1*^{a}, IL10-/- and C57BL/6 RAG2$^{-/-}$ mice were bred and maintained under specific pathogen-free conditions in our animal facility. RAG1$^{-/-}$ backcrossed in to a C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, ME) at 6-8 weeks of age and used within 2-3 weeks of their arrival. All animals were used between 6 and 9 weeks of age.

Antibodies

The following mAbs were purchased from BD Biosciences (San Diego, CA): APC and PE conjugated anti-CD4 (clone RMA-5); biotin Thy1.2 (CD90.2); APC TCRβ (H57-597); FITC and CyChrome Mac-1 (clone M1/70); biotin GR-1 (Ly6G) and PE NK1.1 (Ly-55). CyChrome F4/80; Thy1.1 (CD90.1) biotin and AlexaFluor™488 CD25 (PC61) were produced and labelled inhouse. Biotinylated antibodies were revealed with streptavidin-PE (BD Biosciences).

Cell Purification and Transfer

Pooled LN cells were stained with anti-CD4-PE and CD25-Alexa and cell sorting was performed on a MoFlo High Speed Cell-sorter (Cytomation Inc., Fort Collins, CO). RAG2-/- or RAG1-/- mice were injected in the retro-orbital plexus with 3 x 10^5 of either $CD4^+CD25^+$, $CD4^+CD25^-$ or 3 x 10^5 of each, in 200 μl of PBS. Purity of populations was routinely > 98% for CD4+CD25- and > 99% for CD4+CD25-. The major contaminant in the CD4+CD25+ population consisted of CD4+CD25- cells.

Injection

Mice were injected intra-peritoneally with either 1μg of HPLC purified lipopolyssacharide (LPS) (a gift from Dr. Christopher Galanos, Max-Planck Institute for

Immunobiology, Freiburg, Germany) in 200µl of PBS or PBS alone. PBS was prepared from Double Processed Tissue Culture Water, Endotoxin Tested (Sigma-Aldrich, Spain) and Gibco Tissue Culture 10x PBS solution (Life Technologies, Gaithersburg, MD).

Cell Recovery and Flow Cytometric Analysis

Peritoneal exudate cells (PEC) were obtained by washing the peritoneal cavity with 10mls of FACS buffer, recovered using a 16g needle (volume recovered varied from 7.5 to 9.5 mls). Single cell suspensions from spleen and mesenteric lymph node were prepared by forcing the organs through a nylon mesh. For staining, 1×10^6, when possible, cells were pre-incubated for 5 min with unlabeled mAb to the Fc receptor, washed and then incubated for 20 min on ice in a 96 well plate with 25 µl the relevant antibody diluted in PBS 2% FCS with 0.01% sodium azide. Cells were then washed twice and ressuspended in FACS buffer containing propidium iodide.

Intra-cellular Cytokine Staining

Spleen and PEC cells were surface stained as above for CD4 APC and Thy1.1 biotin-streptavidin-PE. Cells were washed and plated in RPMI 10% FCS at 1×10^6 cells/ml and stimulated with PMA/ionomycin (Sigma-Aldrich) for 5 hours at 37 C in the presence of 10µg/ml Brefeldin-A (Sigma-Aldrich). Cells were then harvested washed in ice-cold PBS, and incubated in 2% paraformaldehyde for 20 minutes at room temperature. Following another wash in ice-cold PBS, cells were incubated in permeabilization buffer (FACS buffer containing 0.5% saponin) for 10 min, RT, pelleted and incubated for 30 min RT in permeabilization buffer containing FITC anti mouse IFNγ (BD Biosciences). Finally cells were washed twice in permeabilization buffer and twice in FACS buffer, and re-suspended in FACS buffer for acquisition.

BrdU

For BrdU labelling *in vivo*, mice were injected i.p. with 1 mg of BrdU (Sigma-Aldrich) in 200 μl PBS and sacrificed 24 hours later. Spleen and peritoneal cavity cells were surface stained with CD4-PE. BrdU staining was perfomed as described previously (7) using FITC labelled anti-BrdU (BD Biosciences).

CFSE labelling and *In vitro* Proliferation Assay

Sort-purified $CD4^{+}CD25^{=}$cells from B6 mice and $CD4^{+}CD25^{-}$ cells from congenic C57BL/6 Thy 1.1 mice were re-suspended at $2.5x10^{6}$ in serum free HBSS and stained with 5μM CFSE (Molecular Probes, Oregon, USA) for 5 min at room temperature. The reaction was stopped by the addition of FCS (20% final) and stained cells were washed once in RPMI-1640 supplemented with 10% FCS.

CFSE labelled $CD4^{+}CD25^{-}$lymphocytes, together with $1x10^{5}$ γ-irradiated (30 Gy) APCs (RBC-lysed, T depleted splenocytes) were cultured for 3 days in the presence of 0.5 μg/ml anti-CD3 mAb (2C11 clone) and sorted CFSE labelled CD4+CD25+ suppressor population (U-bottomed 96-well plate), in RPMI-1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME, 10 mM HEPES, and 1 mM sodium pyruvate (all purchased from Life Technologies). The total number of T cells (CD4+CD25+ and CD4+CD25-) were keep constant (5x104/well). Cultures were supplemented with 10 μg/ml LPS (Sigma-Aldrich On day 3 cells were recovered and analysed by flow cytometry were fluorescent intensity of CFSE on Thy1.1+ cells was scored.

Measurement of TNF-α in the supernatants of *ex vivo* cultures

Pooled peritoneal wash cells of 3 RAG2-/- re-suspended in DMEM supplemented with 10% FCS, L-glutamine, Penicilin/Streptavidin, and HEPES buffer (all from Gibco). Cells were seeded on 96-well plates (10^{5} total PerC cells per well), together with purified T cells in indicated ratios and anti-CD3 overnight. LPS was then added to the culture to a final concentration of 0,1 ng/mL. After 4 h, TNF-α activity was measured in the supernatant by an L929 cytotoxicity bioassay (15). Briefly, L929 fibroblasts were plated at

5×10^4 cells/well into flat-bottom 96-well plates in 100 μl of medium and incubated at 37°C for 24 hours. Media was aspirated and fresh media, containing 15 μg/mL cyclohexamide (Sigma), was added. 50 μL of the recovered supernatant was then incubated with L929 and serially diluted (1:3). Recombinant TNF (R&D Systems, Minneapolis, MN) was included in each assay as a positive control and as standard concentration of TNF-α. After 18 hours at 37°C, live L929 cells were stained with Cristal Violet (Sigma; 0.01% in 20% methanol), for 10 min. at RT. After washing, cells were solubilised with 100 μL of Methanol (Sigma-Aldrich, Seelze, Germany) and absorbance was read at 595nm on an ELISA plate micro-reader.

Results

$CD4^{+}CD25^{+}$ Treg cells inhibit CD4 T cell accumulation at the site of inflammation.

The effects of inflammatory stimuli on Tregs were examined using bacterial lipopolyssacharide (LPS) to provoke inflammation in the peritoneal cavity in the absence of non-self peptides. Ragl-/- mice were reconstituted with 3 x 10^{5} sorted $Thy1.1^{+}CD4^{+}CD25^{-}$ target cells, either alone or together with 3 x 10^{5} $Thy1.2^{+}CD4^{+}CD25^{+}$ Treg cells. Recipients were injected intra-peritoneally (i.p.) with 1μg LPS in phosphate buffered saline (PBS), or PBS alone, 3 times at a 3 day intervals . Mice were sacrificed two days after the last injection, and cells from the spleen, mesenteric lymph nodes and peritoneal cavity were counted and analysed by flow cytometry (Fig. 1). Injection of LPS i.p. leads to increased T cell recovery from the peritoneal cavity. Among mice that received only CD4+CD25- cells, those injected with LPS had, on average, a more than 4-fold increase in peritoneal T cells over those that received PBS alone (average of 45.10^{3} and 10.10^{3} cells, respectively). Injection of LPS did not significantly affect the number of $CD4^{+}CD25^{-}$ cells recovered from spleen or mesenteric lymph nodes (3.5 x 10^{5} and 2.5 x 10^{5} cells; 1.7 x 10^{5} and 1.4 x 10^{5} cells, respectively). Co-transfer of the complementary, $CD4^{+}CD25^{+}$ population abrogated the accumulation of $CD4^{+}CD25^{-}$ cells in the peritoneal cavity in response to LPS (15.10^{3}), indicating that control of T cell numbers by this population operates efficiently at a site of inflammation. $CD4^{+}CD25^{+}$ cells also controlled accumulation of naïve cells in the mesenteric lymph nodes, as in a previous report (16). Naïve cell numbers in the spleen were much less, but still significantly, affected.

Decreased accumulation of $CD4^{+}CD25^{-}$ cells at the site of inflammation associates with inhibition of their migration capacity

Suppression of naïve $CD4^{+}$ T cell proliferation *in vitro* is currently used as a correlate of the *in vivo* regulatory activity of $CD4^{+}CD25^{+}$ Treg cells. We tested the capacity of $CD4^{+}CD25^{+}$ cells to suppress *in vitro* the proliferation of $CD4^{+}CD25^{-}$ cells stimulated by anti-CD3 and APCs in presence of LPS. As above, Thy1.2 and Thy1.1 congenic mice were

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used to purify $CD25^+$ and $CD25^-$ cells respectively. Cells were then labelled with CFSE and plated at various ratios. At day 3 of culture the number of Thy1.1 cells and their CFSE content was determined by FACS analysis (Fig 2A). As expected, LPS enhances the proliferation of $CD4^+CD25^-$ cells maintained in culture alone, presumably through activation of the APCs. In presence of LPS, suppression of proliferation by $CD4^+CD25^+$ cells is readily detectable, as indicated by a CFSE profile progressively skewed to the right and a net decrease in the number of cells recovered as the proportion of $CD4^+CD25^+$ versus $CD4^+CD25^-$ increased in the initial culture. At a 1:1 ratio, inhibition of proliferation by $CD4^+CD25^+$ cells is such that the additional expansion due to LPS is abrogated. These data indicate that $CD4^+CD25^+$ cells are efficient suppressors of the proliferative responses of CD4 cells to inflammatory stimulus in vitro.

We next investigated whether the inhibition of T cell accumulation in the inflamed peritoneal cavity we evidenced above results also from control of proliferation. In a first step, we evaluated the impact of LPS on the number of cycling cells in Rag2-/- mice that received $CD4^+CD25^-$ cells alone. Ten days after adoptive transfer, mice were injected i.p. with 1 mg Bromodeoxyuridine (BrdU) and either 1 μg of LPS or PBS. All animals were sacrificed 24 hours later and incorporation of BrdU in $CD4^+$ cells from spleen and peritoneal cavity was assessed by FACS analysis (Fig.2B). In all animals tested, the fraction of cells incorporating BrdU is very high (20-30%) indicating extensive homeostatic proliferation. While as above, the number of recovered CD4 cell was significantly increased between LPS and PBS injected animals (not shown), LPS did not induced increased proliferation of CD4 cells, either in the spleen nor in the peritoneal cavity.

As increased T cell proliferation is not responsible for the accumulation of lymphocytes in the peritoneal cavity, Tregs must be altering T cell homing to inflamed site. Additional support for this hypothesis is provided by the analysis of mice submitted to prolonged LPS treatment. Upon adoptive transfer, mice were injected ip every 3 days for 30 days with either LPS or PBS. As a result of continuous homeostatic expansion, the number of T cells recovered in each animals and in all tissues, is at least 10 fold higher than in the previous experiments. As shown in Fig. 2C, under these conditions, LPS treatment still leads to increased number of peritoneal T cells, indicating that these doses

are not inducing tolerance to LPS. Moreover, this increase is still efficiently inhibited by the presence of Tregs. In contrast, the number of T cells recovered from the mesenteric LN is reduced about 3 fold in LPS treated animal when compared to controls. Consequently, the total number of cells recovered from both mesenteric LN and peritoneal cavity in each animal is constant independently of the treatment. Mesenteric LN have been shown to be the major site of T cell accumulation in adoptive transfer into alymphoid animals bearing enteric bacteria. Since our animals tested helicobacter positive at the time of these experiments, we conclude that the inflammation induced by LPS lead to preferential migration of the CD4 cells to the peritoneal cavity instead of the mesenteric LN. This finding strongly supports the idea that Tregs inhibit migration of CD4 cells to the site of inflammation.

$CD4^+CD25^+$T cells do not affect $CD4^+CD25^-$ cells differentiation into helper cells.

Previous reports indicate that Tregs may also control the differentiation of naïve T cells into effectors helper cells in an allergy model (9). In our experimental system, a large proportion of splenic CD4+CD25- cells produce IFN-γ (79%) as determined by intra-cytoplasmic staining and FACS analysis 10 days after transfer in PBS treated animals (Fig 3). Co-transfer of $CD4^+CD25^+$ cells leads to a decrease in the frequency of IFN-γ producing cells (45.8%), which corresponds to a dramatic reduction in the total number of TH1 cells . As expected, injection of LPS does not significantly alter the proportion of IFN-γ producing cells in the spleen in either single or co-transferred recipients. Surprisingly, peritoneal inflammation does not induce preferential recruitment of TH1 cells, as the proportion of IFN-γ producing cells in the peritoneal cavity is indistinguishable between single transfer recipient that receive either LPS or PBS. Moreover, there is no significant effect of Tregs on the frequency of IFN-γ producing cells at this site, although the severe reduction in the number of Thy1.1 cells result in a very low representation of TH1 cells. When assessing the expression of other cytokines such as TNF-alpha, IL-4 and IL-10 by Th1.1 cells, in the different experimental groups, similar results were obtained, namely that neither Tregs nor LPS per se affected the frequency of effector CD4 cells (not shown). These results are consistent with those obtained in the IBD

model (17), and argue against control of helper cell differentiation by Tregs in inflammatory systems.

Control of local inflammation by $CD4^+CD25^+$ cells associates with reduced responses of innate components

We have shown above that $CD4^+CD25^+$ cells limit the number of T cells secreting inflammatory cytokine at the site of LPS injection. We next investigated how this regulation would influence further the responses of components of the innate immune system. Innate inflammatory responses are characterised by large amount of TNF-alpha production, and intense migration of various cellular subset to or out of the inflamed site.

TNF-alpha production in the animals analysed above was monitored by measuring serum content. No significant differences could be established between animals that receive d LPS or PBS. We attribute this result to the fact that induction of TNF-alpha upon LPS injection is transient : serum level peaks at 90min after injection and return to normal 6 hours later (not shown). The lymphocyte analyses presented above were conducted 36 hours after the last injection of LPS, a time at which production ofTNF-alpha is no longer detectable.

In contrast, cellular migration characteristic of local inflammation could easily be evidenced in the peritoneal cavity of LPS injected animals (Fig. 4). Thus, LPS injection provoked a large reduction in the number of peritoneal $F4/80^+Mac1^{high}$ macrophages, a phenomenon not significantly enhanced by transfer of $CD4^+CD25^-$ cells, neither affected by the presence of $CD4^+CD25^+$. A population of lymphoid cells, composed of a large majority of $CD4^-NK1.1^+$ natural killer cells was preferentially accumulating in LPS injected mice. This subset was slightly increased in$Rag2^{-/-}$ mice, greatly augmented in recipient of $CD4^+CD25^-$cells, and significantly reduced in co-transferred animals. Finally, similar results were obtained when analysing an SSC intermediate subset mainly composed of $GR1^{high}Mac1^{int}$, a population previously described as neutrophils producing high levels of interleukin-12 (18)

Although these results do not allow to discriminate between a direct or indirect regulation of the innate responses they demonstrate the capacity of Tregs to lower local inflammatory responses.

$CD4^+CD25^+$ Treg cells control LPS-induced TNF-α production by cells of the innate immune system

We next assessed whether $CD4^+CD25^+$ T cells directly control the inflammatory responses of components of the innate immune system . In a first step, we set ex vivo assays to monitor the production of TNF-alpha by LPS stimulated peritoneal cells prepared from $Rag2^{-/-}$ animals. These cells were set in culture for 18 hours, alone or together with sorted $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells in the presence of anti-CD3 antibody to allow for cellular conjugates to be formed. LPS was then added to the media and TNF-alpha produced in the supernatant measured 4 hours later (Fig. 5). Co-culture in the presence of $CD4^+CD25^+$ T cells but not of $CD4^+CD25^-$ efficiently reduces the amount of TNF-α produced by peritoneal cells upon LPS stimulation. This reduction is proportional to the number of $CD4^+CD25^+$ cells added to the culture, and result in a 60- 80% suppression at a 1:1 ratio. When added to the culture, IL-10 dramatically suppress TNF-α production (data not shown). Since anti-inflammatory function of CD4+CD25+ in the IBD model has been shown to be dependant on IL-10 (16), the experiment was repeated using $CD4^+CD25^+$ T cells purified from $IL\text{-}10^{-/-}$ mice. In this condition suppression is reduced but not abolished showing that suppression of TNF-α production is partially IL-10 dependent. As Rag2-/- mice are completely deprived of the cellular components of the adaptive immune system, these results indicate that $CD4^+CD25^+$ T cells can directly regulate cellular components of the innate immune system to limit inflammatory responses .

Interleukin-10 is necessary for in vivo control of LPS induced inflammatory reactions.

Production of interleukin-10 (IL-10) by Tregs has been shown to be necessary for the control of IBD as well as for control of naïve T cell expansion (16, 17). To assess whether IL-10 also plays a role in the control of local inflammatory reactions we repeated the co-transfer experiments described above, using Thy1.2 $CD4^+CD25^+$ T cells sorted from healthy IL10-/- mice (Fig. 6). The number of CD4 cells recovered from the peritoneal cavity of animal recipient of co-transfer that received LPS is much higher than in PBS controls, indicating that CD4+CD25+ cell from IL-10-/- mice do not inhibit accumulation

of naïve T cells at the site of inflammation (Fig6 A). Moreover, Thy1.1 cell numbers were also increased in the spleen and mesenteric LN of LPS treated mice. Analyses of the innate compartment in this set of experimental animals reveal also that IL-10 is necessary for $CD4^{+}CD25^{+}$ control of NK and neutrophils infiltration at the site of inflammation (Fig 6B).

In another system, IL-10 has been proposed to act indirectly on T cells, by controlling the innate immune system (19, 20). According to this finding and given the dependency on IL-10 for all the $CD4^{+}CD25^{+}$ effects we monitored here, our results can therefore be interpreted as $CD4^{+}CD25^{+}$ cells control primarily the innate immune response and consequently inhibit CD4 cell migration to a site of a much lowered inflammation.

Discussion

The present work demonstrates that under inflammatory conditions $CD4^+CD25^+$ T cells are efficient regulatory cells in the control of numbers of naïve/effector T cells. Tregs also interact with the innate immune system, and reduce inflammation, which may be an important factor in reducing the damage to host tissues that accompanies inflammatory responses.

Inflammation has been proposed as a state that would abrogate dominant immune regulation, to allow for efficient priming and immune responses. The recently described expression of the glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR) on $CD4^+CD25^+$ cells and the demonstration that antibodies to this receptor abrogate suppression (21, 22) has been advanced as evidence of a mechanistic basis for this hypothesis. Contrary to this, although LPS is a potent adjuvant it was shown previously that depending on the time of administration, LPS can greatly down-regulate immune responses by promoting suppressor T cell function (23-25). But if efficient regulation of immune responses occurs in inflammatory conditions, this raises an important issue: what allows priming and initiation of immune responses? While we do not know the repertoire diversity and specificity of responder cells in our experimental system, $CD4^+CD25^-$ cells purified from anti-MBP (myelin basic protein, an antigen unlikely to be found in the peritoneal cavity) TCR transgenic mice did not infiltrate the peritoneal cavity, with or without LPS (data not shown), indicating that specificity of T cells is also important in this context. The specificity of $CD4^+CD25^+$ cells themselves is so far unclear; but in broad terms they appear to be self reactive cells (7, 26-28). Recent evidence indicates that they are, at least in part, the product of positive selection by self peptides expressed on cortical thymic epithelium (26, 29), as had been previously demonstrated for regulatory CD4 T cells before the use of CD25 marker (30).

We believe that the key factor in the decision to mount an immune responses may be the self-reactive repertoire of Tregs, and that the self/non-self discrimination can not be attributed to activation or not of germ line encoded receptors. The system we used is heavily biased towards the recognition of self-antigens, as cells are expanding in the

absence of external stimuli, albeit not in the absence of the enteric flora. It is known from the work of others that expansion of T cells in lymphopenic hosts is driven by host antigens, and due to their self-reactive repertoire, Tregs are likely to undergo higher affinity interactions with APCs presenting self peptides. Finally, the ratio of $CD25^{+}$:$CD25^{-}$ is also important, and the 1:1 ratio used in the present work is a marked increase over the 1:10 found in most lymphoid tissues in normal mice. Thus, it has recently been shown in a model of *Leishmania major* infection that while Tregs at a 1:1 ratio suppress anti-parasite responses, at the physiological ratio (1:10) they regulate the immune response, but allow it to take place efficiently (11).

Tregs suppress the proliferation of CD4+CD25- cells (as well as CD8 (31) and B cells (32)), and IL-2 production (33, 34), but other effects, such as modulation of antigen presenting cell (APC) (35) activity are controversial. Here we demonstrate suppression of the innate response to LPS *in vivo*, which may be attributed to reciprocal interactions with the $CD4^{+}CD25^{-}$ population. *In vitro* however, we show that Tregs can interact directly with peritoneal macrophage and reduce TNFα production in response to LPS. TNFα is a key mediator of damaging inflammatory side effects, particularly of endotoxic shock. Naturally activated B lymphocytes and natural serum immunoglobulin have been shown to regulate TNF-α production *in vivo* (36), and our results suggest that naturally activated T cells may also be important in this regard. In support of a role for Tregs in directly modulating the innate immune system, TNFα produced by non lymphoid cells is also crucial to intestinal pathology when $CD45RB^{high}$ cells are transferred to RAG mice, a classic assay for immune regulation by Tregs (37). Direct suppression of the innate immune system by Tregs is evident in two recent papers, one showing that Tregs can avoid pathology in a model of lymphocyte independent colitis brought on by *Helicobacter hepaticus* infection (38), and the other demonstrating a lower response to *Leishmania major* in Rag- mice reconstituted with Tregs alone (11).

The role of IL-10 in Treg function is controversial. While IL10 is needed not only for the control of IBD, but also for the control of cell numbers *in vivo,* as well as in the peritonitis system shown here, it is clearly not essential for suppression of proliferation *in vitro*. This may be due to the fact that a large part of the *in vivo* proliferation of naïve cells is in fact driven by inflammatory immune responses to enteric bacteria, as has been

suggested before (10). Thus, IL-10 through its anti-inflammatory properties may be affecting proliferation by reducing inflammation in the gut and other tissues and its role may be most important in allowing Tregs to interact with the innate immune system. In contrast, *in vitro* assays with α-CD3 driven proliferation would require another, cell contact dependent form of regulation, possibly dependent on either TGF-β or CTLA4, or other unidentified molecules. The IL-10 dependent effect of Tregs in the present work, however, appears to be independent of control of cell numbers and to target effector T cell migration instead. Kohm *et al* have also found that in a murine model of experimental autoimmune encephalomyelitis (EAE) Tregs operate in part by modulating infiltration of pathogenic T cells in to the central nervous system (39). Tregs may utilize their specific pattern of inflammatory chemokine receptor expression (32, 40) to arrive before naïve cells at sites of inflammation and reduce chemoattractant stimuli, or, alternatively they may modulate the up-regulation of chemokine receptors on other T cells.

Figure Legends

Figure 1: $CD4^+CD25^+$ cells limit lymphocyte accumulation at the site of inflammation in vivo. $Thy1.1^+CD4^+CD25^-$ cells were transferred either alone (CD25-) or together with $Th1.2^+CD4^+CD25^+$ lymphocytes (Co-Transfer) into $Rag^{-/-}$ mice. Animals received in addition either PBS or LPS i.p. On day 12 post-transfer, number of $CD4^+Thy1.1^+$(closed circles) and $CD4^+Thy1.2^+$ (open circles) cells in Peritoneal Cavity, Spleen and Mesenteric Lymph nodes were evaluated by FACS analyses.

Figure 2 : $CD4^+CD25^+$ cells control augmented proliferation and migration of CD4+CD25- cells induced by LPS. A Proliferation of $Thy1.1^+CD4^+CD25^-$ upon *in vitro* stimulation with anti-CD3 in presence of irradiated APC, LPS and various numbers of $Thy1.2^+CD4^+CD25^+$ cells (indicated as ratio $CD25^-$:$CD25^+$). Proliferation of $Thy1.1^+CD4^+$ cells was monitored by evaluating their CFSE content (left) and their cell number (right) after three days in culture . The geometric mean of CFSE intensity is indicated in each panel. The number of Thy1.1 cells recovered after 3 days culture is expressed as fold increase over single culture set in media alone. Data are representative of 4 independent experiments. **B** Homeostatic proliferation of $CD4^+CD25^-$in vivo is not affected by LPS treatment. Mice that received $CD4^+CD25^+$ cells were injected with either PBS (closed bars) or LPS (open bars) and one day later with BrdU. 24 hours later animals were sacrificed and incorporation of BrdU by $Thy1.1^+CD4^+$ cells from spleen and Peritoneal cavity assessed by FACS analyses. Similar animals that did not receive BrdU were used as controls for the staining. Results represent the mean and standard deviations of 3 animals for spleen and 4 animals for peritoneal cavity . **C.** Number of $TCR\beta^+Thy1.1^+$ cells recovered from the peritoneal cavity (open bars) and mesenteric lymph nodes (closed bars) of individual mice transferred with $Thy1.1^+CD4^+CD25^-$ cells and submitted to PBS or LPS treatment for 1 month .

Figure 3: Differentiation into IFN-γ producing cells is not affected by $CD4^+CD25^+$ cells. Mice received $Thy1.1^+CD4^+CD25^-$ alone or together with $Thy1.2^+CD4^+CD25^+$ cells, treated with PBS or LPS and analysed at day 12 as in Figure 1. Cells recovered from the

spleen and Peritoneal Cavity were incubated 6 hours in PMA/ionomycin and Brefeldin-A before staining for intracellular cytokine. Plots shown are gated on CD4+ Thy1.1+ cells.

Figure 4: $CD4^+CD25^+$ cells limit the increased infiltration of non lymphocytes at the site of induced inflammation. Mice received no cells, $Thy1.1^+CD4^+CD25^-$ alone or together with $Thy1.2^+CD4^+CD25^+$ cells, treated with PBS or LPS and peritoneal cells analysed by FACS at day 12. **A**. Typical FACS profile of peritoneal cells after PBS or LPS treatment. Mice were recipient of $Thy1.1^+CD4^+CD25^-$ cells alone. **B**. All animals were analysed as in A, and total number of Macrophages ($F4/80^+Mac1^{high}$), NK cells ($CD4^-$ $NK1.1^+$), Neutrophils ($GR1^{high}Mac1^{int}$) for each animal is plotted .

Figure 5: $CD4^+CD25^+$ T cells suppress TNF-α production by peritoneal cavity cells from Rag2-/- mice stimulated with LPS. Production of TNF-alpha by Peritoneal cells from Rag2-/- mice upon LPS induction was measured by the L929 bioassay. Cells were previously maintain in culture for 18hours in presence of anti-CD3 and CD4+CD25+ purified from WT or IL-10-/- mice at the indicated ratio. For each condition, TNF-alpha production is plotted as the % detected in single culture (150-180 u/ml) from 2 independent experiments

Figure 6: IL-10 production by $CD4^+CD25^+$ cells is necessary for the control of inflammatory reactions induced by LPS in vivo. $Rag^{-/-}$ animals received $Thy1.1^+CD4^+CD25^-$ cells purified from WT animals and $Thy1.2^+CD4^+CD25^+$ cells isolated from $IL\text{-}10^{-/-}$ mice. **A.** Number of $Thy1.1^+$ (filled circles) and $Thy1.2^+C$ (open circles) $CD4^+$ T cells recovered in Peritoneal cavity, Spleen and mesenteric LN of individual mice, 12 days after PBS or LPS treatment . **B**, Peritoneal cavity cells from the same animals was fractionated according to FSC, SSC, CD4, NK1.1, F4/80, Mac-1 as in Fig. 4.

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Figure 1

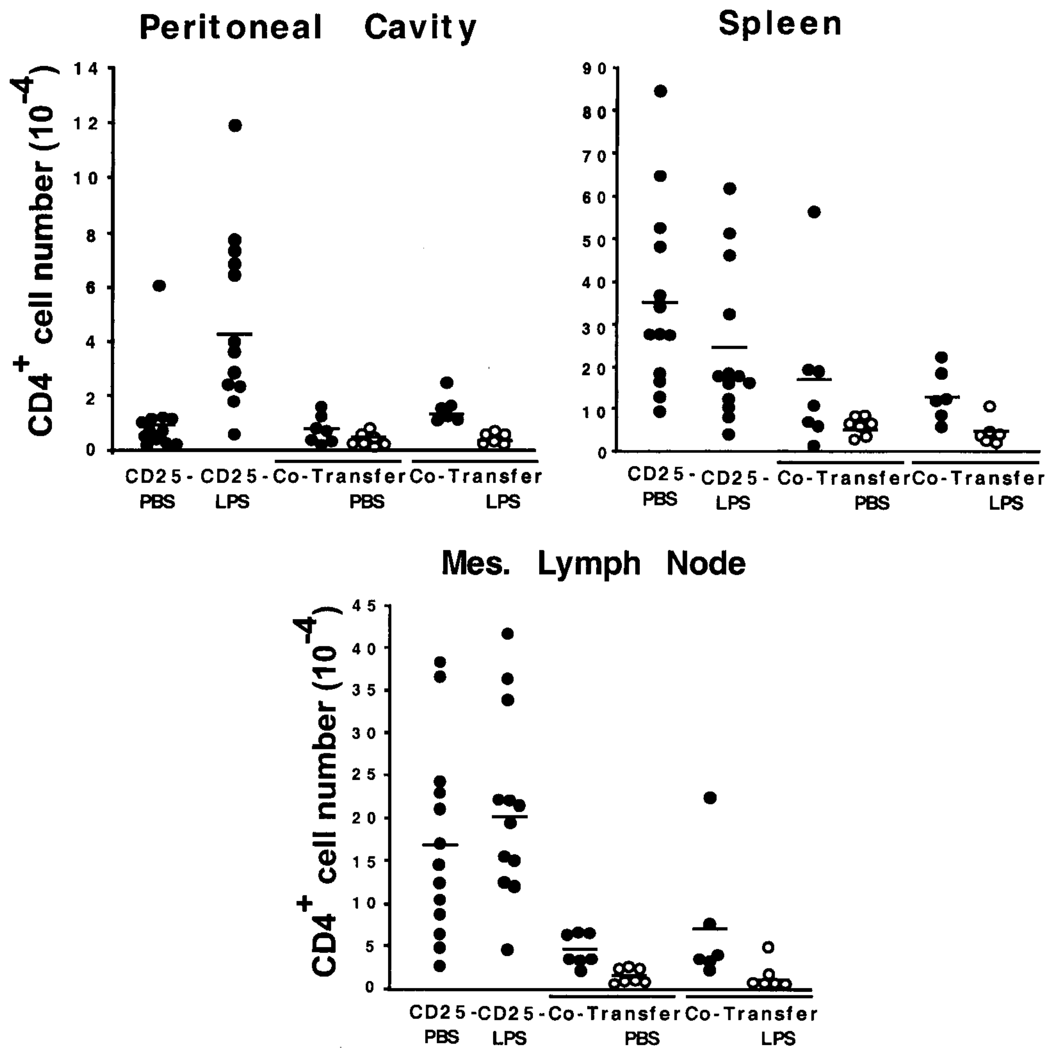
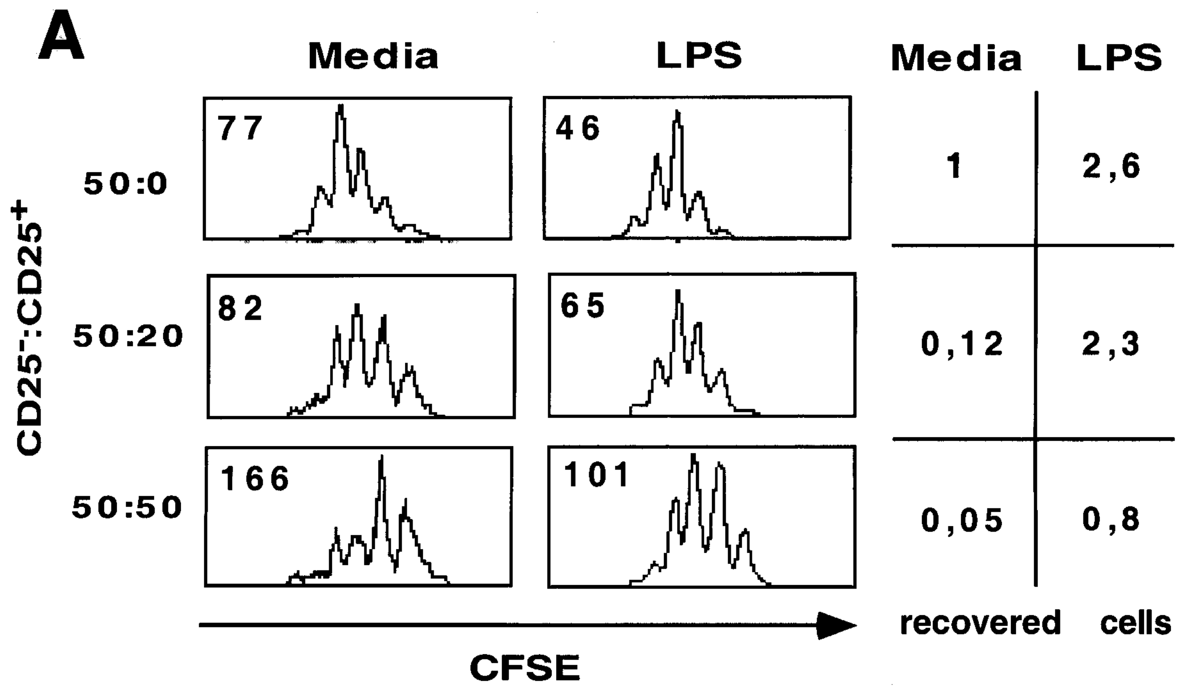
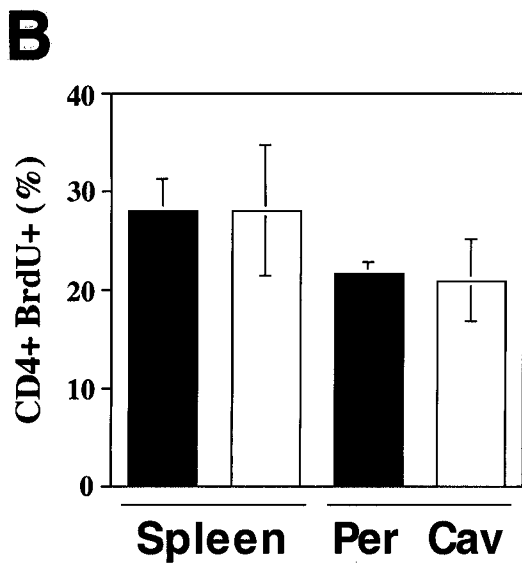


Figure 2





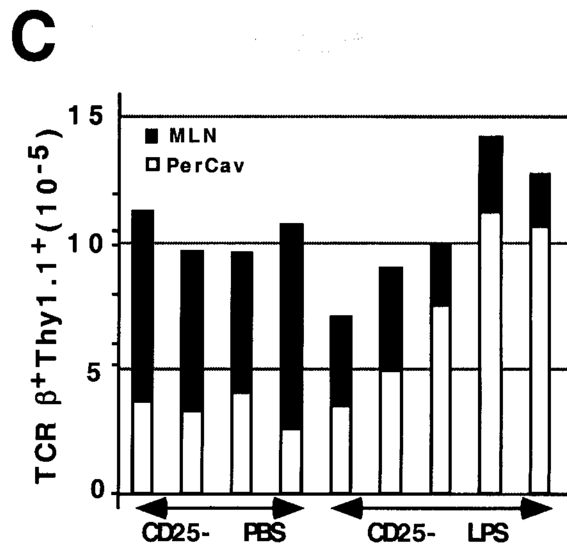


Figure 3

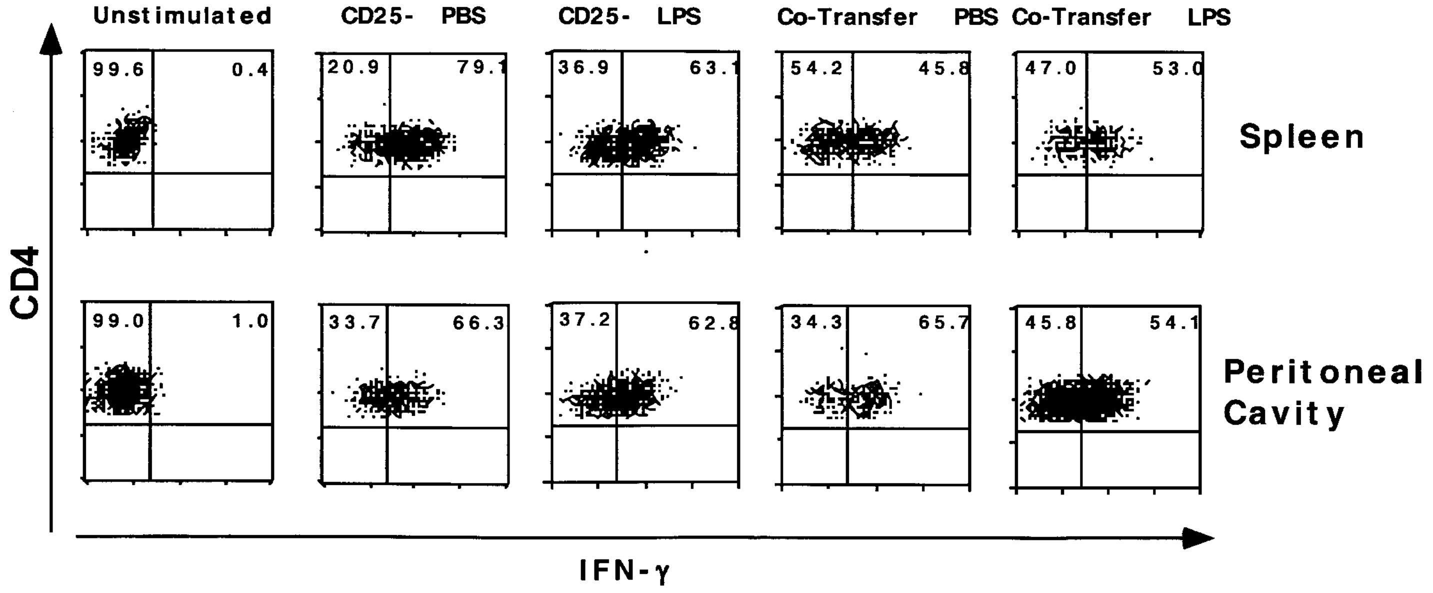
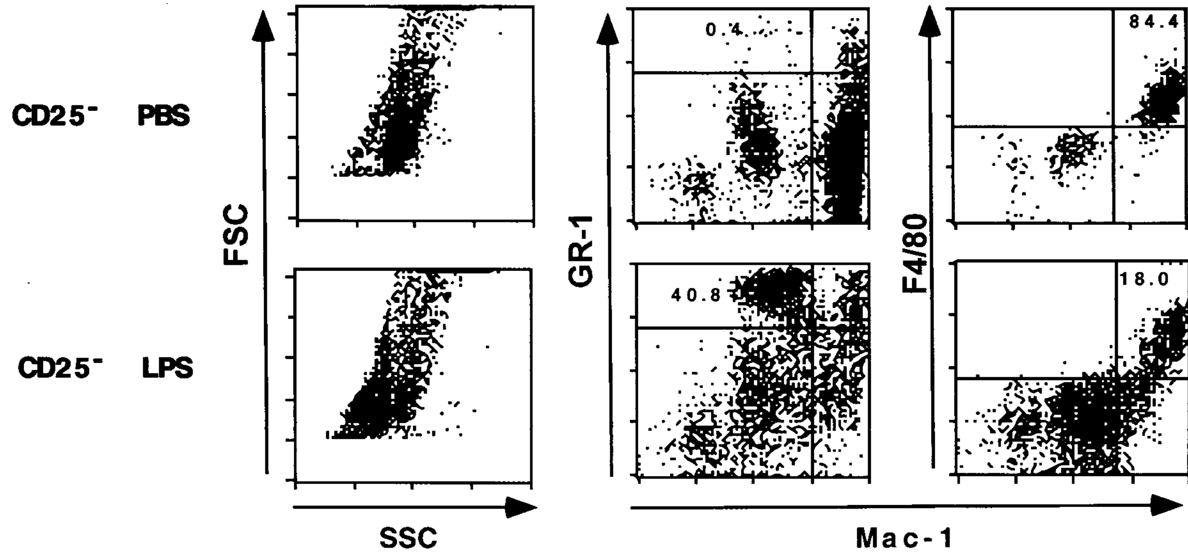


Figure 4

A



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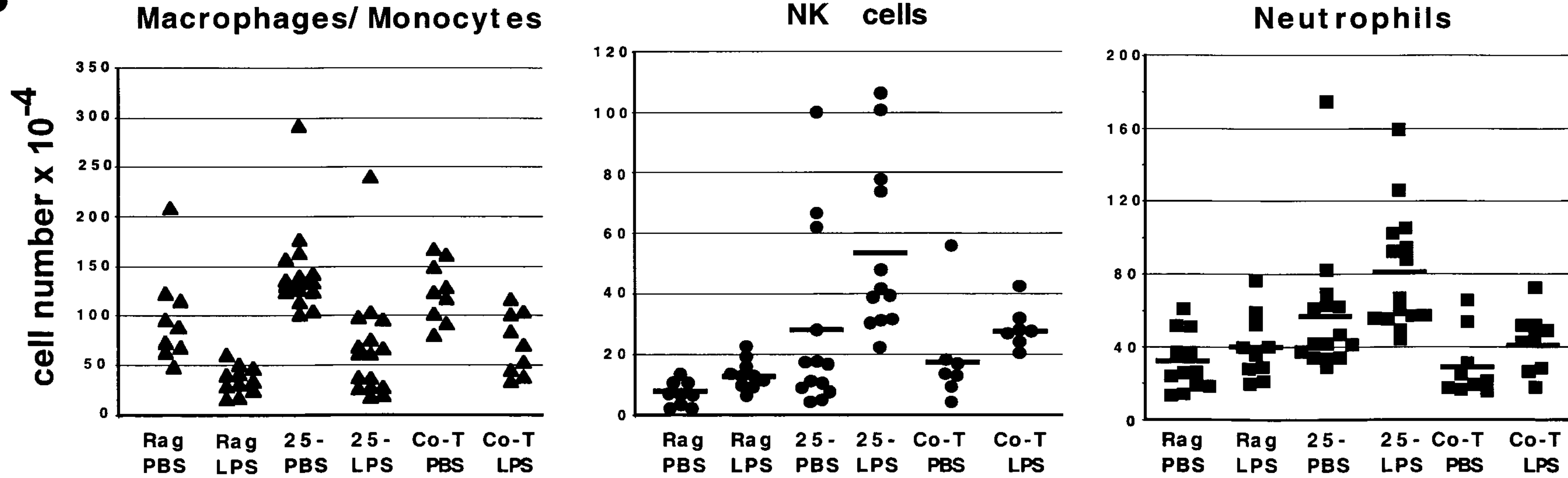


Figure 5

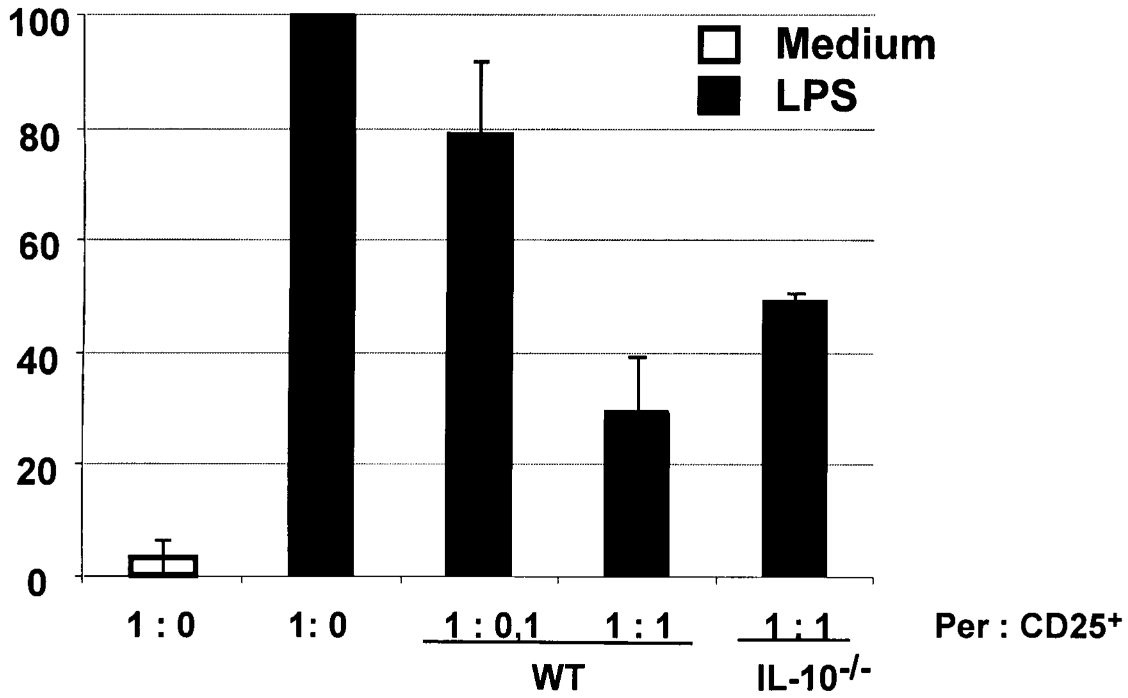
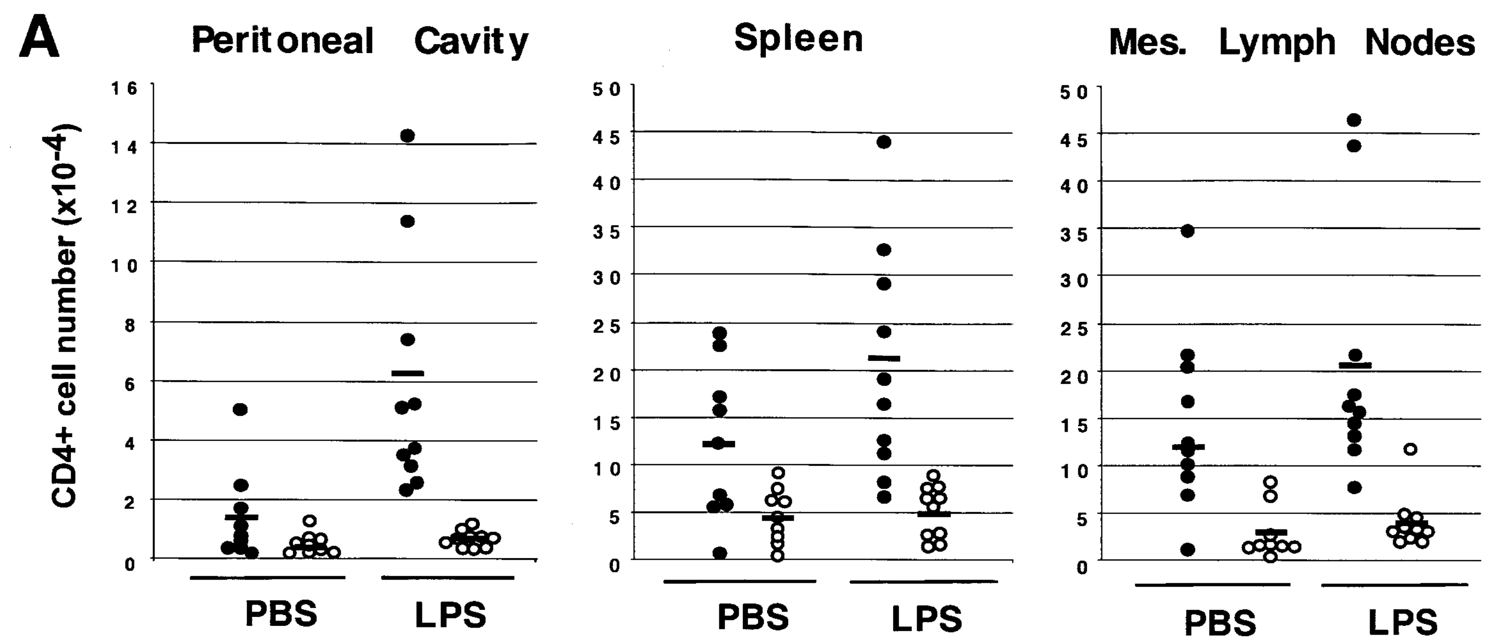
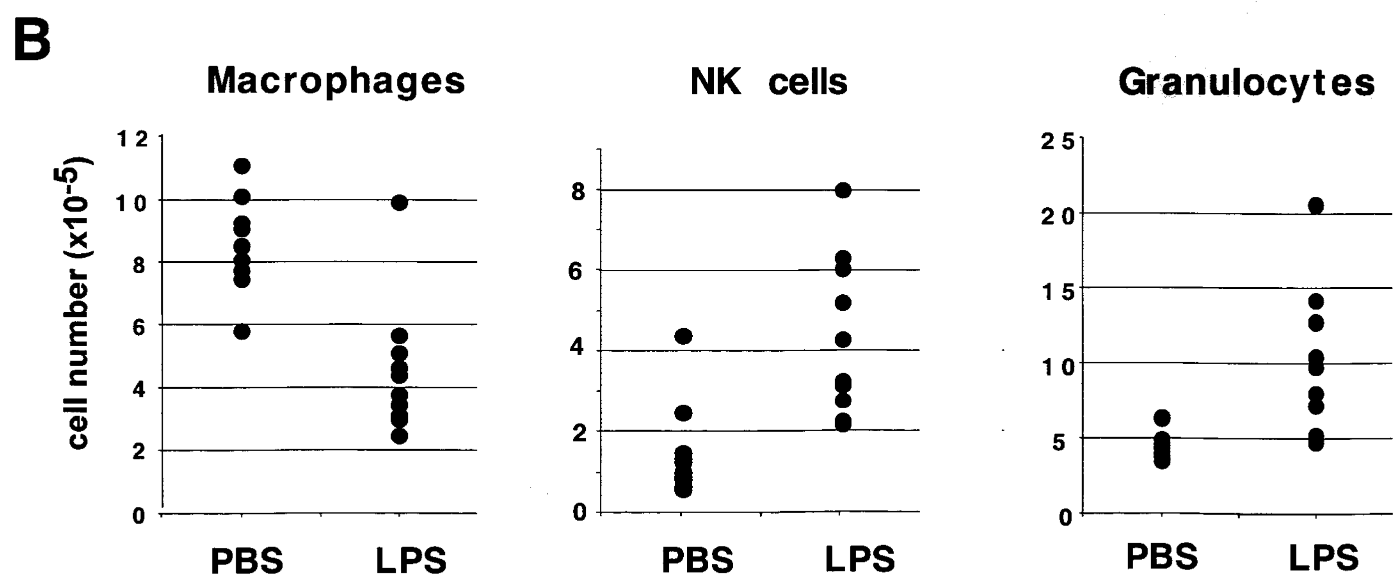


Figure 6





Manuscript IV.

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Short paper

CD4+CD25- Regulate CD4+CD25+ Regulatory T cells

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Abstract

Expression of the IL2 receptor α chain (CD25) on CD4 T cells is a marker for T cells with regulatory functions (Tregs). CD25 expression is, however, a hallmark of recent TCR engagement and a labile phenotype. Here we reassess Treg homeostasis and stability of CD25 expression upon adoptive transfer in alymphoid mice in the absence or presence of various numbers of the complementary CD4+CD25- population. Using differentiating allelic markers for each population of lymphocytes, we show that Tregs expand poorly and undergo a dramatic down-regulation of CD25 expression when transferred alone. In contrast, both the frequency of CD25 expressing lymphocytes and the total number of Treg is increased by the presence of CD4CD25- cells. Moreover expression of CD25 on Tregs transferred alone is re-established upon de novo contact with CD4+CD25- cells *in vivo*, and associates with regulatory function. Taken together these results indicate that the homeostasis and phenotypic stability of Tregs is strongly dependent on the CD4+CD25- population, which itself has a homeostatic regulatory function. The significance of CD25 expression as a phenotypic marker of Tregs is further discussed.

Introduction

Over the past decade, cell surface markers used to characterise memory/effector T cells were reported to be expressed constitutively on a significant fraction of T cells in un-manipulated mice. These naturally activated T cells are enriched for cells with regulatory function, and their absence or deficiency leads to harmful responses triggered by both self and non-self antigens (1, 2). In the case of CD45RB expression, $CD45RB^{high}$ CD4 T cells are mainly antigen inexperienced, while the $CD45RB^{low}$ phenotype represents antigen experienced/previously activated cells (3). Transferring $CD45RB^{high}$ CD4 cells alone into T and B deficient mice leads to an aggressive inflammatory bowel disease (IBD). Conversely, $CD45RB^{low}$ CD4 cells are not only not pathogenic themselves, they prevent the pathology caused by the naïve population (4). Upon transfer to immunodeficient hosts, however, more than 90% of the original $CD45RB^{high}$ population becomes $CD45RB^{low}$ (5), a conversion that has been proposed to be largely driven by activation by enteric antigens (6). The pool of naturally activated T cells is largely independent of stimulation by non-self antigens, both functionally and phenotypicaly, as they are present in, and prevent disease when purified from germ free mice (7) (6). A subset of CD4 cells naturally expressing another activation marker, the IL2 receptor α chain (CD25), has been shown to avoid autoimmunity and limit immune responses in several systems (2, 8-10). CD25+ cells are contained in the $CD45RB^{low}$ fraction of CD4 cells, of which they represent roughly one third, and are also responsible for most of the protective effect of this population in the IBD system (11). Transfer of CD4+CD25- cells into lymphopenic hosts does not lead to high surface expression of CD25, making this marker more stable than the $CD45RB^{low}$ phenotype (12).

While the effects of Treg cells on the activation and proliferation of CD25- have been clearly demonstrated, little is know about the reciprocal end of this interaction, i.e., do CD25- have a role to play in the physiology of Tregs, other than as passive targets of regulation? Although Tregs have been characterized as un-responsive to proliferative stimuli *in vitro*, they divide well upon transfer to lymphopenic hosts (12, 13). Moreover, in normal un-manipulated mice Tregs are dividing at a higher rate than CD25- cells (14). The nature of the factors that govern the proliferation of Tregs *in vivo* is still to be

established, even though IL2 is thought to be crucial. In the present work, we assess the effects of CD25- cells on the expansion of Tregs and on the stability of the CD25+ phenotype. We show that CD25- cells enhance the recovery of Tregs, as well as maintain their surface expression of CD25. Furthermore, loss of CD25 expression upon transfer is not due to expanding contaminant fractions and is readily recovered upon contact with CD25- cells *in vivo.*

Materials and Methods

Mice

C57BL/6, C57BL6/-*Thy1^a^*, *Igh^a^*, *Gpi1^a^*, IL10-/- and C57BL/6 RAG2-/- mice were bred and maintained under specific pathogen-free conditions in our animal facility. RAG1-/- backcrossed in to a C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor, Maine) at 6-8 weeks of age and used within 2-3 weeks of their arrival. All animals were used between 6 and 9 weeks of age.

Antibodies

APC and PE conjugated anti-CD4 mAb (clone RMA-5), CD45RB-PE (clone 16A) and Thy1.2 biotin (CD90.2) were purchased from BD Biosciences. Thy1.1 (CD90.1) biotin and AlexaFluor™ 488-CD25 (PC61) were produced in the laboratory. Biotinylated antibodies were revealed with streptavidin-PE (BD Biosciences).

Cell Purification and Transfer

Pooled LN cells were stained with anti-CD4-PE and CD25-Alexa, and cell sorting was performed on a MoFlo High Speed Cell-sorter (Cytomation Inc., Fort Collins, CO). RAG2-/- or RAG1-/- mice were injected in the retro-orbital plexus with 3 x 10^5 of either CD4+CD25+, CD4+CD25- or 3 x 10^5 of each in 200 μl of PBS. Purity of populations was routinely > 98% for CD4+CD25- and > 99% for CD4+CD25-. The major contaminant in the CD4+CD25+ population consisted of CD4+CD25- cells. For CD45RB$^{hi/low}$ sorting, cell were stained with CD4-APC, CD45RB-PE and CD25-Alexa. The 30% lowest and 60% brightest (CD25-) expressers of CD45RB in the CD4 population were sorted.

Cell Recovery and Flow Cytometric Analysis

Single cell suspensions from spleen and mesentheric lymph nodes were prepared by forcing the organs through a nylon mesh in PBS 2% FCS. For staining, 1 x 10^6, when possible, cells were incubated for 20 min on ice in a 96 well plate with 25 μl the relevant antibody diluted in PBS 2% FCS with 0.01% sodium azide. Cells were washed 2x in FACS buffer and analysed on a FACSCalibur (Becton Dickinson, Mountain View, CA) instrument using Cell Quest™ software. Live lymphocytes were discriminated through the FSC/SSC channels and propidium iodide exclusion. Cells counts were obtained by acquisition of a known number of 10μm latex beads (Coulter Corp., Miami, FL) mixed with a know volume of the cell suspension.

Results

Regulatory T cell numbers are determined by CD25- cells.

To determine whether CD25- cells play a role in the expansion of Tregs *in vivo*, Rag-/- mice received 3x 10^5 Thy1.2+CD25+ cells in the presence or absence of an equal number of Thy1.1+CD25- cells. the original freshly sorted CD4+CD25+ CD4+CD25- populations will be referred to by their Thy1.2 and Thy1.1 allotypic markers,respectively. Twelve days after adoptive transfer, the number of Treg (Thy1.2) cells recovered from the spleen of co-transferred mice was 2 fold increased when compared to those of recipients of single transfer (Fig. 1A). Cell recovery in the mesentheric lymph nodes was much lower and the difference in numbers was less significant between the two groups of animals (Fig. 1B).

For each cellular subset, the number of cells recovered in the spleen of individual mice in the co-transfer group varied significantly. However, when analysing each mouse, a positive correlation can be established between the number of Thy1.2 and Thy1.1 cells recovered (Fig1C), further supporting a role for naïve cells in Treg homeostasis. Interleukin-10 (IL10) is a key cytokine in the control of homeostatic expansion by Tregs, and IL10 deficient CD4+CD25+ cells are unable to effectively control CD4 T cell numbers (12). Co-transfers using CD25+ cells purified from IL10-/- mice also show a strong correlation between the expansion of Thy1.2 and Thy1.1 cells (Fig 1D), indicating that this property is independent from the Treg ability to control homeostatic expansion.

Finally, while all co-transfers above were performed at an initial ratio of 1:1, Thy1.2:Thy1.1, this ratio was reduced 2-3 fold in spleen and 3-4 fold in mesentheric LN at the time of analysis (Table 1). This result further supports the idea that CD4CD25- cells promote survival and or expansion of Tregs while being regulated by them. To assess whether this ratio is dependent on interactions between Treg cells and other naturally activated T cells, we excluded CD45RB^{low} CD4 cells by sorting CD25- CD45RB^{high} cells. Table 1 shows that the ratio in this set of mice is not significantly different from that obtained using unfractioned CD25- (although increased in the spleen). This reduction in the frequency of Tregs may reflect either a difference in cell

proliferation/survival capacity or dependence of Tregs on naive cells. If the former is true, reducing the transfer ratio of Thy1.2:Thy1.1 should still lead to a decrease in the recovered ratio, while if the latter is the case this should not happen, and depending on the transfer condition the recovery ratio may even increase. The fact that transferring at a ratio of 1:10 leads to a similar (spleen and mesentheric lymph nodes) or higher (in other lymph nodes) recovery ratio argues against reduced survival/expansion capability as the explanation. Thus we conclude that CD25- T cells play a role in the homeostasis of Tregs.

CD25 expression is dependent on CD25- cells.

The specificity of the CD25 marker is an important unresolved question. As it was initially characterised as an early activation marker, the concern exists that the CD25+ population analysed is a mixture of Tregs and conventional activated T cells. As for CD25 expression on the Tregs themselves, a previous report indicated that CD25 expression on regulatory T cells is unstable, particularly in the absence of naïve T cells (12, 13, 15). Similar results were obtained in our system. As seen in figure 2A (top), CD25 expression is lost on 80% of the Thy1.2 cells recovered from single transferred animals. Furthermore, the remaining positive cells express very low levels of CD25 and do not exhibit a similar expression level to the original sorted population. The expression level of CD25 on these Thy1.2 cells most closely resembles the weakly positive phenotype acquired by CD25- T cells upon single transfer (Fig.2A, middle). Homeostatic expansion driven activation is not enough to generate CD25 bright T cells from naïve precursors. On the other hand, a significant fraction (around 60% in lymph nodes, figure 2A, bottom) of Thy1.2 cells maintain their CD25+ phenotype in recipients of co-transfers with CD25- cells. This is lower than the 80% reported by Annacker *et al.* (12), but these cells are expressing CD25 at a similar level to the original population. This difference may be due to the different time points analysed*?????*. Interestingly, as shown in figure 2B, the percentage of CD25 bright cells in recipients of co-transfers is similar to that of normal mice, about 10%. This is in spite of the fact that co-transfers were done at initial 1:1 ratios, and that the ratio of Thy1.2 to Thy1.1 cells at this point is between 1:4 to 1:5.

Conventional activated cells may become CD25+, even if transiently. We next analysed CD25 expression on lymphocytes infiltrating the lungs of reconstituted Rag-/- mice infected with *Pneumocystis carinii.* As we have shown previously, in this model naive cells when transferred alone drive a lethal hyperinflammatory reaction (16), and these cells are highly enriched for producer of IFNγ and TNFα (data not shown). However, figure 2C shows that the vast majority, about 90%, of these cells remains CD25- (data from control un-infected reconstituted Rag-/- mice is not shown, as virtually no T cells could be recovered from their lungs at this time point). Pulmonary inflammation also does not lead to maintenance of CD25 expression on Tregs in single transfer (fig. 2C, left). These results demonstrate that on Tregs CD25 expression is mainly maintained by the presence of naive cells. Furthermore, in accordance with what has recently been shown by Belkaid *et al.* (17) in *Leishmania major* lesion infiltrating lymphocytes, even in infected tissues were T cell activation reaches high levels, CD25 still reliably distinguishes Tregs from effector cells.

Treg loss of CD25 expression is not due to differential expansion.

We next ascertained that loss of CD25 expression in single transfer is not due, at least partially, to an expansion of contaminating CD25- in the sorted CD25+ population. As shown in figure 3A, the largest impurity in our 99% pure sorted CD25+ population consisted of CD25- cells (0,6%). Sort purified Thy1.2+CD25+ cells were mixed at 100:1 ratio with Thy1.1+CD25- cells before transfer in Rag -/- animals. At 12 days post-transfer 95 and 80 % of T cells were CD25- in spleen and LN respectively (Fig. 3B). The representation of Thy1.1+ and Thy1.2+ cells in the spleen was essentially the same as in the initial preparation while in the mesenteric lymph node, Thy1.1 cells did expand preferentially relative to the Thy1.2 cells, but this expansion (corresponding to less than 10% of TCRβ+ cells, Fig3B bottom) was not sufficient to account for the 80% of CD25-. Cells recovered from the peritoneal cavity (data not shown) were similar to those of mesenteric lymph nodes. Thus, the alteration of CD25 cell surface expression of Tregs when present alone is wholly due to phenotypic change in this population, and not to expansion of contaminating CD25- cells.

Tregs reacquire CD25 expression upon renewed contact with CD4+CD25- cells.

We next examined whether renewed contact with naive CD4 T cells would rescue CD25 expression on Tregs that had lost it when transferred alone. 3 x 10^5 Thy1.2+ Tregs were transferred to Rag-/- deficient mice and allowed to expand for 12 days, which as shown in Fig.2A leads to loss of CD25 expression on 80% of the cells, with the remaining fraction expressing low levels. On day 12, mice received an additional 3 x 10^5 Thy1.1+ CD25- cells and sacrificed 10 days after this secondary transfer. Spleen and mesenteric lymph node T cells were analysed for expression of CD25 and Thy1.1. The original Treg population (Thy1.2+) reacquires CD25 expression when put in *de novo* contact with CD25- cells (Fig.4A). This effect is specific to the Thy1.2 cells, as Thy1.1 T cells did not acquire CD25 expression. The surface level of CD25 on Thy1.2+ is comparable to that of CD25+ in the initial co-transfer experiments.

We next assed whether Tregs that had lost and reacquired CD25 expression had preserved their ability to control homeostatic expansion of CD25- cells *in vivo*. Un-manipulated mice and animals transferred 12 days before with Thy1.2 CD4CD25+ cells received 3 x 10^5 Thy1.1 CD25- lymphocytes. The number of CD25- cells recovered from the spleen, mesentheric lymph nodes and peritoneal cavity (data not shown) 10 days later was significantly reduced in the recipients of sequential transfer (Fig. 4B) indicating that the "parked" Treg population was still efficient at regulating CD25- cell numbers.

Discussion

The present work demonstrates that CD4+CD25- cells participate in the regulation of CD4+CD25+ cell numbers and phenotype. By showing that CD25- cells are not only passive targets of regulation, but also play a central role in Treg physiology we therefore establish a novel feed back loop in the dynamics of these two cell populations. This finding is consistent with several studies implicating IL2 as the cytokine necessary for maintenance of CD4CD25+ cells in peripheral tissues. We show also that loss of CD25 expression by CD4CD25+ cells is quickly reversed in presence of CD4CD25- cells, supporting the idea that CD4CD25+ cells may constitute a distinct lineage of lymphocytes.

Tregs do not normally secrete IL2 and are potent suppressors of *in vitro* IL2 secretion (18, 19). Paradoxically, Tregs have also been shown to be dependent on exogenous IL2 for their generation/survival (20). IL2 was initially characterized as a potent T cell mitogen but was later shown to also trigger activation induced cell death (AICD) (21). Mice with targeted deletions of the IL2, IL2Rβ and IL2Rα genes develop immune-pathology and uncontrolled lymphoproliferation (22-25), a finding first attributed to defective AICD. It now seems more likely that deficiency in regulatory T cells is the dominant cause of disease all in these models. Thus, pathology in mice deficient for IL2 or IL2 receptor components can both be prevented by restoring the Treg compartment (15, 26, 27). In contrast, one study contradicts a role for IL2 in the peripheral maintenance of Tregs claiming it is only important for thymic development of these cells (27). Aside from its effect on the generation and proliferation of Tregs, IL2 may also play a role in triggering their function (28). The dependence of Tregs on CD25- shown here may represent an *in vivo* equilibrium between their need for IL2 and their suppression of its secretion. That the ratio CD25:CD25 found in the present work is higher than the natural ratio in unmanipulated mice may simply reflect higher production of IL2 and other, as yet unidentified growth factors, by CD25- undergoing homeostatic expansion and activation- it does not represent a *de facto* equilibrium state.

Still, CD25+ cells expand and survive well in both T cell deficient and alymphoid recipients. Some of this survival/expansion may nevertheless be dependent on other IL2

sources, such as dendritic cells (29). IL2 independent factors have also been implicated, such as a positive effect of B cells (30) on the number of Tregs, and we have recently shown that pro-inflammatory stimuli can also expand Tregs through engagement of Toll Like Receptors which can lead to IL2 secretion by Tregs themselves (31). Finally, constant high-affinity stimulation through the T Cell Receptor itself may help to maintain CD25 expression, and this may be responsible for example for the high percentage (46%) of lesion associated CD25+ cells reported in single transfer in a recent report on control of responses to *Leishmania major* (17), although in this case there is also a local inflammatory stimulus provided by the infection.

Importantly, Tregs that lose and then regain CD25 expression are still efficient regulators of homeostatic proliferation of CD25-, indicating a functional as well as phenotypic conservation (fig4). Consistent with this, Gavin *et al.* (13) showed that Tregs that underwent homeostatic expansion alone not only retained the ability to suppress *in vitro* proliferation of CD25- cells, but that this function may even be augmented relative to freshly isolated Tregs. The independent lineage hypothesis is supported both by the recent description of a transcription factor, Foxp3, involved in the commitment of Tregs (32), and the fact that they are mostly derived from a pool of single positive thymocytes (33). We demonstrate here that although CD25+ cells are a separate pool of T cells, they are nonetheless involved in reciprocal interactions with other lymphocyte populations.

Figure Legends

Fig 1) CD25- determine the expansion of CD25+ cells. A) Number of Thy1.2+ cells recovered from spleen and lymph nodes of C57Bl6 Rag-/- mice that received this population alone or co-transferred with CD25- cells. B) Thy1.2+ cells recovered in the mesenteric lymph nodes of single and co-transferred mice. C) Correlation of the number of Thy1.2 and Thy1.1 cells recovered in the spleen of co-transfer experiments. D) Correlation of the number of CD25+ and CD25- cells recovered in the spleen of co-transfer experiments where IL10-/- mice were used as donors of CD25+ cells.

Fig 2) Cell surface expression of CD25 depends on the presence of CD25- cells. 2A, histogram plots of CD25 expression of Tregs in single or co-transfer (top and bottom, respectively), and naive cells in single transfer (middle). Light line indicates CD25 expression level on Tregs of normal control mice. 2B) Percentage of CD4 cells expressing CD25 in wild type and co-transfer recipient Rag-/- mice. 2C) CD25 expression on lung infiltrating cells.

Fig 3) Loss of CD25 expression does not reflect cell population dynamics. A) The largest contaminating fraction in our sorted CD25+ cells consisted of CD25- cells. B) Analysis of mice reconstituted with Thy1.2+CD25+ cells plus 1% fraction of Thy1.1+CD25-, 12 days after transfer, for spleen (top) and mesenteric lymph node (bottom). Cells were gated for TCRβ+ as shown in the left, and the frequency of Thy1.1+ cells was analysed (right).

Fig 4. Analysis of secondary transfer. Mice received 3 x 10^5 CD25+ cells which were allowed to expand for 12 days before receiving an equal number of CD25-. Mice were analysed 12 days after secondary transfer. A) Spleen and mesenteric lymph node T cells (gated on TCRβ+ cells). Gating on Thy1.2+ and Thy1.2- T cells shows that sequential transfer leads to substantial recovery of CD25 expression on Thy1.2+ cells, while Thy1.2- remain negative. B) Number of Thy1.1+ TCRβ+ cells recovered in the spleen and mesentheric lymph nodes of single or co-transferred mice.

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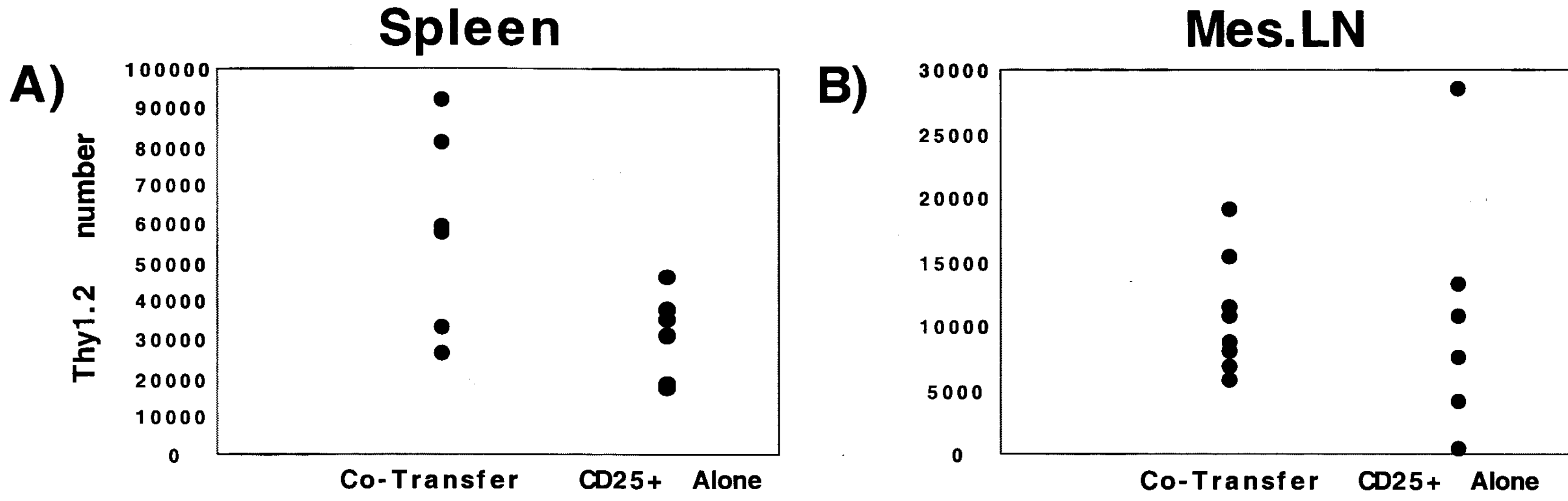
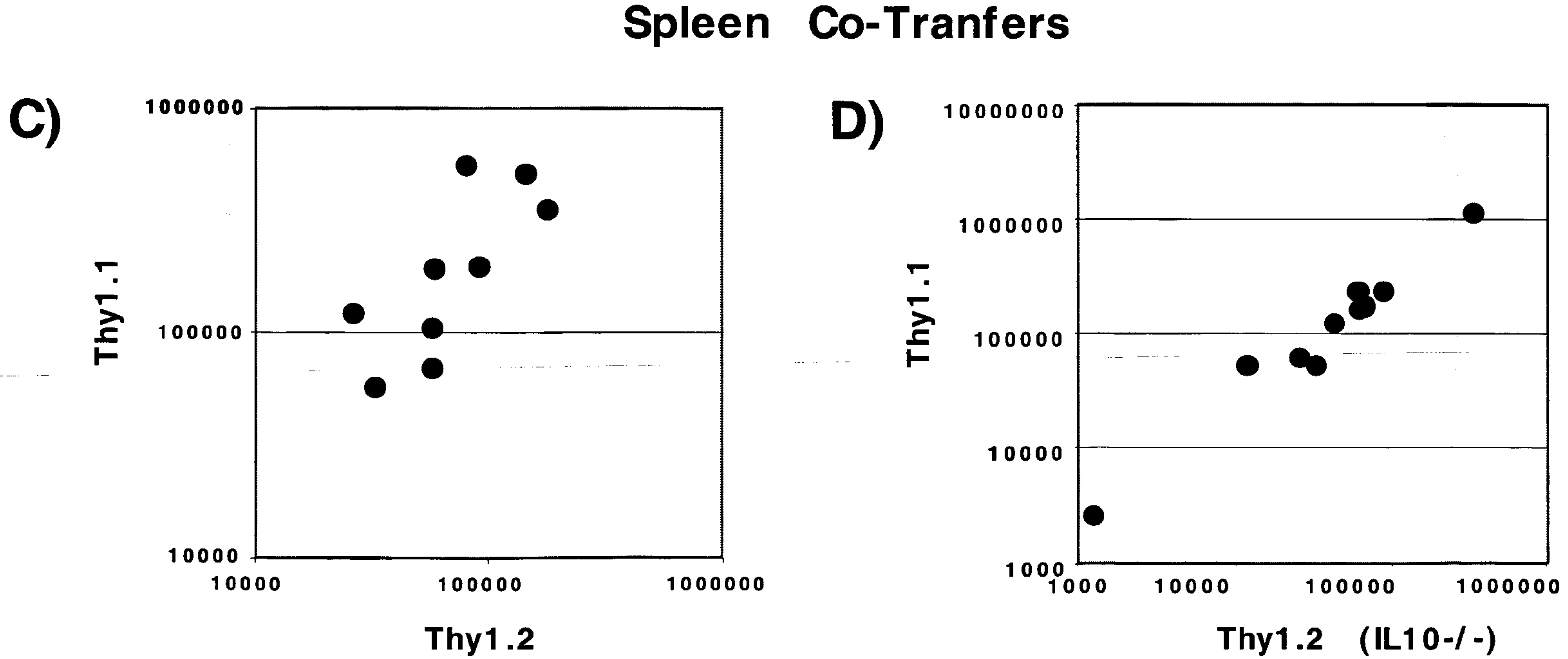
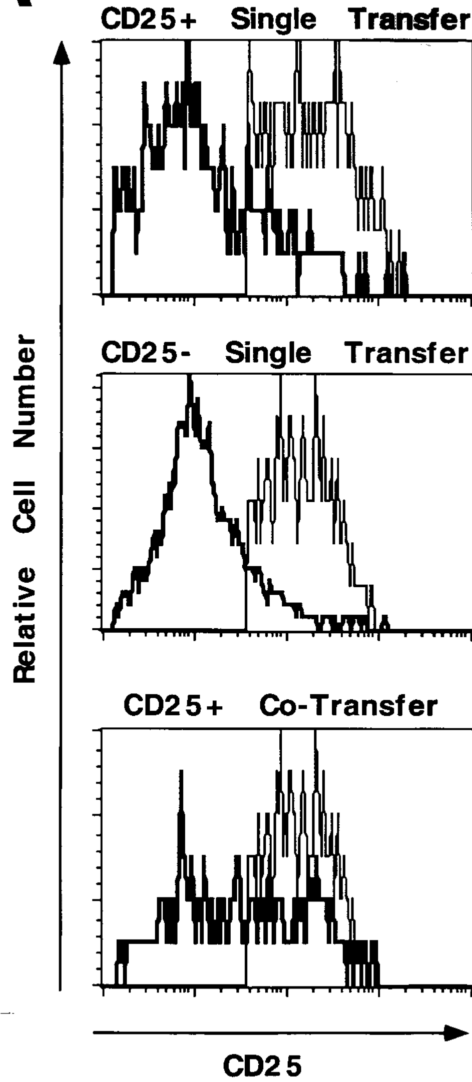
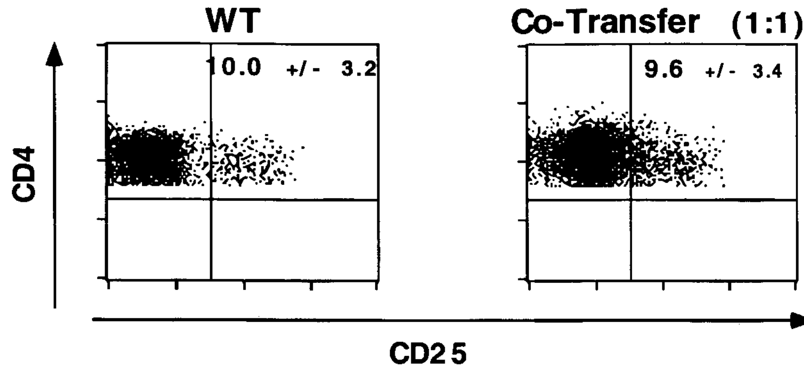
Fig 1






2A
CD25+ Single Transfer
CD25- Single Transfer
CD25+ Co-Transfer
Relative Cell Number
CD25


2B
WT
10.0 +/- 3.2
Co-Transfer (1:1)
9.6 +/- 3.4
CD4
CD25

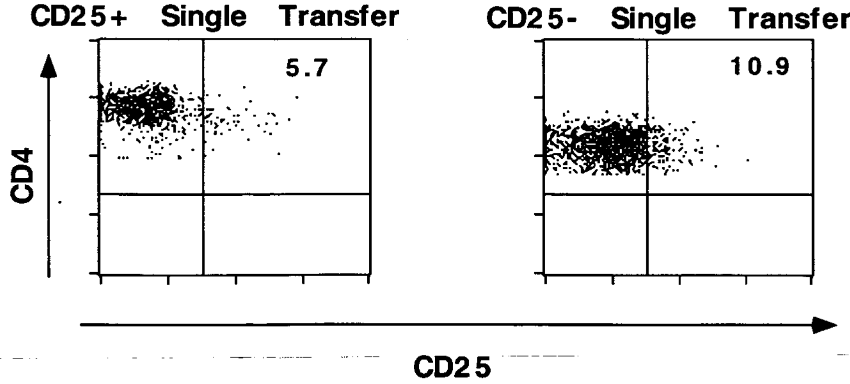
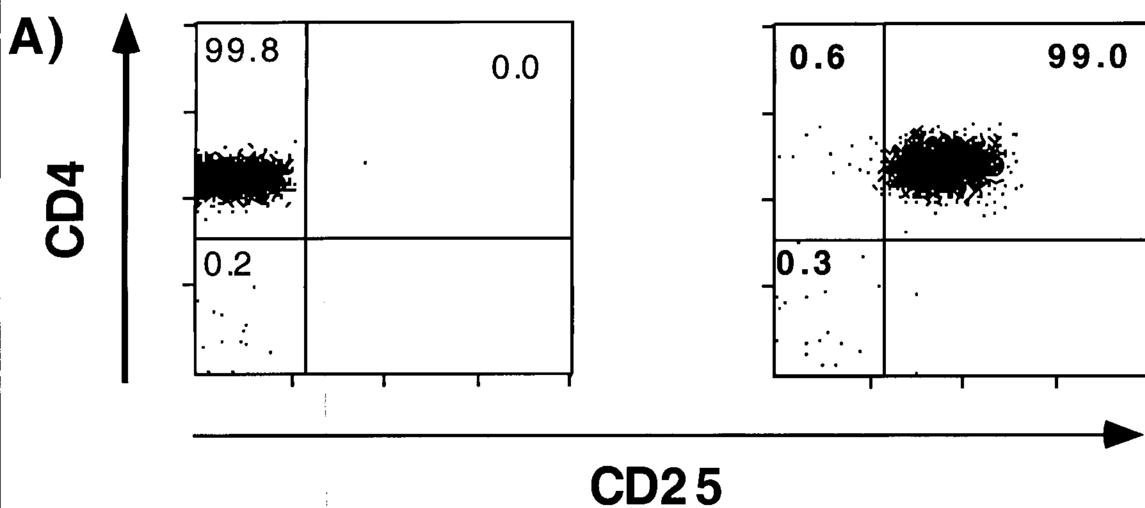

2C
P. carinii infected lungs
CD25+ Single Transfer
5.7
CD25- Single Transfer
10.9
CD4
CD25

Fig 3



B)

12 Days Post-Transfer

TCRβ

3.6

0.2

3.4

Spleen

16.9

3.5

9.1

Mes. LN

CD25

Thy1.1

Fig. 4A

TCRβ

Spleen

97.4 2.6

13.5 86.5

Thy1.2

CD25

Mes. LN

78.4 21.6

23.6 76.6

Thy1.2

CD25

Fig. 4B

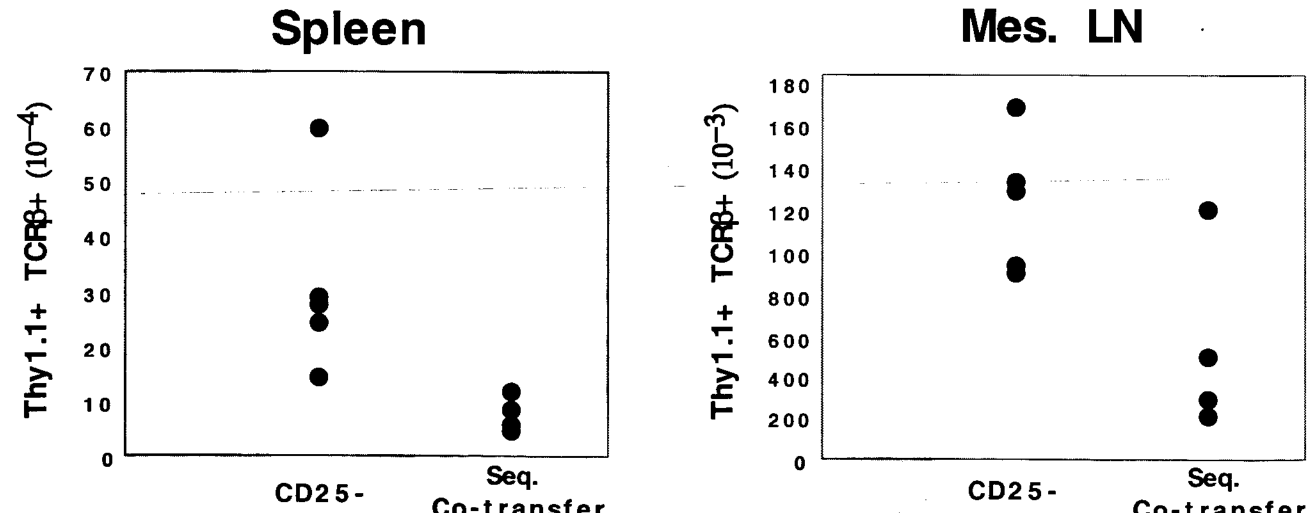


Table 1: **Ratio of Thy1.2:Thy1.1 T cells 12 days post-transfer**

Group	Spleen	Mes. LN	LN
Thy1.2:Thy1.1 **1:1** (n= 9)	0.43 +/- 0.22	0.26 +/- 0.10	N/D
IL10-/- Thy1.2:Thy1.1 **1:1** (n= 10)	0.38 +/- 0.12	0.26 +/- 0.13	N/D
Thy1.2:Thy1.1(CD45RB^{high}) **1:1** (n= 3)	0.57 +/- 0.09	0.31 +/- 0.09	0.43 +/- 0.12
Thy1.2:Thy1.1(CD45RB^{high}) **1:10** (n= 3)	0.07 +/- 0.05	0.07 +/- 0.04	0.21 +/- 0.07

Supplementary Results: CD25+ cell expansion

One of the main problems in the study of regulatory cells is how to expand them. This is true both of basic science and application concerns in this field. As they were initially characterized as "anergic", they were promoted as cells with severely limited proliferation capacity. However, *in vivo* studies have shown that they do in fact proliferate when transferred to lymphopenic hosts, the so-called homeostatic expansion. Furthermore, recent work has shown that CD25+ cells are in fact proliferating more than CD25- cells in normal, unmanipulated mice, as measured by BrdU labeling.

In manuscript II (Caramalho et al., 2003) we have shown that *in vitro* inflammatory stimuli through TLRs can promote division of Tregs, and that this stimulation synergizes with IL2. IL2 itself had already been shown by others to be a mitogen for Tregs (as indeed for all T cell types). We further examined the differential effects of inflammation on the recovery of Tregs *in vivo*. Rag-/- mice were reconstituted with CD25+ cells either alone or in combination with CD25- (co-transfers) and mice were then injected 3 times at three day intervals with either LPS or PBS i.p. At the end of this period, mice were sacrificed and peritoneal wash cells were recovered.

As shown in figure 1, LPS increases the recovery of Tregs from the peritoneal cavity. Furthermore, it has an additive effect with the presence of other T cells (CD25- cells). This may be analogous to the synergistic effect seen *in vitro* (man.II) between LPS stimulation and IL2. Contrary to what others have reported for higher doses of LPS, we do not see a significant rescue of the loss of CD25 surface expression in treated mice (fig2). Thus this re-enforces what we report in manuscript IV, that the only reliable way so far to maintain the original Treg surface phenotype is to have naive cells present, possible again through their production of IL2. Fig.1 also supports the interdependence of the two population that we report in man. IV. Finally, we tested if a clear TLR4 role on T cells could be seen in this response. To this end, we sorted Tregs from B10ScCr mice, a natural mutant strain which has a genomic deletion spanning the TLR4 locus, rendering these mice unresponsive to LPS and resistant to endotoxic shock. As shown in figure 3, when B10ScCr cells were used to reconstitute TLR4 wild type Rag deficient mice a similar accumulation of Tregs in the peritoneal cavity was seen. This indicates that at the dose used and time point analysed, the effect seen in figure 1 may be entirely attributed to indirect stimulation of Tregs by the host innate immune system's response to LPS.

This does not however discard a role for TLR4 on Tregs at other doses or time points. Furthermore, the differential effect of TLR4 ligation on these cells *in vivo* may be more subtle, involving for example a modified or enhanced effector function. More studies will be needed to clarify this issue.

Fig.1:Inflammation locally expands CD4+25+ T cells

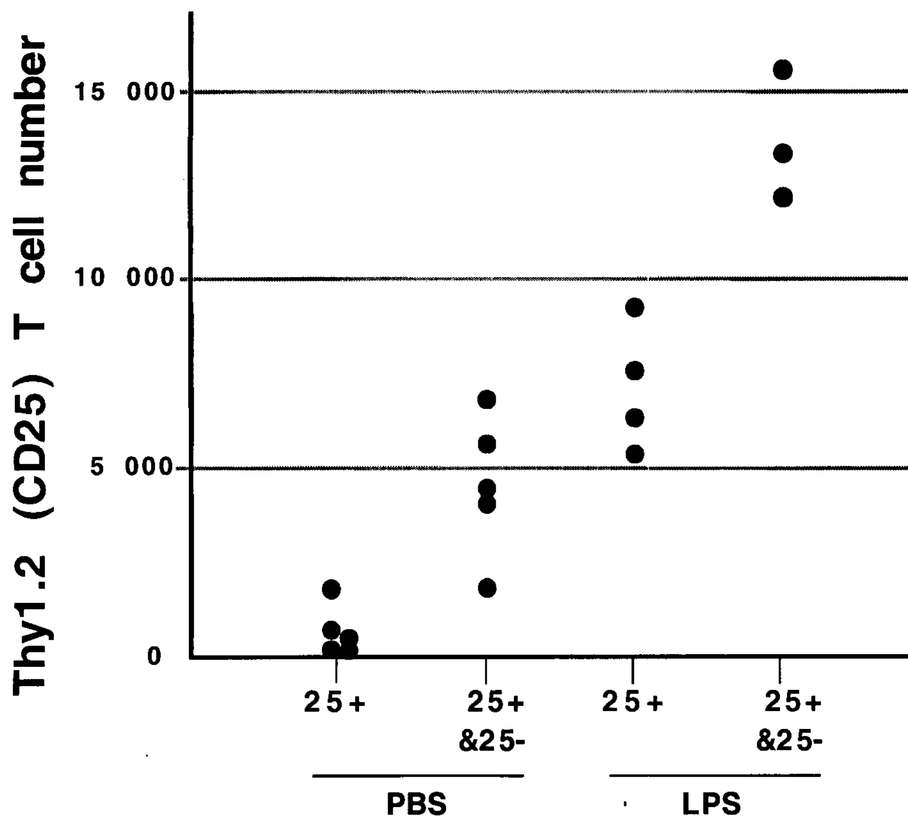
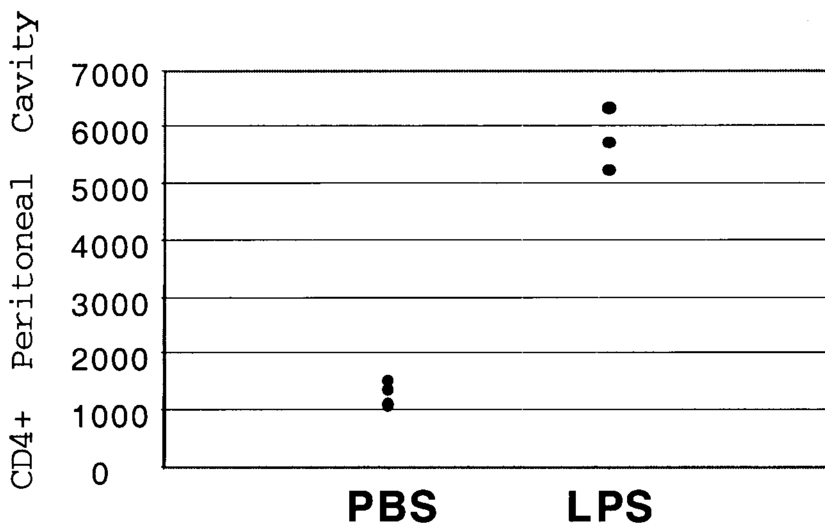


Fig.2:In vivo indirect effect.



Rag-/- mice reconstituted with CD25+ cells from B10ScCr mice.

Supplementary Results:

CD25+ T cell repertoire

A major component of the dominant tolerance model proposed by Coutinho and collaborators was that it contained an ontongenic component. Tregs would be generated at higher frequencies in the newborn/perinatal thymus because due to delayed colonization by bone marrow APCs negative selection is very efficient at this stage. Taking it one step further, as antigen receptor diversity is restricted in early life, this model would allow for germline selected specificities to enter the Treg pool. Although in accordance with the facts when it was proposed, this aspect of the model has proven extremely difficult to demonstrate.

We reasoned based on evidence that particular systems may highlight developmentally regulated deletion in the Treg pool. In particular, it has been demonstrated that deletion of maturing thymocytes by endogenous (retroviral) superantigens is deficient in newborn mice. Recently it was reported that in cells expressing Vβ genes targeted for superantigenic deletion that survived, a marked enrichment for CD25+ cells that produced IL10 occurred (Papiernik et al., 1997).

An older observation by Augustin and collaborators (Rajasekar et al., 1993)reported that T cells surviving deletion in DBA/2 mice had very particular repertoires. Specifically, they were enriched for germline sequences, i.e, they had few or no n-nucleotide addition at the junction of TCRβ V,D, and J gene segments. This is a hallmark of early ontogeny T cells, and derives from the low expression of the enzyme terminal deoxynucleotide transferase (TdT) *in utero* and perinatally.

Thus we reasoned that following CD25+ superantigen non deleted CD4 T cells might provide a window to study early Tregs. Unfortunately, while we do see a mild enrichment in the rare surviving cells for CD25 expression (usually around 30% CD25+ as compared to 10% for other Vβ expressors in the same mice, we failed to detect in either the CD25+ or CD25- superantigen non-deleted cells any evidence for low junctional diversity. This may be due to the use of a different strain (Balb/c as opposed to DBA/2) but it nonetheless argues against this as a general tool to study perinatal Tregs.

This data is presented in annex, with the germline V, D and J segments identified for each Vβ gene sequenced, as well as the N nucleotides.

Annex: TCRβ sequences from CD25+ and CD25- cells.

V Beta 14 CD25-

Sequence –11

Vb14	N	TCRBD2	N	J2s5
TGT GCC TGG AGT CT		GGACT		ACC AAG ACA CCC

Sequence –12

Vb14	N	TCRBD2	N	J2s3
TGT GCC T	ATC	GGGGGG	CA	A GTG CAG AAA

Sequence –13

Vb14	N	TCRBD2	N	P	J2s3
TGT GCC TGG AGT CT	C	CTGGGGGG		T	A GTG CAG AAA

Sequence –14

Vb14	N	TCRBD2	N	J1s5
TGT GCC TGG AG	CGTG	GGAC	AA	A ACA ACC AGG CTC

Sequence –15

Vb14	P	N	TCRBD2	N	J2s5
GCC TGG AGT CT	A	T	CTGGGGG	TCC	CC AAG ACA CCC AGT

V Beta 14 CD25+

Sequence +11

Vb14	N	TCRBD2	N	J2s5
TGT GCC TGG AG	ACT	GGGACTGGGG		A ACC AAG ACA

Sequence +12

Vb14	N	TCRBD2	N	J2s3
Unreadable				

Sequence +13

Vb14	N	TCRBD1	N	J1s3
TGT GCC TGG AG	CCAA	GACA	T	TT CTG GAA ATA

Sequence +14

Vb14	N	TCRBD1	N	J2s7
TGT GCC TGG AG		GGACAG		CT CCT ATG AAC

Sequence +15

Vb14	P	N	TCRBD2	N	J2s3
TGT GCC TGG AGT CT	AG	TTC	GACT		TG CAG AAA CGC

Vß11 Sequences **<u>CD25-</u>**

Sequence -1

Vbeta 11	**N**	**TCRBD**	**N**	**Jbeta2S5**
aag cag ctt ag	GC	GC		CC AAG ACA CCC

Sequence -2

Vbeta 11	**N**	**TCRBD2**	**N**	**Jbeta2S4**
aag cag	TTTC	GAC		A GTC AAA ACA

Sequence- 3

Vbeta 11	**N**	**TCRBD1**	**N**	**Jbeta2S5**
aag cag ctt	TC	CAGG	TCCT	A ACC AAG ACA

Sequence-4

Vbeta 11	**P**	**N**	**P**	**TCRBD2**	**N**	**Jbeta2S7**
aag cag ctt aga	T	TCTTT	CC	GGGACTGGGGGG	T	TG AAC AGT ACT

Sequence-5

Vbeta 11	**N**	**TCRBD2**	**N**	**Jbeta1S4**
aag cag ctt ag	T	A		G ACG AAA GAT

Sequence-6

Vbeta 11	**N**	**TCRBD1**	**N**	**Jbeta2S4**

aag cag ctt a		CAGGG	CT		TC AAA ACA CCTT

Sequence-8

Vbeta 11	**N**	**TCRBD2**	**N**	**Jbeta2S5**
aag cag ctt ag		GGGAC	AT	CC AAG ACA CCC

Sequence-9

Vbeta 11	**N**	**TCRBD2**	**N**	**P**	**Jbeta2S3**
aag cag c	C	GGGAC	AA	T	A GTG CAG AAA

Vß11 Sequences **<u>CD25+</u>**

Sequence -1

Vbeta 11	N	TCRBD1	N	Jbeta2S5
aag cag ctt ag	TC	CAG	AG	AG ACA CCC AGT

Sequence -2

Vbeta 11	N	TCRBD1	N	Jbeta2S5
aag cag ctt ag	TC	CA	T	C AAG ACA CCC AGT

Sequence- 3

Vbeta 11	N	TCRBD2	N	Jbeta2S7
aag cag ctt ag	T	GGACT	TTT	ATG AAC AGT ACT

Sequence-4

Vbeta 11	N	TCRBD2	N	Jbeta2S5
aag cag ctt aga		GACTGGGGGGGC	TCG	AG ACA CCC AGT ACT

Sequence-5

Vbeta 11	N	TCRBD2	N	Jbeta2S7
aag cag ctt ag	T	GGACT	TTT	ATG AAC AGT ACT

Sequence-6

Vbeta 11	**N**	**TCRBD1**	**N**	**Jbeta2S4**
aag cag ctt aga		GGGACAGG		ACA CCT TGT ACT

Sequence-8

Vbeta 11	**N**	**TCRBD1**	**N**	**Jbeta1S6**
aag cag ctt a		CAGGGGGC	GG	ATT CGC CCC TCT

Sequence-9

Vbeta 11	**N**	**TCRBD1**	**N**	**Jbeta1S6**
aag cag ctt a		CAGGGGGC	GG	ATT CGC CCC TCT

Conclusions and Perspectives

"The inevitable never happens. It is the unexpected, always."
John Maynard Keynes

The experimental findings of this thesis demonstrate that Tregs operate well in inflammatory conditions, even when these are brought on by an infection (with resulting down modulation of protective immunity. Tissue inflammation seems to attract Tregs, which is accordance with findings of other groups on their particular pattern of chemokine receptor expression. Furthermore, pro-inflammatory PAMPs such as LPS promote Treg expansion via the Toll Like Receptor family, which also have a particular expression pattern for Tregs. Regulation of effector T cell infiltration to sites of inflammation is also achieved by Tregs, and there is some indication that this may be at least in part due to down modulation of the innate inflammatory response, thus reducing chemotaxis promoting molecules such as chemokines. LPS stimulation of Treg proliferation synergizes with IL2. As we show that CD25- cells promote viability of Tregs *in vivo*, an effect which may be strongly dependent on IL2, sites of ongoing inflammatory immune responses may be particularly stimulating for Tregs as they will then encounter both types of stimulation. That this happens *in vivo* is suggested by our first set of supplemental data, although here the indirect effect (of systemic inflammation) does not allow us to point to a direct effect of TLRs in this case. Finally, in the last set of supplemental data, we join other groups in failing to report particular repertoire characteristics for Tregs (when examining TcR diversity, and not reactivities), even though we use a trick that should have enhanced any such difference by focusing on Vβ genes that interact with endogenous superantigens.

Manuscript one extends the finding of the IBD model of intestinal inflammation to other mucosal surfaces, in this case the lung. Hyper-inflammation in this site has more acute consequences than in the bowels, which may show that the pulmonary environment is more sensitive, and in particular the accumulation of inflammatory cells and their products may rapidly compromise function. The notable new finding in this study is the inhibition of immune responses against non-self, as shown by the reduced rate of clearance of *Pneumocystis carinii* in mice reconstituted also with Tregs. This extends the range of Treg activity from mediating tolerance to self to general immune regulation. *P. carinii* induced pneumonia is one of the major complications observed in patients suffering from AIDS. Up to now this was thought to reflect a simple consequence of immunodeficiency, failure to control an innocuous infection. Our results indicate that disregulated T cell responses brought on by lymphopenia may be the main cause of disease. Indeed in AIDS patients many of the more frequently observed life threatening opportunistic infections are not caused by exotic pathogens, but by the resident commensal flora. Thus the observations in I,

together with older findings principally on intestinal flora driven immune pathology in the absence of proper dominant regulation suggest that other harmful immune reactions may exist in AIDS and other immuno-compromised patients.

The results in manuscript I do not allow us to distinguish whether the regulatory T cells in this model of acute pneumonia are self or pathogen reactive, or a mixture of both. In the recent literature, a few other models of regulatory T cell inhibition of responses to non-self have been reported, and in some of these the Tregs involved have been shown to be pathogen specific (Kullberg et al., 2002; McGuirk et al., 2002; McGuirk and Mills, 2002). How these cells develop is not clear, in particular their relationship to Tregs that develop in the thymus. They may be differentiating wholly in the periphery upon contact with pathogens, and some have proposed that they may derive from some form of terminally differentiated Th2 responder cell. Alternatively, they may be direct descendents of thymic Tregs that are cross reactive with pathogen antigens, or multi-reactive as whole. A hybrid model would be one where thymic, anti self, Tregs direct the peripheral differentiation of anti pathogen Tregs, a slightly different mode of what has been termed "peripheral education". It should also be noted that the mucosal sites where a lot of these phenomena occur have also been proposed to be sites of extra-thymic T lymphopoesis, thus there may be local differentiation of pathogen specific Tregs during development, in a mode analogous to that of CD25 cells.

When looking at the CD25+ cells themselves in this model we find that they migrate to inflammatory sites, and their presence there was proportional to the level of infection. What is not clear from this work is what are the activating elements for Tregs in the inflammatory reaction. Previous work by other groups showed that Tregs are well equipped to migrate to inflammatory sites, and they display expression of receptors for inflammatory chemokines, and respond promptly to inflammation by chemotaxis (Bystry et al., 2001; Iellem et al., 2001). But once they are there, what do they recognize? In this sense, integrating the chemokine work of others with our results from manuscript II we may yield another piece of the puzzle. Tregs migrate fast to inflammed tissues, and once there they can be activated and expanded by microbial products through their TLRs. This may already have a beneficial effect by helping to regulate inflammation. If in addition we accept the proposal that Tregs are enriched for self-reactive cells, then they may also prevent that the response propagates to host antigens. We do not yet know the proportion of Tregs that express specific TLRs, nor if the receptors are evenly distributed, clonally expressed, or fall

somewhere in between these two extremes. An attractive possibility is that TLR expressors are a subset of semi-innate regulatory T cells, representing maybe a primitive state. The TcR repertoire of cells that expand in response to mitogens should prove illuminating in this matter, but at the present time we have no data on this. Interestingly, *in vitro* treatment with LPS enhances suppressive capability, and an *in vivo* correlate of this phenotype is needed.

Manuscript III suggests that once the cells arrive at the site of inflammation, they make it less attractive for effector cells. The cytokine implicated in the regulation of the LPS response, IL10, acts mainly on cells of the innate immune system to dampen inflammation. This scenario is in turn consistent with our demonstration that Tregs can inhibit peritoneal macrophage activation directly *in vitro*, and that the *in vivo* innate response to LPS is also lessened in their presence. Whether Tregs can also attenuate endotoxic shock *in vivo* should also be examined. Immune deficient mice are more susceptible to negative effects of LPS, and although natural antibody has been shown to be important in this respect (Reid et al., 1997), it does not fully recover the wild type phenotype, and a role for Tregs may exist. Alternatively, cooperation between naturally activated T and B cells may occur and have a beneficial regulatory role, and in this respect LPS provides a good experimental model. These results show that while IL10 is not a player in the regulation of *in vitro* T cell proliferation, its production by Tregs is central to their immuno-regulatory function. Data from other models support this assertion, while some *in vivo* models, such as autoimmune gastritis (AIG) seem to be regulated fine in the absence of IL10 (Suri-Payer and Cantor, 2001). IL10 is most probably crucial where reducing inflammation is necessary to promote tolerance or to avoid immune pathology. In cases of abnormal apparently spontaneous activation of autoimmune responses, it may not be necessary, and direct interference with T cell activation, possibly in a cell contact dependent manner may be required. It should be noted that in this model there are no non-self antigens, and the LPS injection leads to innate immune activation and presentation of self in an activating context.

The final set of experimental results shows that far from being an isolated, untouchable, regulatory T cell subset, CD25+ Tregs are dependent on their complementary T cell population. This had already been implied by detailed studies of mice deficient in components of the IL2/IL2R pathway. Manuscript IV also argues for the idea that CD25+ cells are at least in part constituents of a distinct lineage, as activation does not lead CD25- to a similar cell surface phenotype, even in an infectious (IV) or purely inflammatory (Sup.1, Fig 2) context (and certainly not in homeostatic

expansion induced activation as in IV, which does lead to a CD45RB^{low} CD62L^{low} phenotype, for example. CD25+ that have lost surface expression appear to have some "phenotypic memory", as expression is selectively restored upon renewed contact with target cells. Integrating these last results with the previous ones, we could propose that *in vivo* a similar situation to what is described for the synergistic effect of IL2 and LPS on Treg proliferation observed *in vitro* in AnnexII. If CD25- cells promote Treg survival/growth and inflammation promotes naïve cell activation and proliferation, sites of ongoing immune responses may be the optimal environment for Tregs.

Our results argue against models where the self/non-self discrimination is left as the responsibility of the Innate Immune system and germline encoded receptors, such as the TLR family of genes. There is no question that co-stimulation is crucial both to the induction of the immune response, and to the effector class decision for the response. It does not, however, make the self- non self discrimination, it merely sets the stage where this decision will have to be taken.

Tregs are gaining increasing acceptance, but the view that tolerance can be achieved through the use of modified two-signal model and deletional mechanisms is still alive and well, and one can read in highly influential journals a synthesis like (Medzhitov and Janeway, 2002) that read:

> *"Because the co-stimulators are induced by PAMPs, their expression on APCs flags the antigenic peptides presented by the same APC as being of microbial origin and activates antigen-specific T cells. Self-peptides expressed and presented by APCs are not recognized as nonself, because T cells specific for these peptides are eliminated during negative selection in the thymus. Thus negative selection and microbial induction of costimulatory molecules together ensure that the adaptive immune response is generated against infecting pathogens but not against self-antigens."*

This like the most extreme case of a germline self/non-self discrimination model, Matzinger's "danger" (Matzinger, 1994) assumes that central tolerance leaves a harmless pool of peripheral specifities (or that peripheral anergy finishes the job). In effect, these hypothesis deal not with tolerance (and Matzinger has the courtesy to dismiss self/non-self considerations from her speculations altogether) but with when and how to induce an immune response- and innate signals are certainly the key here. The "danger" formulation is particularly ominous, as here the predominant signal is damage to self, a situation where the need to balance tissue repair

with proper assessment of the presence or absence of an infectious threat is vital- and which, together with imperfect central tolerance would make the immune system prone to self destruction.

Evidence that innate immunity plays a master switch role in tolerance is severely lacking. Even if we look at the absolute limit case, injecting optimal doses of self proteins emulsified in complete Freund's adjuvant in most cases we fail to induce autoimmunity. One can often see a transient burst of autoreactivity that does not degenerate into disease. Furthermore these episodes often re-inforce tolerance, rendering the host less susceptible to subsequent immunization, even if the secondary immunization is repeated with adjuvant. We would argue that this is not in any case the correct test of the hypothesis- physiological concentrations of self antigen must be used- or more accurately, adjuvants should reveal anti-self activity based on the presentation of only the endogenous antigens. Here a few cases do lead to disease, most notably in adjuvant arthritis. These are however rare and very strain dependent phenomena. In contrast, as was discussed in 1.10, a variety of infectious/inflammatory stimuli re-inforce tolerance. A recent report by Medzhitov's group claims to show that ligation of Toll Like Receptors on APCs leads to the abrogation of suppression by rendering the effector cells refractory to regulation, via an IL6 (and an as yet unidentified factor(s)) dependent mechanism (Pasare and Medzhitov, 2003). Examination of the experimental system they used however, shows that the conclusions that can be drawn are limited, as the response they monitor is a response to ovalbumin. This is in fact down modulated by the mechanism they propose- but what is the relevance of this to tolerance? In this case they would have to show that their mechanism will lead Tregs to relax their control of anti-self responses. We discuss the role of Tregs in modulating immune responses in general in Annex V. Regulation of responses to non-self in also important, but avoid excessive immune pathology from an appropriate response is a distinct problem from avoiding autoimmunity. In this case, the interplay of soluble factors may be much more important, as an analysis of the relative importance of IL10 in immunepathology **X** autoimmunity would indicate. In modulating immune responses, control of the general level of inflammation APC activation and a higher level of non-specific suppression could be important, and may effectively be counterbalanced by effector cytokines such as IL6. So what then would allow for immune responses in the presence of Tregs? We would argue that the two key factors are the natural ratio (at least as low as 1:10) of Tregs to naïve/effector T cells, and differences in TcR repertoire. The combination of the two factors would favor induction of responses to non-self, while the self reactive repertoire of

Tregs would work to prevent responses to self. A natural consequence would be that as responses to non-self will always occur in the context of presentation of some self antigens, every immune response is in fact reduced by Tregs. In the particular case of their responsiveness to LPS and other TLR ligands, at least in the case of LPS their optimal response dose is 2 orders of magnitude lower than that of dendritic cells. So interference with initial priming in an infectious context would be inefficient, which is consistent with the transient bursts of autoimmunity that follow immunization with self antigens in adjuvant. However at higher doses, turning off deleterious inflammatory responses and reducing the risk of anaphylaxis and shock may have a higher survival value.

The results presented here on the immunological basis of pathology in *Pneumocystis carinii* infection adds to previous results in suggesting that currently available immuno-supressive therapy based on the use of corticoids or radiotherapy should be approached with renewed caution. The growing literature on lymphopenia induced autoimmunity and immunepathology (Gleeson et al., 1996) indicates that interruption of treatment may lead to the appearance of new and aggressive auto-immune syndromes in patients. In addition most, if not all, lymphopenia inducing approaches target activated/cycling cells, and it should be noted that Tregs are not only clearly activated cells, they are also cycling at a higher rates than naive cells *in vivo* (Hori et al., 2002).

The previous history of suppressor T cells should strongly encourage that results inconsistent with the need for, or existence of, regulatory T cells be examined with great care. Perhaps the most vulnerable aspect of Tregs is that most models where they have been shown to operate *in vivo* are derived from lymphopenic states, and evidence for their function in the intact immune system is still very thin. Stockinger (Stockinger et al., 2001) has argued that in an intact immune system, competition for space and resources will prevent the expansion of pathogenic clones without the need to invoke a special subset of regulatory T cells. Thus, such factors as availability of APCs and antigen, as well as growth factor like IL7 are know to influence the expansion of cells in lymphopenic hosts. Experimentally, her group recently demonstrated that T cells from TcR transgenic mice that expand well in Rag mice also prevent IBD induced by transfer of $CD45RB^{hi}$ cells, even though they have a naive ($CD45RB^{hi}$ CD25-) phenotype. Conversely, poorly expanding T cells from different transgenic mice fail to protect co-transferred recipients. Finally, they show that transfer of a high number of wild type $CD45RB^{hi}$ cells does not induce disease (Barthlott et al., 2003). Against the sufficiency of an "ecological" model of immune regulation are

two main sets of data. The first are the still relatively rare studies that show specificity requirements for Tregs. This is most clear in the EAE model, where LaFaille and co-workers have shown that space-filling activated monoclonal T cells do not avoid disease (Olivares-Villagomez et al., 2000). For CD25 regulatory T cells in this model work of Hori in our group has clearly shown that T cells specific for the nominal self antigen (MBP) are necessary in this model (Hori et al., 2002). Another fact that is hard to reconcile with competition for peripheral space is the finding that Tregs appear to derive from a differentiated thymic lineage with its own rules of selection, i.e., that they have a separate developmental program. Finally, while it may be said that CD4 cells compete with each other for space, particularly in the activated T cell compartment (and this may be extended to CD8 cells, which are also influenced by the presence of CD4 cells in a homeostatic manner)(Freitas and Rocha, 2000), it is harder to reconcile with descriptions of regulation of other cell types that are not directly competing with T cells for resources. In this category is our own finding in Manuscript III of regulation of macrophage function (Caramalho et al., 2003), together with Powrie's report of direct regulation of innate responses *in vivo* (Maloy et al., 2003) and the direct regulation of B cell responses (Bystry et al., 2001) On the other hand, in favor of the proposal that a stable, diverse immune system does not require regulatory T cells are reports that CD25 antibody depletion *in vivo* is not sufficient to induce autoimmunity (McHugh and Shevach, 2002), even when combined with thymectomy and extended over a long period (Laurie et al., 2002).

If we take these observations at face value, could we arrive at a consistent role for Tregs in the healthy, polyclonal immune system? Let us suppose that it will be confirmed and extended that effective deletion of Tregs in the adult does not lead to disease. Attributing, for simplicity, only one function to Tregs, to limit the proliferation of effector/naïve cells, they could avoid that the developing immune system becomes excessively oligoclonal (La Gruta et al., 2000) by limiting the expansion of thymic emigrants. This way the immune system while it is growing would do so mainly by accumulating a diverse pool of thymic emigrants (as opposed to the steady state system, where incorporation of thymic emigrants is relatively rare). The result would be a diverse and stable system, where different clones would keep each other in check. As shown before by others, this would be important not only for tolerance, but also to allow for appropriate responsiveness. Now the system is mature and stable. Why pay the cost of feeding and housing 10-15% of Tregs? Well, what task do we want this system to perform? Host defense. We have argued in the

Introduction that the main advantage of the adaptive immune system is to have efficient and diverse secondary responses. By limiting T cell proliferation, Tregs could also help to achieve this. At the time of the primary response some T cells must terminally differentiate into effector cells, but other must stick around to become memory cells. The process of terminal differentiation is apparently linked to the extent of cell division, and by limiting this process, Tregs may prevent exhaustion of responder cells. But we could go a step further and propose that what we want to avoid in the course of the immune response is precisely what we wanted to avoid in the course of development: excessive oliclonality. Thus by limiting the rate of growth of the fastest growing clones, Tregs may preserve the ability to target diverse epitopes in the secondary response. This would be a bad idea in principle, but given that T cells recognize linear short peptide sequences, oligoclonality could prove disastrous to effective memory. The mechanism may even selectively target the highest expanding clones. Taking into account the data in Annexes III and IV and previous data on both the dependence of Tregs on exogenous IL2 a potential enhancement of their suppressor function by this cytokine it is arguable that they would prefer to be in the neighborhood of fast growing clones secreting in large amounts.

A more pervasive criticism of regulatory T cells admits their existence, and even their relevance for immune regulation, but denies them a role in natural tolerance. Here the debate is less straightforward and precedes the current Treg era- disagreement starts at how to define self-antigens and what exactly, besides death of the host one wants to control. Cohn stated this most clearly in the time of suppressor T cells: *"(a) the self-nonself discrimination is determined by paralysis (negative unresponsiveness) not suppression (positive unresponsiveness) and (b) suppression plays a role in determining the class of the response (cell mediated or humoral not the self-nonself discrimination"* (Cohn, 1981). It should be noted that Stockinger *et al.* are also in accordance with a regulatory T cell in this context- as they put it *"to control the magnitude of immune responses, irrespective of whether they are directed at self or foreign (for example gut bacteria) antigens"*, and propose that this may happen by competing for IL2. Arguments against IL2 competition as the main *in vivo* mode of regulation have been presented above. This seems to fit very well with the Th1/Th2 paradigm (Mosmann et al., 1986), which does not call for a specific lineage of Tregs. In some models of immune pathology induced by infection, the Th1/Th2 mode of regulation is clearly important. If we take the prototypical case of *Leishmania major* infection, making the right choice between Th1/Th2 is vital, but in this case integrity of the host is threatened by the pathogen and

not the immune system, and the key is to avoid visceral colonization by the bug (as opposed to the relatively mild cutaneous legion). But for the experimental systems dealt with in this thesis a role for either of these subsets has in general been discarded. Another model of dominant tolerance that may conform well with Cohn's definition is mucosal administration of antigen, where systemic unresponsiveness mediated by T cells may be accompanied by vigorous IgA responses in the gut, for example. Tregs however have been shown to regulate both cellular and humoral responses, without any evidence so far of deviation of response as a mechanism. However, it is difficult, if not impossible, to prove to everyone's satisfaction that some mode of immune response is not happening somewhere in the body.

As the late Rod Langman always pointed out, a consistent model for the evolution of regulatory T cells is completely lacking. This is only slightly ameliorated by the fact that a satisfactory model for the evolution of the adaptive immune system as a whole has yet to be put forward. One can think of the pressure driving the evolution of regulatory T cells simply in terms of an "aseptic" self/non-self discrimination, i.e., to avoid the spontaneous occurrence of (primarily) organ specific autoimmunity. Alternatively, it may be thought that in nature infections are so frequent as to be almost constant, and that any selectable role for Tregs will have to be apparent in this context. Langman and Cohn (Langman and Cohn, 1996) point out that most mortality happens in childhood and is due to infectious disease. As they state *"Thus, as a guess, autoimmunity may well be expressed concomitantly with infection, serving to exacerbate the course of disease and thereby rendering lethal an otherwise only debilitating infection."* Consistent with this, in immune deficient mice reconstituted with naive T cells mortality derives from disregulated T cell to the normal or pathological mucosal flora. In contrast, when transfer recipients are T, but not B cell, deficient (as in nude or CD3 KO mice, for example) a complex set of organ specific autoimmune syndromes develop- but acute mortality from IBD or IPD does not ensue. Natural antibodies and activated B cells may be mediating this difference by keeping infections in check, as has already been demonstrated in models of bacterial infections. Furthermore, in the case of B cells, evolutionarily selected specificities play a key role, as some of these protective natural antibodies are derived from germline genes, and positive selection is also important. Considering an evolutionary scenario for regulation, circumstantial evidence points to a mucosal origin. Apart from the arguments above, the immune system is believed to have originated from a primordial gut associated lymphoid tissue (GALT), and

ontogenically this is reflected in the early colonization of these tissues by waves of stereotypical T cells (and, more controversially, by local, thymus independent generation of T cells). B cells are also selected to make protective specificities at the mucosal surfaces. Immune responses in the gut are tightly regulated by networks of local cells, as well as by compartmentalization. Thus, one can imagine a scenario where a cell type arose to limit mucosal immune responses and then was co-opted to perform regulatory functions in the rest of the organism. This cell may even have started as a local helper cell. TGFβ is an important suppressor cytokine in the periphery. It has however, another important function: TGFβ is necessary for B cells to switch to and produce IgA the main protective immunoglobulin isotype in the gut (Cazac and Roes, 2000). T cells producing suppressor cytokines may have been selected initially as mucosal helpers. Locally, stromal cells in the gut also produce TGFβ and may help to differentiate Tregs (Fagarasan et al., 2001). Likewise, IL10 which can also drive cells to differentiate in to suppressors is produced by freshly isolated dendritic cells from Peyer Patches, but not from peripheral organs (Iwasaki and Kelsall, 1999).

In practical terms, the most important perspective in the field of regulatory T cells is of course that they will be useful in the treatment of autoimmune disease, transplantation, allergies and immunotherapy of cancer, among others. The Grail is to be able to manipulate the regulatory population in an antigen specific way, avoiding the current approach of general immune-suppression that leaves the patient vulnerable to opportunist infections (and to the effects of treatment induced lymphopenia on immune-regulation). The identification of positive and negative regulators (the latter for inducing tumor immunity, in particular those that work in concert with TcR signaling is an area of intense research. To this end several encouraging results exist in the recent literature. An essential first step was identification by several groups of a population of regulatory T cells in humans with similar phenotype and *in vitro* function as murine Tregs (Baecher-Allan et al., 2001; Dieckmann et al., 2001; Levings et al., 2001; Ng et al., 2001; Stephens et al., 2001; Taams et al., 2002). Regulatory T cells also add an important cautionary note to current therapeutic approaches which induce lymphopenia- and some clinical situations such as bone marrow transplantation could benefit greatly from this field.

The feasibility of using Tregs in therapy will also benefit from improvements in markers for defining this cell population, most importantly, markers that will set them apart from conventional activated T cells – which

may be present in large amounts in patients suffering from autoimmune disease, or insufficient in cancer patients. Thus, the ability to manipulate separately Tregs and activated T cells is of central importance. In that sense, our results demonstrating that Tregs selectively express TLRs are a step in the right direction, all the more so as more than just another marker, TLRs may provide an efficient to selectively expand Tregs. Some of the results presented here also put the good old CD25 marker itself in a good light to this end.

Annex to conclusions and perspectives: Manuscript V

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Regulatory T cells: the physiology of autoreactivity in dominant tolerance and "quality control" of immune responses

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Summary: Little progress has been achieved over the last 20 years on the clinical management of several conditions that relate to self-tolerance and to the regulation of immune responses: autoimmune diseases, transplantation tolerance, tumor immunity, allergy and vaccine development in chronic infections. These failures, it is argued, are due to the inability of the prevalent "recessive tolerance" concepts to accommodate physiological autoreactivity and the regulatory potential it embodies. In this review, the advantages of "dominant tolerance" models are underlined in the light of critical evidence and in the general context of the natural autoimmune activities. The role of regulatory T cells is discussed, notably in the regulation of inflammatory reactions and, more generally, in the "quality control" of immune responses. It is anticipated that progress will be brought about by dominant tolerance approaches, and through an increased knowledge of the differentiative pathways, repertoires, mechanisms of activation and effector functions of autoreactive, regulatory T cells.

Introduction: the poor performance of recessive tolerance models and how regulatory T cells can solve some of the problems

Natural tolerance to the body tissues remains the major unsolved problem in immunology. There is, today, no satisfactory theoretical framework that accommodates all the relevant observations and yet "makes sense" within current theory. This is necessary to orient clinical research aimed at developing better diagnostic tools and therapeutic interventions. Thus, we have no specific or curative treatment for any autoimmune disease (AID), and no autoimmune patient has ever been cured of disease. We can only diagnose AID *a posteriori* after functional or structural lesion of the target organ, and we are quite incapable of establishing a prognosis with an acceptable degree of certainty. In addition, we cannot specifically tolerize the recipients of organ or tissue grafts to the antigens of the donor, and our current methods for managing allergy remain poor and empirical. Finally, current efforts in the development of effective vaccines to boost tumor immunity or to prevent chronic infections are yet to be successful.

It does not escape anybody's attention that all these unsolved problems relate to self-tolerance and to the regulation of immune responses. We take this state of affairs as the unfortunate demonstration that, in spite of many a claim for discoveries of mechanisms and of a general hypothesis on tolerance, we have achieved little or nothing in the solution of the problem. At this stage, therefore, it would seem appropriate to start by setting aside and going beyond the conceptual frameworks, theories and approaches that have occupied the scene for the last 20 years or so and have been the object of enormous investments for such a poor result. This is, first of all, the prevalent notion that natural tolerance is based on "recessive" mechanisms: lack of reactivity due to "ignorance", inactivation, or physical elimination of autoreactive lymphocytes.

It is long since that we have listed "some reasons why deletion and anergy do not satisfactorily account for natural tolerance" (1), and 12 years have passed since we proposed that natural tolerance is based on "dominant" mechanisms instead (2). Our intellectual dissatisfaction was centered on three serious limitations of the conventional theory (see (3) for details), all applying to peripheral, tissue-specific antigens. Current models of "recessive tolerance" do not account for the facts that: a) transplantation tolerance is only inducible before or around birth, adult individuals being completely or clearly more refractory; b) thus far, successful induction of transplantation tolerance has only been obtained with two types of tissues or antigen-presenting cells (APCs), namely hemopoietic cells (HC) or thymic epithelium (TE); c) acquisition of peripheral tolerance to tissue-specific antigens is somehow related to intrathymic selection, as so elegantly demonstrated in the series of experiments conducted by Le Douarin and colleagues.

Let us consider each of these problems in some detail. First of all, we take experimental models of tissue/organ transplantation as appropriate models for physiological tissue-specific tolerance, and this may be unwarranted. On the other hand, as discussed below, evidence for "dominant tolerance" has also been produced in many other systems (e.g. in AID models), and we shall always give more weight to graft rejection/acceptance *in vivo* than to *in vitro* tests that isolate components from the immune system and the body, and are limited to measuring proliferation or only a few of the many different types of effector activities that lymphocytes can mediate. The principle that "natural tolerance is a developmental question" has been experimentally established by Billingham and colleagues (4), and repeatedly confirmed ever since as, for example, in one of the first experimental systems for the tissue-specific expression of a viral transgene in mice (5). Yet, current ideas on natural tolerance often ignore this central problem, perhaps revealing their origin in the Lederbergian modification of Burnet's theory, which confuses the development of the organism with the development of single lymphocytes. It is worth recalling that Burnet, when proposing his model of recessive tolerance along with the clonal selection hypothesis, also postulated that all lymphocytes were produced during embryonic life (6). This "time problem" apparently worries very few colleagues – in a recent forum on self/nonself discrimination (7), only Langman & Cohn gave it serious consideration (8), explaining that tolerance cannot proceed on a "one cell by one cell basis", but requires some sort of "memory" of the immune system's development (be this "negative", as they propose, or "positive", as we defend; see below for further discussion).

To the time problem, Medawar & Woodruff added a "cell-type problem" by showing that while hemopoietic cells (spleen and bone marrow) could induce tolerance in newborn semi-allogeneic hosts, cells from other tissues (e.g. skin, liver) could not (9). This basic observation was extended by the work of Le Douarin's group and colleagues, who demonstrated that, again, several xenogeneic and allogeneic peripheral tissues were unable to induce full tolerance to themselves, even in early chicken embryos, before any lymphocyte development whatsoever (10). Moreover, they described that TE has the ability to induce tolerance to peripheral tissues if grafted in the same embryos, and made the surprising observation that it sufficed for tolerance establishment that a fraction of all T cells in the animal were selected ("educated") on the appropriate TE (11, 12). Most interestingly, therefore, both HC and TE display the ability to induce tolerance to many different (all?) tissues and organs from the donor, implying, for recessive tolerance views, that TE and HC each express all tissue antigens in the body. In a dominant tolerance frame, however, a partial overlap between peripheral and thymic antigens suffices to derive a set of mechanisms that results in tissue-specific tolerance. As previously argued, dominant mechanisms can explain the ability of the system to tolerize all "embryonic self", while rejecting virus-infected cells, for example. If we add to these major questions the recurrent observations on the development of AID in immunodeficient animals, as well as the overwhelming evidence for the physiological occurrence of autoreactivities in all individuals, the requirement for "dominant mechanisms" in natural tolerance becomes, in our view, indisputable.

Dissatisfied with "recessive models", we followed the ground-breaking work of several other groups (see this vol-

ume) and joined Le Douarin's efforts in deciphering TE-dependent, developmentally acquired tissue tolerance. Together, we transposed the avian experimental system to mice, and confirmed all the original conclusions, as reviewed elsewhere (13): all day 16 fetuses grafted with allogeneic skin *in utero* acutely reject the graft by 3 weeks after birth, upon acquisition of immunocompetence (14); grafting of allogeneic TE to athymic mice induces life-long, robust tolerance to grafts of various tissues from the donor (15). Incidentally, however, as recently shown by R. Araujo et al. (personal communication), TE is not intrinsically tolerogenic and is rejected if grafted to adult, immunocompetent animals. Moreover, we produced evidence for the thymic selection of specific regulatory T cells (Tregs) that imposed tolerance to minor and major histocompatibility antigens in a dominant manner, to a full repertoire of competent, reactive T cells (16). Finally, we could show that such thymic Tregs, once in the periphery, can recruit naïve recent thymic emigrants in the presence of the specific tissue antigens for further regulatory functions (17).

What remains and what needs to be corrected in our 1996 model of thymic generation of Tregs

These results led us to propose a model of dominant tolerance that was based on the thymic generation of Tregs and attempted to account for the developmental constraints in acquisition of tissue-specific tolerance and for the essential role of TE in this process (3). Let us now critically revise this model in the light of the findings that were produced, by us and others, since its publication. In short, the model postulated that:

- Tregs are generated in the thymus upon high affinity T-cell receptor (TCR) interactions with antigens presented in TE. This postulate has recently been confirmed (18), and the associated commentary by Sakaguchi (19) actually reproduces quite closely our original affinity distribution curve (3).
- T cells acquire a regulatory phenotype within the thymus, not because they constitute a developmentally independent cell lineage, but as a result of TCR selection: their high affinity results in intrathymic activation on TE, concomitantly with positive selection. This has not been directly demonstrated, but a population of mature T cells with activation markers and regulatory functions was shown to be induced (18), and regularly present inside the thymus (20, 21). In contrast to Sakaguchi (19), however, we continue to think that activation (and induction of regulatory effector functions) corresponds to a threshold of avidity (single-site TCR affinity and numbers per cell, together with ligand densities) which is below that necessary for deletion. This view is supported by the finding of CD25 expression and some Treg characteristics in cells that bear TCR Vβ chains reactive with self superantigens but nevertheless escape deletion, both in the thymus and in the periphery (22).
- The "developmental window" of tolerance acquisition could be explained through two converging mechanisms, only one of which concerned thymic selection. Thus, it was postulated that differentiating T cells undergo distinct fates if they are selected on TE APCs (poor in deletion but excellent in positive selection and activation) or on HC APCs (excellent in deletion). In embryonic and perinatal life, the thymic stroma composition would be predominantly epithelial, favoring, therefore, positive selection and activation to regulatory functions of all differentiating T cells with TCRs reactive to intrathymic antigens; in contrast, once development is completed, the thymic stroma composition would shift to a predominance of HC, leading to the deletion of most autoreactive T cells (3). This postulate is probably wrong, at least in part. Thus, the abundance of Tregs in the adult thymus shown by Mason and colleagues (20, 21) indicates that a significant production of Tregs in the thymus continues well beyond developmental times, probably throughout adult life. Furthermore, a clearly higher representation of Tregs in early postnatal life has not been demonstrated (18).
- Starting from the conviction that the thymus does not contain all self antigens, the model explained the generation of tissue-specific Tregs by peripheral recruitment of recent thymic emigrants – that had exited the thymus after positive selection just like cells that are specific for foreign antigens – once these were engaged by tissue antigens in the presence of Tregs that had been generated in the thymus. This scheme required some degree of overlap between the set of antigens expressed in the thymus (responsible for the generation of thymic Tregs) and in each of the peripheral tissues (that should simultaneously engage thymic Tregs and recent thymic emigrants). This requirement could be ensured, however, if thymic Tregs were assumed to display multireactive or promiscuous TCRs, in line with their predominantly embryonic origin (23), actually contributing a developmental genetic control for tolerance acquisition, and providing a coherent, if putative, explanation for perinatal multireactivity. On the other hand, the

model also accounted for the time problem in self/non-self discrimination, by postulating a "recruitment" window for recent thymic emigrants in the periphery (3). This part of the model has received support from the following observation: given the original demonstration by McCullagh that peripheral tissue antigens are required for tolerance acquisition (24), Seddon & Mason showed that thymic Tregs do not depend on the presence of the peripheral tissue-specific antigens, while peripheral Tregs with a similar phenotype do (25). On the other hand, while our earlier experiments (17) as well as those of Waldman & Cobbold (26) clearly demonstrate peripheral, extrathymic recruitment of specific Tregs, neither set of observations was produced in physiological conditions: education of Tregs was observed either along lymphoid reconstitution (17) or in the presence of inhibitory anti-CD4 antibodies (26). Furthermore, we have been unable to demonstrate education of T cells expressing autoreactive TCR (S. Hori, M. Haury, A. Coutinho, J. Demengeot, in preparation) in Lafaille's anti-myelin basic protein TCR transgenic mice, which spontaneously develop severe encephalomyelitis only if they are Rag deficient, but remain healthy if transfused with T cells of other specificities (27). Others have met with similar conclusions after thymic transplantation in reconstituted adult recipients (R. Araujo, personal communication). The possibility remains, therefore, that peripheral education of Tregs is limited to, or maximized in, the early post-natal period of peripheral T-cell colonization or in the corresponding periods of lymphoid reconstitution following T-cell removal. If it is assumed that peripheral education is of no physiological significance, however, this would imply that all Tregs are produced "as such" in the thymus and, therefore, that the thymus expresses all relevant tissue-specific antigens. On the other hand, the thymus may well be a mosaic of cells, each presenting different combinations of self antigens, such that all tissue-specific antigens are represented as a whole. Each differentiating T cell would thus be exposed to only a subset of tissue-specific antigens, and many tissue-specific cells would consequently exit the thymus as naïve. It follows that the thymic mosaic model is ineffective in the deletion of all self-reactive T cells, but it could effectively diversify the thymic Treg repertoire. On the other hand, in the context of dominant regulation, this is roughly equivalent to having no tissue-specific antigen expression in the thymus (3), for it suffices to educate as Tregs only a fraction of all T cells, whatever their specificity. The thymic mosaic notion is attractive, however, as it could dispense with peripheral education (dominant suppression of naïve cells in the periphery would be enough), but it does not account for the developmental window of tolerance acquisition (tissue-specific antigen expression was claimed in the adult thymus), and it provides no explanation why such autoreactive T cells are not deleted intrathymically.

Anti-inflammatory activity of Tregs and their role of quality control in adjusting immune responses to appropriate levels

The activities of Tregs may not all be reduced to their ability to dampen inflammatory reactions in the tissues; for example, deficits of tissue-specific Tregs are systematically accompanied by the production of autoantibodies to the same target tissues (28). Whether or not, in this case, B-cell activation results from disruption of direct Treg–B-cell interactions is an issue that we discussed previously (29). On the other hand, most experimental systems that have been used in the analysis of Tregs deal with their ability to control inflammation: this is the case with inflammatory bowel disease (IBD) (30), but it may also explain much of the protective effects of Tregs in chronic organ-specific AID, effected by inflammatory infiltration. In line with this notion, Treg activity has been described to operate, at least in part, through deactivation of tissue APCs (31) and to be mediated by two notoriously anti-inflammatory cytokines, transforming growth factor (TGF)-β and interleukin-10 (32–35). TGF-β seems to play a more fundamental physiologal role, as suggested by the comparison of the autoimmune phenotypes in the respective knockouts (KOs) (36, 37).

Let us first consider the specificities of "effector" inflammatory T cells, and their putative physiological role which is the target for the activity of Tregs. Acute IBD is readily induced in immunodeficient animals by the transfer of naïve T cells depleted of Tregs (30). Since no IBD ensues after the same transfers into germ-free animals (38), it would seem that we are dealing here with a problem of immunopathology i.e. an adverse inflammatory reaction provoked in the tissues by the expected, even desirable, reaction of normal, non-self-specific T cells. We recently produced the first evidence of inhibition of "protective" response by Tregs in immunodeficient animals carrying a lung infection by *Pneumocystis carinii* (PC), a non-pathogenic microorganism for immunocompetent hosts (S. Hori, T. Carvalho, J. Demengeot, submitted). These Rag-deficient animals display no clinical signs of disease and very minor tissue inflammation or other pathology. If reconstituted by either naïve CD4 T cells or by limited

numbers of total CD4 cells, however, they undergo a violent inflammatory pulmonary reaction (IPD) that kills them in less than 2 weeks. Tregs (defined by the expression of CD4 and CD25) from syngeneic donors do not cause such a reaction and totally control IPD induced by naïve CD4 cells. The system is thus quite similar to IBD, but we have proceeded to quantitate PC in the tissue, and to demonstrate that Tregs, while dampening inflammation and avoiding the lethal, acute reaction, also markedly inhibit the protective response and the clearance of the microorganism, thus contributing to the maintenance of the infection. In other words, while a normal T-cell compartment ensures resistance to commensal microorganisms in the absence of adverse inflammatory reaction, deficits in Tregs result in severe local inflammation, and excess Tregs result in no protective response and no resistance to infection.

These observations indicate that the physiology of immune protection is a narrow equilibrium between immunopathology and no immunity at all, and underline the fundamental importance of the quantitative relationships between Tregs and effector T cells. This balance is, by definition, maintained in the normal individual, but this may depend on secondary adjustments between the two cell populations as the response proceeds. Thus, while there is no doubt that Tregs inhibit the proliferation of naïve T cells at appropriate ratios, we have recently observed that limited numbers of Tregs are actually expanded either by co-stimulation of naïve CD4 T cells (K. Leon, V. Oliviera, I. Caramalho, J. Carneiro, in preparation), or by inflammatory signals (I. Caramalho, J. Demengeot, unpublished). These are results obtained *in vitro* and must, therefore, be taken as mere indications of physiology. At any rate, Tregs that fail to proliferate in the presence of APCs and anti-CD3 antibodies will do so in co-cultures with an excess of naïve CD4 cells or in cultures where APCs are activated by innate immunity mechanisms. If confirmed in physiological conditions, these results add another layer of complexity to the control of immunopathology and to the regulation of appropriate levels of protective immune responses to non-self. Other factors must influence such responses to commensal microorganisms and non-cytophatic viruses, be these local and related to the nature of tissue alterations or to innate immunity mechanisms, or else, systemic and related to acute phase reactions accompanying infections. Treg activities would thus appear as a "quality control" mechanism, regulated by feedback processes that monitor the end-result of immune responses through levels of inflammation, tissue integrity and systemic homeostasis (39).

From the above observations on IBD and IPD, we take it that the inflammatory naïve cells are directed at the pathogenic microorganisms. It could be argued, however, that naïve T cells cause lesions because they are induced to proliferate (and further promote inflammation) in a local context of pre-existing inflammation due to microbial colonization, rather than by specific stimulation by microbial antigens. Thus, we have recently shown that a similar local expansion of "inflammatory" naïve CD4 cells can be obtained by inducing "sterile" inflammation in the peritoneal cavity of recipient mice (T. Carvalho, I. Caramalho, J. Demengeot, in preparation). On the other hand, since Tregs from germ-free animals are competent in controlling IBD pathology (40), it would seem that Tregs are self-specific. Our recent finding that Tregs also regulate sterile inflammation provides further evidence for their autoreactive specificity.

Being self-specific and part of the naturally activated pool of lymphocytes, these cells could be in a permanent effector stage, and constantly scrutinize body tissues (41, 42) in order to set appropriate levels of immune responses. Alternatively, they could selectively detect sites of inflammation (for example, via elevated expression of cellular markers, or of innate immunity activities including natural killer cell activation), and rapidly accumulate there. Once at the site, while an APC that has lost threshold levels of self-markers (e.g. because of viral infection) elicits no reaction from Tregs, and naïve T-cell activation may proceed, inflammation-induced levels of self markers would turn on Tregs effector activities that result in the control of inflammation and limitation of the naïve T-cell response (even to non-self determinants). This notion is in line with recent observations by R. Araujo et al. (submitted) who demonstrated, in a system of allogeneic tissue transplantation, that the first cells to be found at the tissue are naturally activated T cells that were present in the recipient prior to grafting. On the other hand, Tregs could preferentially localize to sites "marked" by the very activity of responding naïve T cells. It is very plausible that this pathway represents only one of the multiple aspects involved in the initiation and regulation of inflammatory reactions. The ancient evolutionary origin of such reactions and their fundamental functional relevance in defense and tissue physiology make it likely that other non-specific and natural (e.g. natural antibodies) components are at play, and that autoreactive Tregs are only a late addition to a complex control.

The natural immune activity and the physiology of autoreactivity

The rule of multireactivity in the primary immune repertoire has been well established for both B and T cells (23, 43), and

it is known to be ensured by invariable genetic developmental programs, notably, the absence of N-sequence additions (44, 45). The evolutionary advantage of a multireactive repertoire early in life, however, has remained elusive. Other than the general hypothesis of "completeness" in a small repertoire for defensive purposes – that seemed inappropriate given the fact that embryos and newborns are protected by maternal antibodies in most species, from fish to mammals – the only other alternative in conventional views is the higher efficiency of thymic positive selection (46). On the other hand, once physiological autoreactivity is accepted, multireactivity seems to provide the best strategy to ensure an autoreactive repertoire from the very start of the system. This is made more plausible by the fact that adults counterselect for multireactivity in a very marked manner (23, 47). If applied to T-cell promiscuity in the newborn repertoire (23), this developmental/evolutionary strategy could ensure the primary generation of Tregs in the developing thymus, by providing the first autoreactive (multireactive) T cells, as recently discussed in detail (29).

Normal individuals, even if secluded from environmental antigens, maintain a set of activated T and B cells in the periphery (48, 49). These can only be autoreactive, for they are only exposed to self antigens. Hence, the naturally activated lymphocyte compartment embodies the physiological autoreactivity. Many different types of observations have suggested that, even in adults if cell numbers are limiting, this compartment is the first and/or the only one to be established, indicating its predominant functional relevance at all developmental stages. This notion is further reinforced by its early establishment in the embryo through a developmental program that can only have been evolutionarily selected.

From *horror autotoxicus* and recessive tolerance to the present dominant tolerance model, concepts of autoreactivity have thus evolved through five major steps, prompted by the following observations listed in a chronological order: a) the evidence for the availability of autoreactive lymphocytes in the normal repertoire; b) the demonstration that autoreactivity is ensured from early development through a complex genetic "program", which can only indicate its functional relevance in evolution; c) the evidence that many autoreactive T and B cells, rather than being eliminated from the organism, are actually activated in a selective manner in the "undisturbed" immune system; d) a number of heterogeneous clinical observations showing that normal natural (auto) antibodies display marked beneficial effects in various AID (e.g. (50)); and e) the evidence discussed in the present volume that such naturally activated autoreactive T cells are regulatory and mediate many a protective function to the organism, inhibiting pathogenic autoimmunity, regulating inflammatory reactions and dampening immunopathology associated with protective immune responses. In other words, not only does the system physiologically accept autoreactive cells, but it also selects them for activation in a developmentally programed manner, and it uses such activated autoreactivities to somehow control pathogenic autoimmunity and immune responses in general.

We are thus very far from the old schemes of recessive tolerance by "ignorance" – be this active deletion or antigen seclusion – and dominant tolerance by "assertion" of self's composition is surfacing in current concerns. It has taken too long, however, to achieve the intellectual satisfaction of dealing with coherent models and, hopefully, for our understanding to benefit patients. Above all, the notions of dominant tolerance solve the old time problem and explain the developmental window for tolerance acquisition that had remained largely ignored since Lederberg's hypothesis. This imposes the requirement for some sort of memory of the "developmental self that is present all the time" that could be positively provided by the Treg compartment. The only alternative is the suggestion by Langman & Cohn (8), who propose to solve the problem in a symmetrical way: instead of accumulating a regulatory self-specific memory, they suggest that the system is leaky for a sort of non-self memory, thus providing inducers for immune responses to any new antigens. This postulate, on the other hand, imposes a small and highly specific repertoire, two properties that seem incompatible with the available evidence. As the developing immune system combines multireactivity with poor negative selection, Langman & Cohn's model would have deleterious effects not only on natural tolerance but also on the specificity of immune responses. It continues to make more sense to us that the system naturally selects its repertoires on the basis of what is in the organism, and it proceeds by building up a memory of the developmental self in a dominant manner, starting at developmental times when tissue scrutinity by T cells is selectively favored. Enhanced lymphocyte circulation through tissues may provide another fundamental developmental aspect of natural tolerance.

We believe that, with these initial observations on the protective role of natural antibodies and of naturally activated Tregs in AID and immunopathology, we are only at the start of a whole new period of developments in this field. The repeated observations, often produced unexpectedly in the analysis of various KO mice, that general or selective immunodeficiencies result in AID, should help to convince the

defenders of conventional, recessive tolerance models that something is rotten in the kingdom of Denmark.

Clinical potential of the regulatory T-cell approach beyond AID: transplantation, allergy, tumor immunity and chronic infections

The potential interest of manipulating Tregs in clinical approaches to AID and to the immunopathology associated with infections is today well accepted: organ-specific AID such as diabetes, thyroiditis and multiple sclerosis, but also systemic lupus erythematosus and other systemic conditions, as well as inflammatory bowel and pulmonary diseases, may all gain from the development of these views. We look forward to seeing the results of clinical trials launched on these principles. In addition, as previously discussed (29), it is also clear that Tregs may contribute important clues to some basic questions, such as the regulation of conventional immune responses, clonal dominance and affinity maturation, peripheral lymphocyte expansion and control of total cell numbers.

Most interestingly, work in this field is now extending to other major problems in clinical immunology that had found no solution in the conventional schemes. Discussions on dominant versus recessive tolerance and the choice of the correct alternative are far from academic; rather, they determine very concrete clinical decisions, as the introduction of dominant tolerance notions is bound to drastically depart from conventional practices. For example, dominant tolerance principles, while accommodating anti-inflammatory therapies in AID, strictly exclude immunosuppression, for they postulate that disease is already the result of immunodeficiency. Immunosuppression, rather than correcting the dysfunction, is likely to jeopardize irreversibly the remaining ability of the patient to recover physiological (dominant) tolerance, thus evolving from remission to a relapsing-remitting clinical course, or from the latter to a progressive disease. The reverse is true in tumor immunity: the antigenic composition of cancer cells, regardless of all tumor-specific epitopes they might express, remains composed by a majority of normal self antigens, making it likely that tumor cells are protected from (auto)immune attack by the physiologic activity of Tregs. It follows that: a) rejection of the tumors must include the inactivation or elimination of Tregs (immunosuppression, eventually through antimitotic therapies, is indicated in this case, although it also removes effector responses); b) vaccination with tumor-specific epitopes, while often resulting in a transitory phase of (auto)immune tumor regression, is likely to prime and recruit novel tumor-specific Tregs that, in similarity with AID vaccination (as in experimental allergic encephalomyelitis) establish a state of resistance to further immune attack. In short, relevant progress in current difficulties is to be expected from this complete turnaround in the consideration of physiological tolerance. The following is a very short summary of current advances.

Full, long-lasting dominant tolerance to minor and major transplantation antigens has been demonstrated by Cobbold & Waldmann after administration of non-depleting anti-CD4 antibodies that allow for the peripheral recruitment and activation of specific Tregs (51). After many years of interesting observations on suppressor cells mediating neonatal tolerance induced *à la* Medawar (52), as well as on the beneficial effects of donor-specific blood transfusions in graft acceptance, those observations bring new hopes for potential clinical applications (53).

As originally shown by Hoyne et al. (54), the immune response to allergens is modulated by cells with characteristics of Tregs. Recent work by M. A. Curotto de Lafaille and colleagues (personal communication), using a strictly biclonal mouse, demonstrates that IgE antibody responses are profoundly inhibited by Tregs in a dominant manner. Much is to be expected from this entirely novel approach to the old question of allergy. This may be a paradigmatic case: we seem to know everything on the components and mechanisms of the allergic reaction, from the Th1/Th2 dichotomy to the molecular and cellular basis of isotype switching, to IgE-receptors and the cytokine mediators, but are, nevertheless, unable to predict and solve the problem. It did look as if we missed a fundamental aspect of the process: this may well be the Tregs.

Onizuka et al. (55) have recently called our attention to the role of Tregs in inhibiting effective tumor immunity. Following the early work of North on tumor-specific suppressor cells, all conducted *in vivo* (56), and in accordance with several suggestions from clinical observations, these authors have shown that removal of Tregs is fundamental in promoting immune responses that eliminate tumors. In many of their cases, tumor rejection is obtained by simply eliminating Tregs, without specific antitumor priming (55). This is particularly relevant, in view of the potential problem of priming Tregs when administering "tumor vaccines". Here again, we witness an entirely new perspective of dealing with an old question that had remained open, in spite of much effort in the alternative approaches aiming at reinforcing effector responses.

One last aspect of immunological difficulties may perhaps find novel solutions through Tregs. This is the problem of chronic infections. In a variety of situations (viral, bacterial

and protozoal infections), the immune system does not seem capable of eliminating the microorganism, while maintaining, nevertheless, a significant level of response. Chronic infections seem often close to equilibrium, but slowly result in progressive debilitation the host. Our contention is entirely hypothetical, but it would seem worthwhile to investigate the role of Tregs in the context of situations such as AIDS, tuberculosis and malaria, to mention only the great killers of today, as well as manipulating them in the control of severe clinical situations provoked by the effective immune response, as is the case in infections by non-cytopathic viruses.

Conclusion

In the end, we all wonder why Tregs and the respective phenomenology have been missed for so long. Thus, even if only some of the above expectations are to be confirmed, even partially, it is striking that so many excellent investigators did not see them. There were, of course, all the negative aspects of the suppressor T-cell saga of the 1970s and early 1980s, but there was no valid reason to throw away the baby with the bath water. We should, nevertheless, retain the fact that essentially all the suppressor cell phenomenology of that time was constructed from highly artificial *in vitro* culture systems, in order to avoid repeating the mistake. There are now, of course, novel experimental systems that make it unavoidable "to find Tregs", a cell population that operates in very small numbers and actually "looks like" conventional T cells. We have little doubt that the availability of novel techniques for marking and isolating small cell populations, as well as the use of Rag-KO mice and other immunologically deficient mutants as hosts for transgene expression or for defined cell transfers, have all been instrumental, as first demonstrated in the original experiments of Lafaille and colleagues (27). And we are quite certain that other surprises will turn up whenever conventional phenomena are analyzed in the complete absence of Tregs. On the other hand, many of the fundamental observations described in this volume have been available for more than a dozen years and have been generally ignored, for they did not fit with the prevailing concepts that sold so much better. This is a good lesson, particularly in the context of today's scientific practice. Not wishing to engage in epistemological analyses, it may be worth recalling that we can only see what we are looking for. Hence the fundamental importance of accepting ideas and approaches that are alternative to the prevalent *status quo* but yet serious and solid, of fostering originality and diversity beyond the limited frames arrogantly imposed by the establishment and the fashions created by journals. As in one of the commandments in Russell's Liberal Decalogue: "Do not ever discourage thinking, for you are sure to succeed". Hence the relevance of conceptual frameworks, the value of serious theoretical analysis and model building, the need to encourage hypothesis-driven research and the continuous revision of "established" truths. Jerne said it often: "Science is more than mixing things in test tubes".

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