MODULATION OF REGULATORY T CELLS IN AUTOIMMUNE DISEASE

A dissertation submitted for the degree of Doctor of Philosophy in Medicine, Specialization in Immunology

Supervisors:
Doutora Jocelyne Demengeot, Ph.D.
Instituto Gulbenkian de Ciência
Professor Doutor António Coutinho, MD, PhD.
Faculdade de Medicina da Universidade de Lisboa

Maria Francisca Botelho de Gusmão de Moraes de Brito Fontes
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À memória da minha Avó, Maria Leonor Beltrão de Seabra Teixeira de Lemos Botelho de Gusmão
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SUMMARY

Foxp3+ regulatory T cells (Treg) are a specialized group of lymphocytes that constitute the basis of control of physiological auto-reactivity. These cells prevent autoimmune disease (AID) by inhibiting the activation of potentially pathogenic effector cells and, in mice and humans, their absence causes a lethal form of autoimmunity. In order to contribute to the study of Treg in AID three different experimental layouts were designed to appraise: (i) the impact of immunosuppression (IS) on the number and function of Treg and on immune regulation; (ii) the response of Treg to inflammation and; (iii) the effect of an inflammatory immune response during the course of an AID.

It was hypothesized (because of their generalized effect on the immune system and inability to cure AID) that steroids (the most commonly used form of non-specific IS) may damage Treg. To determine the impact of IS on Treg number and function, a 5 day course of hydrocortisone (HC) was administered to the anti-myelin basic protein (anti-MBP) T cell receptor (TCR) transgenic (Tg) mouse (TR+ mice) which are naturally protected by natural Treg from developing experimental autoimmune encephalomyelitis (EAE). A transfer of Treg prevents the EAE which develops when these Tg mice are set in a RAG−/− background (TR− mice). We concluded that IS caused by a short course of steroids was not deleterious to Treg number or function; however, higher doses, formulations of different potencies and/or longer therapies remain to be formally tested.

To assess the effect of IS on immune regulation we evaluated the impact of several conditions of lymphopenia on EAE onset and severity using the anti-MBP TCR Tg system. The effect of HC alone was compared to irradiation, cyclophosphamide (Cyp), pertussis toxin (Ptx) and CD25 depletion, each alone or in combination therapy with HC. Generalized lymphopenia (steroids or irradiation) was compared to selective Treg depletion alone (mAb mediated CD25 depletion) or to lymphopenia associated with Treg depletion (Cyp). Our results indicate that lymphopenia is a trigger of EAE in TR+ mice only when associated with selective Treg depletion. In addition, the use of Cyp or Ptx demonstrates a correlation between a harmful effect on Treg
number and function and the development of AID. In this respect, the combination of HC and Cyp, often used in the clinic, was particularly harmful.

Following a symmetrical reasoning, we next tested whether ongoing immune activity promotes immune regulation by stimulating an increase in Treg number. We utilized an experimental layout designed to assess the response of regulatory T cells to inflammation by testing the possibility of conversion of naïve donor T cells to Foxp3+CD4+ T cells. T-cell induced colitis (a mouse system of T cell lymphopenia and severe Treg number reduction that commonly serves as a model of human inflammatory bowel disease) provided the means to study the dynamics of inoculated Treg expansion. We reveal that in ongoing severe colitis induced by the transfer of naïve T cells containing a small Treg contaminant, Treg of donor origin accumulate and reduce the severity of colitis. Transfer experiments in which the Treg contaminant was excluded from the donor cell population unequivocally demonstrated naïve T cell conversion to Treg. These findings support the notion that inflammation and immune responses favour Treg accumulation, through expansion and conversion and allow for Treg function.

Bearing in mind that Helicobacter spp. (H) is a recognized facilitator of T cell-induced colitis, it has not been formally established whether T-cell induced colitis is an AID targeted at intestinal tissues or an immunopathology caused by ongoing immune responses specific to Helicobacter. In the course of these experiments we showed that while T cells isolated from H+ mice presenting with severe colitis were not colitogenic upon adoptive transfer in H− mice, they induce fulminant colitis in H+ recipient mice. Even though a Helicobacter requirement for self Ag presentation by intestinal dendritic cells (DC) was not excluded, these results suggest that colitogenic T cells in this system are Helicobacter specific and not tissue specific, in favour of an infectious rather than autoimmune aetiology of T cell-induced colitis.

Based on the evidence that T-cell induced colitis results from an exacerbated immune response to enteric bacteria we directly tested whether such an inflammatory immune response would affect the course of a bona fide AID. Colitis was induced in TR− mice by transfer of naïve T cells. T cell-induced colitis was shown to completely prevent EAE development but was unable to suppress established EAE. Prevention of EAE only occurred with rapid colitis induction in the presence of Helicobacter colonization of recipient mice. Even though protection occurred within a time window which corresponds to that expected from a Treg transfer, these results indicated that the protective immune response that follows the acute phase of T cell-induced colitis is mediated by donor CD4+CD25− T cells.

This work has contributed to further awareness of the mechanisms of lymphopenia as a trigger of AID and has advanced knowledge on the biologic behaviour of Treg in response to an
ongoing inflammatory immune response. Technical advances in the methodology to define and better identify Treg, in conjunction with the recent burst of information describing the number and function of Treg and polymorphisms of genes implicated are expected to strengthen the knowledge about the association of Treg to the pathogenesis and genetic susceptibility of human AID.

**Key words:** Autoimmune disease; Regulatory T cells; Steroids; T cell-induced colitis; Experimental autoimmune encephalomyelitis
RESUMO

As células T reguladoras (Treg) são um grupo de linfócitos especializados que constituem a base do controlo da auto-reactividade fisiológica. Estas células previnem a doença auto-imune através da inibição da activação de outros linfócitos T potencialmente patogénicos e, tanto em humanos como no modelo experimental, a sua ausência causa uma forma letal de auto-imunidade. De modo a contribuir para o estudo das Treg na doença auto-imune, foram utilizadas diferentes abordagens experimentais concebidas para avaliar especificamente: (i) o impacto da utilização de imunossupressores (IS) sobre o número e a função das Treg e sobre a regulação do sistema imunitário, (ii) a resposta do organismo à inflamação, no que se refere às Treg e (iii) o efeito de uma resposta inflamatória imune sobre a potencial evolução de uma doença auto-imune.

Foi colocada a hipótese da utilização de corticóides (GC) - a forma de imunossupressão mais frequentemente utilizada - poder ter um efeito negativo sobre as Treg, dado o facto dos IS exercerem um efeito generalizado sobre o sistema imunitário e a sua utilização clínica não levar à cura das doenças auto-imunes. Com o objectivo de determinar o impacto da utilização de IS sobre o número e função das Treg, foi administrada hidrocortisona (HC), diariamente, durante 5 dias, a ratinhos transgénicos. Estes são caracterizados por possuir linfócitos que, na sua maioria, têm um receptor específico para a proteína básica da mielina (ratinhos TR⁺) e estão naturalmente protegidos, pelas Treg, contra o desenvolvimento da encefalomielite experimental auto-imune (EAE). Quando não possuem Treg (ratinhos TR⁻), a transferência deste grupo de linfócitos previne o desenvolvimento de EAE. O presente estudo permite concluir que a IS causada por HC de curta duração não afecta negativamente o número ou a função das Treg, tornando-se necessário estudar o efeito de uma terapêutica mais prolongada.

Para estudo do efeito da utilização de IS sobre a regulação do sistema imunitário, foram induzidas diferentes condições de linfopenia e foi avaliado o seu impacto na capacidade de indução e gravidade da EAE no ratinho TR⁺. O efeito da HC foi comparado ao da radiação gama, da ciclofosfamida (Cyp), da toxina do pertussis (Ptx) e da depleção de CD25, isoladamente ou em conjunto com a HC. A linfopenia generalizada (por efeito da HC, de radiação gama ou
combinação dos dois) foi comparada à depleção selectiva de Treg, induzida pelo anticorpo monoclonal anti-CD25, e à linfopenia associada à depleção selectiva de Treg, induzida pela Cyp. Os resultados indicam que a linfopenia apenas constitui um factor desencadeante da EAE nos ratinhos TR⁺ quando em associação à depleção selectiva de Treg. A utilização de Cyp e Ptx demonstra a correlação do seu efeito nocivo sobre o número e a função das Treg e o desenvolvimento de doença auto-imune, salientando-se o efeito mais prejudicial da HC quando combinada com a Cyp - uma combinação frequentemente utilizada na prática médica.

Seguindo um raciocínio inverso, a pergunta seguinte refere-se à possibilidade de uma resposta imunitária em curso poder fomentar a regulação imunitária, ao promover um aumento das Treg. O método experimental foi concebido para avaliar a resposta das Treg ao estímulo da inflamação e testar a possibilidade de existir conversão de linfócitos T naïve para células T Foxp3⁺CD4⁺. A colite induzida por linfócitos T, um modelo murino de doença inflamatória do intestino, é caracterizada por linfopenia das células T e redução acentuada de Treg, permitindo estudar in vivo a dinâmica da expansão das células inoculadas. Os resultados revelam que, durante o processo de colite induzida pela transferência de linfócitos T naïve contendo um pequeno contaminante de Treg, estas Treg se acumulam e, após transferência, reduzem a gravidade da colite induzida. Através de um tipo de transferência de células naïve, que permite excluir a presença de um contaminante de Treg, foi comprovada a conversão de células naïve para Treg. Estes resultados corroboram a convicção de que a inflamação e consequente resposta imunitária favorece a acumulação de células Treg e, para além disso, permite que estas exerçam a sua função imunossupressora.

Tendo em conta que a infecção por Helicobacter (H) é facilitadora da colite induzida por linfócitos T, pretendeu-se esclarecer se tal tipo de colite era uma doença auto-imune dirigida para os tecidos intestinais ou uma forma de imunopatologia causada por uma resposta imunitária específica para o Helicobacter. O presente estudo demonstra que linfócitos isolados de recipientes H⁺ com colite induzida por linfócitos T não são colitogénicos quando transferidos para recipientes H⁻, contrastando com a colite grave que induzem em recipientes H⁺. Estes resultados demonstram que os linfócitos colitogénicos neste sistema são específicos para o Helicobacter e não reconhecem tecido intestinal, excluindo-se deste modo a hipótese de auto-imunidade.

Finalmente, foi testada a hipótese da colite induzida por linfócitos T (caracterizada por uma resposta imunitária inflamatória a bactérias entéricas) poder exercer influência sobre uma doença auto-imune. Se por um lado ficou demonstrado o papel supressor da EAE através da indução de colite em ratinhos TR⁻, por outro verificou-se não existir inibição se a EAE já estiver instalada, de forma espontânea, nos ratinhos TR⁻. Do mesmo modo, nos recipientes H⁻ não se
verificou a prevenção da EAE quando a ocorrência da colite foi mais tardia e menos grave. O aparecimento de EAE apenas foi impedido quando a colite foi induzida precocemente, em recipientes portadores de *Helicobacter* e quando a transferência de linfócitos indutores de colite foi realizada até às quatro semanas, tal como na protecção conferida aos ratinhos TR- por Treg. Os resultados do presente estudo indicam contudo que esta protecção é mediada por linfócitos T CD4+CD25- provenientes do dador e não por Treg.

O presente trabalho contribuiu para o conhecimento das características da linfopenia que podem ser desencadeantes de doença auto-imune e do comportamento biológico das Treg face a uma resposta imunitária inflamatória. Avanços técnicos na metodologia para melhor definir e identificar as Treg, em conjunto com a explosão de informação sobre a caracterização do número e função das Treg e polimorfismos de genes implicados, deverão contribuir para um melhor esclarecimento da associação de Treg com a patogênese e a susceptibilidade genética das doença auto-imunes.

**Palavras chave:** Doença auto-imune; células T reguladoras; glucocorticóides; colite induzida por linfócitos T; encefalomielite auto-imune experimental
# ABBREVIATIONS

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<tr>
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<td>Knock out</td>
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<td>APC</td>
<td>Antigen-Presenting Cell</td>
</tr>
<tr>
<td>APECED</td>
<td>Autoimmune PolyEndocrinopathy Candidiasis Ectodermal Dystrophy</td>
</tr>
<tr>
<td>APh</td>
<td>AlloPhycocyanin</td>
</tr>
<tr>
<td>APS 1</td>
<td>Autoimmune Polyendocrinopathy Syndrome type 1</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood Brain Barrier</td>
</tr>
<tr>
<td>CD</td>
<td>Crohn's Disease</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freund Agent</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>CsA</td>
<td>Cyclosporin A</td>
</tr>
<tr>
<td>CS</td>
<td>CorticoSteroid</td>
</tr>
<tr>
<td>CSF</td>
<td>CerebroSpinal Fluid</td>
</tr>
<tr>
<td>CTLA4</td>
<td>Cytotoxic T-Lymphocyte Antigen 4</td>
</tr>
<tr>
<td>Cyp</td>
<td>Cyclophosphamide</td>
</tr>
<tr>
<td>D</td>
<td>Day</td>
</tr>
<tr>
<td>D0</td>
<td>Up to 24 hours post last injection</td>
</tr>
<tr>
<td>D1 to D10</td>
<td>First to 10th day after D0</td>
</tr>
<tr>
<td>D-5 to D-1</td>
<td>Fifth to first Day before D0</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic Cell</td>
</tr>
<tr>
<td>DEX</td>
<td>DEXamethasone</td>
</tr>
<tr>
<td>DM</td>
<td>Diabetes Mellitus</td>
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Abbreviations

DP  Double Positive (thymocyte)
DSS  Dextran Sodium Sulphate
EAE  Experimental Autoimmune Encephalomyelitis
FCS  Fetal Calf Serum
FDA  Food and Drug Administration
GC  GlucoCorticoid
GF  Germ Free
GFP  Green Fluorescent Protein
H&E  Haematoxylin & Eosin
H+  Colonized with Helicobacter spp
HC  HydroCortisone
HC0.5  HC 0.5 mg/kg
HC5  HC 5 mg/kg
HC50  HC 50 mg/kg
HC5x5  HC 5 mg/kg/day x 5 days
H Ct  Healthy Control
HPF  High Power Field
i.p.  intra-peritoneal
i.v.  intra-venous
IBD  Inflammatory Bowel Disease
IEL  IntraEpithelial Lymphocytes
IFN  InterFeroN
IgG  Immunoglobulin G
IL  InterLeukin
IL-12R  IL-12 Receptor
IPEX  Immune dysfunction, Polyendocrinopathy, Enteropathy, and X-linked inheritance
Irrad.  Irradiation
IS  ImmunoSuppressant
iTreg  induced Treg
IVIG  IntraVenous Immunoglobulin
LFB  Luxol Fast Blue
LIP  Lymphopenia Induced Proliferation
Ln  Lymph node
mAb  monoclonal Antibody
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
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<tr>
<td>MACS</td>
<td>Magnetic-Activating Cell Sorting</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin Basic Protein</td>
</tr>
<tr>
<td>MC</td>
<td>Mineralocorticoids</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>mLn</td>
<td>mesenteric Ln</td>
</tr>
<tr>
<td>MOG</td>
<td>Myelin Oligodendrocyte Glycoprotein</td>
</tr>
<tr>
<td>MP</td>
<td>MethylPREDnisolone</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple Sclerosis</td>
</tr>
<tr>
<td>n</td>
<td>number of mice</td>
</tr>
<tr>
<td>NFAT</td>
<td>Nuclear Factor of Activated T cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural Killer</td>
</tr>
<tr>
<td>nmLn</td>
<td>non-mesenteric Ln</td>
</tr>
<tr>
<td>NOD</td>
<td>Non Obese Diabetic</td>
</tr>
<tr>
<td>nTreg</td>
<td>natural Treg</td>
</tr>
<tr>
<td>PB</td>
<td>Peripheral Blood</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffer Solution</td>
</tr>
<tr>
<td>PD-1</td>
<td>Programmed Death receptor-1</td>
</tr>
<tr>
<td>PE</td>
<td>PhycoErythrin</td>
</tr>
<tr>
<td>PLP</td>
<td>Proteolipid Protein</td>
</tr>
<tr>
<td>PRED</td>
<td>PREDnisolone</td>
</tr>
<tr>
<td>Ptx</td>
<td>Pertussis toxin</td>
</tr>
<tr>
<td>RA</td>
<td>Rheumatoid Arthritis</td>
</tr>
<tr>
<td>RAG</td>
<td>Recombination Activating Gene</td>
</tr>
<tr>
<td>RTE</td>
<td>Recent Thymic Emigrants</td>
</tr>
<tr>
<td>SCID</td>
<td>Severe Combined ImmunoDeficiency</td>
</tr>
<tr>
<td>SF</td>
<td>Synovial Fluid</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic Lupus Erythematosus</td>
</tr>
<tr>
<td>SP</td>
<td>Single Positive (thymocyte)</td>
</tr>
<tr>
<td>Sp</td>
<td>Spleen</td>
</tr>
<tr>
<td>SPCD4+</td>
<td>Single Positive CD4+ thymocyte</td>
</tr>
<tr>
<td>SPF</td>
<td>Specific Pathogen Free</td>
</tr>
<tr>
<td>T1DM</td>
<td>Type 1 Diabetes Mellitus</td>
</tr>
<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
</tr>
<tr>
<td>TEC</td>
<td>Thymic Epithelial Cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>Tg</td>
<td>transgenic</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming Growth Factor-β</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>Thy</td>
<td>Thymus</td>
</tr>
<tr>
<td>TLI</td>
<td>Total Lymphoid Irradiation</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-Like Receptors</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor Necrosis Factor-α</td>
</tr>
<tr>
<td>TR-</td>
<td>Anti-MBP TCR Tg on a RAG deficient background</td>
</tr>
<tr>
<td>TR+</td>
<td>Anti-MBP TCR Tg on a RAG sufficient background</td>
</tr>
<tr>
<td>Treg</td>
<td>regulatory T cell</td>
</tr>
<tr>
<td>UC</td>
<td>Ulcerative Colitis</td>
</tr>
<tr>
<td>wt</td>
<td>wild type</td>
</tr>
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1. GENERAL INTRODUCTION

Diabetes mellitus, Graves’ disease, multiple sclerosis, rheumatoid arthritis and systemic lupus erythematosus are among a group of diseases of unknown aetiology that affect very different organs or organ systems but share a universal pathogenesis. Common to all is an immune response directed to self, referred to as autoimmunity, the aetiology of a broad spectrum of human illnesses collectively known as autoimmune diseases (AID). This thesis aimed to study: (i) the effect of steroid treatment upon the number and function of regulatory T cells (Treg) in mice; (ii) the response of Treg to inflammation and; (iii) the effect of T cell-induced colitis on the repression of encephalomyelitis in transgenic mice. Even though the study of these three aspects required different experimental layouts - and was therefore included in separate chapters - an effort was made to coherently consolidate them within the common objective namely the understanding of the effects of modulation of Treg in AID.

1.1. Relevance of fundamental research to autoimmune diseases

Some of the most prevalent auto-immune diseases (AID) in the USA are rheumatoid arthritis (RA) with a rate of 0.6%, systemic lupus erythematosus (SLE), with a prevalence of 0.1% in Caucasian and 0.4% in afro-American women (Helmick et al., 2008; Lawrence et al., 2008) and multiple sclerosis (MS) rating 0.04 to 0.17% (Jacobson et al., 1997). In industrialized nations overall prevalence is increasing (Bach, 2002) and this fact is amply illustrated by diabetes mellitus (DM), estimated to rise from a global incidence of 2.8% in the year 2000 to 4.4% in 2030 (Wild et al., 2004). Some AID are actually considered Orphan Diseases, which by definition: (i) affect less than 0.05% of a population, (ii) affect less than 200,000 people in the USA or (iii) for which treatment is not envisaged as profitable for pharmaceutical development.

AID were traditionally taught to have “a mysterious aetiology” or to be designated as a fascinating but poorly understood group of diseases (Davidson and Diamond, 2001) and, even in present times, the inference is often made that when the origin of a disease is obscure, it
suggests an autoimmune aetiology. The classification of a disease as autoimmune has traditionally been based on the detection of an auto-antibody (auto-Ab) that could be visualized reacting with an affected tissue or cell. Two factors, namely, the rarity of naïve T cells specific for any one “peptide - major histocompatibility complex (MHC)” complex, due to the tremendous diversity of the T cell repertoire, and the much greater technical ease with which auto-Ab are identified in comparison to auto-reactive T lymphocytes, actually constitute an important historical bias in the clinical characterization of AID. The development of microscopes that allow for more sensitive detection of cell surface-bound auto-Ab, the capacity to use the auto-Ab to precipitate the auto-antigens (auto-Ag) and the availability of more sophisticated techniques that allow for the exclusion of infections (such as PCR based assays) are technological advances that resulted in a proliferation of newly recognized autoimmune disorders. Auto-Ag sequencing, purification and studies of uptake and processing, and, conversely, Ab isolation, sequencing and isotype determination, allow for a more certain assessment of the possibility that a specific auto-Ag is the driver of the immune response and auto-Ag that are commercially available can be used for identification of auto-Ab for diagnostic means.

Pathogenic antibodies are directly responsible for the immunopathology that derives from Ag recognition and their presence is usually demonstrated experimentally in vivo or in vitro. In a disease such as lupus, pathology is most likely due to deposition of immune complexes irrespective of their reactivity. Lernmark (2001) presents a review of many auto-Ag which are commercially available as recombinant proteins and can be used for specific autoantibody assays in the diagnosis of AID.

The recognition of specific Ag or Ab is not equivalent to the identification of the defect that may underlie pathological autoimmunity. Indeed, with the exception of a few AID associated with immune-deficiencies (Carneiro-Sampaio and Coutinho 2007), and despite powerful genetic studies, no single gene mutation or aberrant gene expression has been identified as a causative factor in any of these diseases (Spurkland and Sollid, 2006). Ongoing work suggests that instead, polymorphisms of normal genes of the immune system (including MHC), that are usually present in the general human population, contribute to the susceptibility for AID development. These polymorphisms may constitute the basis of (i) heterogeneity in clinical presentations, (ii) clinical overlap between AID and (iii) familial clustering of AID. Some of the identified genes are involved in lymphocyte differentiation while others code for proteins of an unknown function, the precise role of these polymorphisms in disease pathogenesis remaining to be understood (Maier and Hafler, 2008). The possibility also remains that genes involved in organ development, protection or repair are implicated. Risk factors cannot be identified in clinical practice and consequently, at
the present time, preventative measures cannot be implemented. AID are incurable and remain responsible for considerable morbidity and mortality, justifying need for ongoing research.

Careful phenotyping of cells and lesions using cytometry, advanced molecular biology and imaging techniques (in order to map pathways controlling lymphocyte physiology) should accompany genetic studies providing important clues. In this regard, humans are very poor study material. Because of obvious ethical constraints, the only cells which are generally available for research are from the peripheral blood (PB) and, rarely, from accessible tissues, and these may not necessarily reflect a pathological process in the lymphoid or target organs. In addition, by the time an AID appears in humans, it is very likely that the initial event or the primary immune abnormality have been diluted or masked by the presence of an immune response. In addition, autoimmunity leads to inflammation which in turn stimulates the immune response, further masking the distinction between primary and secondary events.

Rodent models are preferred because they are affordable, allow for mass production, represent almost all the major AID, can be genetically manipulated to study susceptibility and enable the study of early events, disease severity and organ damage, allowing for therapeutic screening. In an ideal model for a specific AID, the animal develops the disease spontaneously and not through immunization, in an effort to mimic natural mechanisms operating in the human disease. The major drawback for the use of rodent animals is the very limited number of mouse strains, each representing one specific MHC haplotype and in practical terms equivalent to one human individual. There are over 100 alleles at several loci within the MHC, the most polymorphic gene within the human genome. As antigen presentation by the MHC and its recognition by the immune system is a central question, MHC variability may contribute to clinical sub-phenotypes of human disease, differentiated by factors such as the group of target organs affected, the frequency of relapses and disease severity. As an example, the programmed death receptor-1 (PD-1) knock out (-/-) mouse presents with a dilated cardiomyopathy in a Balb/c background and with a glomerulonephritis in a B6 background, remaining to be seen if this is due to a change in the MHC or due to another strain-specific gene that confers a completely different phenotype for the same genetic abnormality (Nishimura et al., 1999; Nishimura et al., 2001). In human AID, an environmental factor is brought into the equation, because concordance disease rates in monozygotic twins are far from 100% (Ebers et al., 1986; Silman et al., 1993; Wandstrat and Wakeland, 2001). In this respect, experimental models do not allow for a re-creation of environmental agents or pressures to which human are subjected in their daily life but, on the other hand, facilitate the study of the presence or absence of specific environmental agents in a
very controlled fashion, having been helped, in this regard, by the development of germ free facilities.

There has been a major effort for experimental models to phenotypically resemble human disease as completely as possible and animal models, such as that of MS, have originated thousands of reports (Steinman and Zamvil, 2006). Ideally, knowledge of disease pathogenesis gained from experimental models should result in the design of rational therapy and consequent pre-clinical studies and clinical trials, going back and forth to experimental models to check new beneficial observations, critical clues from patient studies, unexpected side-effects and combination therapies, in a very dynamic interaction between the laboratory and the clinic. In practice however, advances in treatment of AID in the past 10 to 15 years are not always a direct consequence of experimental research but rather due to a combination of careful marketing and anecdotal evidence of disease improvement of which chronic interferon-β (IFN-β) treatment for MS is illustrative, with only a 30% reduction in relapse rate and disability progression. In contrast, on the basis of a trial with a similar effect to IFN β (Johnson et al., 1995), glatiramer acetate - a polymer of tyrosine, glutamate, alanine and lysine, believed to bind to MHC molecules and block T cell recognition of myelin basic protein (MBP) - carefully designed and first tested in animal models of MS in the 1970’s (Teitelbaum et al., 1971), took 25 years to be approved by the Food and Drug Administration (FDA) for MS treatment. The fact that the use of both IFN-β and glatiramer acetate has been recently challenged in systematic meta-analysis (Filippini et al., 2003; Munari and Filippini, 2004) only accentuates the urgent need for ongoing research into the pathogenesis of AID.

There are some recent examples as to why differences between man and mouse may explain why a specific therapy hailed as promising in mice produces unexpected side-effects in humans. Based on the knowledge that T cell help - mediated by the interaction between CD40L (CD154) on T lymphocytes and CD40 on the cognate B cell - is critical for the production of pathogenic auto-Ab in lupus nephritis, it was predicted that anti-CD40 ligand antibody would lead to a reduction in the severity of nephritis and prolonged survival in experimental models (Kalled et al., 1998). Despite favourable results in phase I human studies (Davis et al., 2001), a phase II trial was terminated prematurely because of thromboembolic events (Boumpas et al., 2003), related to the fact that CD40L is also expressed by platelets, both in monkeys and humans but not in mice (Kawai et al., 2000). Another example of unpredictable collateral damage occurred with the use of natalizumab, an anti-integrin (α4β1) monoclonal antibody. This integrin is an adhesion molecule present on lymphocytes and responsible for their adhesion to endothelium, found to be effective in MS and Crohn’s disease. This drug was actually associated with three deaths from progressive multifocal leucoencephalopathy, which occurs due to a viral infection that could not
have possibly been detected in murine models, since its causative agent - the JC virus - does not infect mice or rats (Van Assche et al., 2005; Kleinschmidt-DeMasters and Tyler, 2005). The recent disastrous result of a phase I clinical trial with TGN1412, a novel superagonist anti-CD28 monoclonal Ab reported experimentally to expand Treg (Beyersdorf et al., 2005), nearly resulted in the death of 6 healthy volunteers due to a cytokine storm (Suntharalingam et al., 2006). CD28 is a co-stimulatory molecule involved in the activation of all T lymphocytes so that in theory this was a possible complication, independently of the results of pre-clinical studies.

Rather than to separate, we need to join “Science Based Medicine” with what we already know from “Evidence Based Medicine”. Progress in the field depends entirely on the very strictest collaboration between fundamental and clinical science, through the constant exchange of questions and clinical translation of research findings to prevention and therapy.

1.2. Pathogenesis of autoimmune diseases

After strong headed dispute in the beginning of the 20th century, when Paul Ehrlich denied the existence of auto-Ab - reviewed in Coutinho (2005) - the concept of autoimmunity as the actual cause of human disease was established in the 1950’s (Witebsky et al., 1957) and modelled on Koch’s postulates, the latter originally intended to establish a causal relationship between microbes and disease. At that time, the pathogenesis of AID focused on B lymphocytes due to a recognized increase in immunoglobulins and the presence of rheumatoid factor, immune complexes and plasma cells. In the mid seventies, it was realized that T lymphocytes predominated in RA infiltrates (Bankhurst et al., 1976) and that removal of thoracic duct lymphocytes (mostly T cells) improved RA (Paulus et al., 1977), as a result of which T lymphocytes became centre point for AID. The reduced auto-Ab production in MRL-lpr/lpr chimeras submitted to Ab mediated T cell depletion (Sobel et al., 1993) and milder renal disease in TCRα−/− MRL-lpr/lpr mice (Peng et al., 1998) demonstrated the dependence of auto-Ab production on T cell help in the MRL-Faslpr model of lupus, where both the Fas mutation and MRL background genes contribute to disease. In addition, allo-Ab production has been shown to be reduced after CD4 T lymphocyte depletion in a model of skin graft rejection (Steele et al., 1996) and, in SLE patients, T cell activation has been correlated with anti-dsDNA production (Spronk et al., 1996). On the other hand, B lymphocytes are indispensable for the development of lupus in Fas sufficient but B cell deficient MRL mice (Chan et al., 1999) and for collagen-induced arthritis (Svensson et al., 1998) and taken together, these observations support a combined role of both B and T lymphocytes in the pathogenesis of AID.
Evidence that a T lymphocyte transfer alone reproduces AID was shown for EAE (Ben-Nun and Cohen, 1982), thyroiditis (Maron et al., 1983) and arthritis (van Eden et al., 1985). Myelin reactive T lymphocytes have been found in patients with MS (Ota et al., 1990) and, with the help of MHC class II tetramers (i) GAD 65-specific T cells have been identified in pre-diabetic NOD mice (Trudeau et al., 2003) and in pre-diabetic patients (Reijonen et al., 2000; Oling et al., 2005) and (ii) cartilage-specific T cells have been recognized in patients with RA (Kotzin et al., 2000). Importantly, this shift in focus led to T lymphocyte directed therapeutic advances with the use of CTLA-4 (Emery, 2003; Genovese et al., 2005) alongside promising B cell targeted monoclonal Ab treatment (Edwards et al., 2002; Edwards et al., 2004). A well-designed set of experiments involving the transfer of OVA-specific DO11 CD4+ T cells into mice that produce secreted OVA as an endogenous self-protein reveals different outcomes in the absence of specific cellular subsets, namely: (i) severe systemic autoimmune reaction in the absence of T and B cells; (ii) mild disease when T cells are absent but B cells are present and; (iii) no disease in the presence of T cells, suggestive that T cells prevent expansion and maintain homeostasis and B cells limit subsequent effector responses of auto-reactive CD4+ T cells (Knoechel et al., 2005).

Criteria to define a disease as autoimmune correctly included the notion that B and T lymphocytes are involved in disease pathogenesis and were established at three different levels of animal experimentation, namely direct, indirect or circumstantial (Rose and Bona, 1993). Transmissibility of the characteristic lesions of the disease by auto-Ab or pathogenic T cells was considered mandatory for direct evidence of an autoimmune mechanism. Indirect evidence required re-creation of the human disease in an animal either spontaneously or through immunization with an analogous Ag to the putative auto-Ag of the human disease. Circumstantial evidence concerned the finding of T lymphocytes or Ab in the affected organ, with improvement of symptoms after the use of immunosuppressive drugs. A working definition of an AID is therefore a disease caused by reactivity of any component of the immune system to self Ag with subsequent inflammatory damage to one or more organs, in the absence of an ongoing infection or other discernable cause (adapted from Davidson and Diamond 2001). It usually involves T lymphocyte activation and/or Ab/immune complex deposition. Even though at the present time AID are still viewed by most clinicians in the framework of diseases of the adaptive immune system, there is a large group of disorders of the innate immune system in humans, – reviewed in Nathan (2002) - of which auto-inflammatory syndromes characterized by mutations in the apoptotic pathways (Padeh and Berkun, 2007) and inflammatory bowel disease linked to polymorphisms of macrophage receptors (Henckaaerts et al., 2007) are examples. Disorders of
innate immunity are characterized by spontaneous inflammation and subsequent activation of the adaptive immune response and will probably be included in the group of AID in future definitions.

1.3. Development of a regulatory T cell concept

It is estimated that \(1 \times 10^6\) T lymphocytes, each with its own distinct T cell receptor (TCR) develops in the thymus every day in a stochastic manner (Tonegawa, 1983). The potential diversity of TCR recombination is of such magnitude that if every T cell was allowed to respond to MHC:peptide complexes without discriminating self from non-self, this would quickly lead to self-destruction. Negative selection in the thymus is a mechanism for preventing attack of healthy self tissues. The development of autoimmune manifestations in recipients of thymic grafts (Thornton et al., 1998) and of a CD25 negative thymocyte population (Itoh et al., 1999), transferred from healthy donors, led to the important realization that, under normal circumstances, negative selection is not complete and furthermore, that physiological autoreactivity must be controlled in the healthy donor.

Physiological tolerance, a property of a living being, is the absence of immune responses to self and to commensals that are, by definition not pathogenic. As proposed by Coutinho (2005), the only way to conjugate (i) the absence of AID with (ii) the natural occurrence of antibodies and autoreactive T lymphocytes that, despite negative selection, are continuously produced throughout the life of an individual and (iii) the establishment of tolerance solely at the stage of embryonic and perinatal development is, to hypothesize, that a group of cells in the immune system develop in this restricted time window and are responsible for controlling physiological autoreactivity, imparting this memory to cells that develop in later periods of development. Tolerance was shown to be effectuated by lymphocytes selected in the thymus and was called dominant, on the basis that it could be transferred from one living organism to another by a T lymphocyte transfer (Coutinho et al., 1993; Modigliani et al., 1995; Le Douarin et al., 1996; Modigliani et al., 1996). The T lymphocytes responsible for tolerance were named regulatory T cells (Treg). It should be mentioned that rejection of a transplant performed in the perinatal period is only prevented by the co-implantation of a thymic graft from the donor species (Le Douarin et al., 1996) and it remains to be determined if the thymus is active throughout life producing Treg continuously or if there is actually a requirement for ongoing Treg education of other cells. More importantly, the concept of a dominant form of tolerance predominates over recessive, ignorant or anergic models, providing a form of control exerted by a group of cells that can be manipulated and eventually used therapeutically.
Because we are unable to confidently identify trigger factors for human AID, at the present time it is not possible to predict whether healthy individuals with benign physiological auto-reactivity or sub-clinical autoimmunity in the form of physiological auto-Ab or self-reactive T lymphocytes, will always remain healthy. This effectively rules out any chance of AID prevention and furthermore, the clinical diagnosis is only possible late in the course of disease, corresponding to the presence of significant target organ destruction. A frequently encountered situation in the clinic is illustrative of these difficulties. RA, SLE, systemic sclerosis, polymyositis, dermatomyositis, mixed connective-tissue disease, and Sjögren syndrome can present with similar clinical features, particularly during the first 12 months of symptoms. However, these have defined discrete diagnostic criteria and patients who present with symptoms, positive serology, or physical findings consistent but not fulfilling criteria for one of these established diseases are diagnosed with undifferentiated connective-tissue disease (UCTD), an entity suggested by LeRoy (1980) and recently defined as (i) signs and symptoms suggestive of a connective-tissue disease, (ii) positive anti-nuclear Ab, and (iii) a disease that lasts at least 3 years (Mosca et al., 2004). It has been shown that UCTD within 12 months of onset usually remains undifferentiated with only a few patients progressing to frank disease (Williams et al., 1999). Studies are ongoing to identify factors that contribute to progression to frank AID and a longitudinal study of the immune system in these patients and their relatives could provide important clues. Other examples include the presence of auto-Ab, in SLE (Arbuckle et al., 2003) and diabetic patients (Schatz and Bingley, 2001), years before the diagnosis of symptomatic disease. The situation is similar with circulating organ-specific auto-Ab predictive of disease, detected in asymptomatic relatives of affected patients years before diabetes (Bonifacio et al., 1990; Riley et al., 1990) and in autoimmune cardiomyopathy development (Caforio et al., 2007). Of note, no study has been able to causally associate a specific infection with an AID which is not surprising, in the light of the fact that patients have multiple infections during their lifetime and those same infections affect individuals without AID.

It is customary to classify AID in systemic or organ specific but in fact, with the exception of demyelinating diseases, most are multi-organ or are associated with a clinical diagnosis of another AID, arguing in favour of a common underlying defect of the immune system. AID may result from the complex interaction between a defective immune system, physiological autoimmunity, an environmental precipitating factor and a genetically susceptible host (Fig.1.1). Regulatory T cell defects are prime candidates for causing immune dysfunction but the relative contribution of each of these factors to the overall equation remains unknown. It is probable that they must coincide for disease to develop. Repertoire deviations in T lymphocytes recently emerged from the thymus have not been reported in the human AID such as SLE, RA or MS.
Significantly, successful autologous stem cell transplantation in MS patients resulted in a thymic output that generated a new and much more diverse TCR repertoire compared to pre-therapy (Muraro *et al*., 2005). As follows, there is much more evidence that defects in Treg, rather than in repertoire, occur in human AID and contribute to disease pathogenesis.

Fig. 1.1
Pathogenesis of Autoimmune Disease

1.4. Origin and mechanism of action of regulatory T cells
The existence of T suppressor cells capable of restricting the development, function and duration of effector T cell responses was first described over 30 years ago (Gershon *et al*., 1974) and the transfer of such cells described to induce Ag-specific tolerance in naïve animals (Ramshaw *et al*., 1976). The notion of T cell-mediated suppression was revived in the 1980’s and early 1990’s (Sakaguchi *et al*., 1982; Sakaguchi *et al*., 1985; Fowell and Mason, 1993) but little explored due to an absence of specific phenotypic markers. In 1995, Treg were defined by CD25 surface expression, and their suppressive capacity was demonstrated by the spontaneous induction of autoimmunity in mice that were thymectomized in day three of life and, subsequently, inoculated with CD4⁺CD25⁻ T cells (Sakaguchi *et al*., 1995; Asano *et al*., 1996). However, cell surface
expression of CD25 is nonspecific since it is also up-regulated on other CD4+ T cell populations after activation. Treg express the X chromosome encoded transcription factor “Foxp3” shown to be indispensable for natural Treg development and function (Fontenot et al., 2003; Hori et al., 2003). At present, Foxp3 is the best known unique marker of this group of cells. Natural Treg are CD4+ T cells selected in the thymus through high avidity TCR-cognate self antigen interactions (Modigliani et al., 1996). Another major population of Foxp3 expressing Treg has recently been described, peripherally generated from naïve CD4+CD25- T cells, further reviewed in point 3.1.4. of the present work.

The fundamental property that defines a Treg is its ability to transfer immunological unresponsiveness from one animal to another or one cell culture to another. Unresponsiveness in vitro is usually measured by the inhibition of proliferation while in vivo measurements include the inhibition of autoimmune disease, graft rejection, allergic reactions or other immune responses. Naturally occurring regulatory T cells have been identified in non-manipulated rodents and humans, and comprise cells of the adaptive immune system (CD4+CD25-Foxp3+ T cells). The precise mechanisms of Treg mediated suppression of potentially pathogenic T cells are still unclear, with divergent conclusions regarding the importance of cellular interactions, release of soluble mediators and functional modification or killing of APC as summarized in Figure 1.2, reproduced from Mottet and Golshayan (2007) and recently reviewed (Sakaguchi et al., 2008). These discrepancies might be explained by differences in the experimental systems (in vitro versus in vivo), the various disease models studied, the pathogenic effector mechanisms and target organs involved, and the contribution of the genetic background of the mouse strains used.

![Fig. 1.2](image-url)  
*Mechanism of action of Treg*
### 1.5. Regulatory T cells in animal models of autoimmune diseases

As shown in Table 1.I, disease development in the presence of a numerical or functional Treg defect provides proof of concept for their fundamental role in effector control. As expected, there are no Treg in scurfy mice, where the defect is precisely a Foxp3 mutation resulting in fatal autoimmunity. The phenotype of IL-2\(^{-/-}\), IL-2R \(\alpha^{-/-}\) or IL-2R\(\beta^{-/-}\) mice demonstrates the role of IL-2 in the development, survival, and function of Foxp3\(^+\) Treg. The discrepancy in the numbers of Treg between IL-2 R\(\alpha\) or \(\beta^{-/-}\) mice can be explained by the redundant role IL-15 signaling through the IL-2R\(\beta\) and the common \(\gamma_c\) complex to promote the development of Foxp3\(^+\) Treg.

Apart from scurfy, lupus - in the progeny of a cross between New Zealand Black (NZB) and New Zealand White (NZW) mice - and diabetes - in Non Obese Diabetic (NOD) mice - are among the very few natural spontaneous AID diseases of mice. The study of Treg in both diseases has shown that there is a reduction in Treg frequency and number in lymphoid organs, when compared to non-autoimmune prone age-matched wild type animals. In these studies, Treg suppressor function has not been tested but, of note, prior knowledge of the frequency of CD25\(^+\) T cells that are Foxp3\(^+\) is required for test interpretation, as this frequency affects the results of \textit{in vitro} suppression assays that are necessarily based on sorting of CD25\(^+\) T cells.

#### Table 1.I - Spontaneous models of AID and Treg defects

<table>
<thead>
<tr>
<th>MURINE MODEL</th>
<th>TREG DEFECT</th>
<th>CONSEQUENCE</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scurfy</td>
<td>Absent Treg due to Foxp3 mutation</td>
<td>Fatal lymphoproliferation</td>
<td>Brunkow et al. 2001</td>
</tr>
<tr>
<td>CD25(^{-/-})((\alpha)-chain IL-2R)</td>
<td>Absence of CD25 expressing cells</td>
<td>Lymphoproliferative syndrome</td>
<td>Papiernik et al., 1998</td>
</tr>
<tr>
<td>CD25(^{-/-})((\alpha)-chain IL-2R)</td>
<td>Slightly reduced Foxp3(^+) Treg</td>
<td>Haemolytic anemia</td>
<td>Antony et al.; 2006</td>
</tr>
<tr>
<td>CD122(^{-/-})((\beta)-chain IL-2R)</td>
<td>Profound reduction Foxp3(^+) Treg</td>
<td>Hyper-reactivity to commensals</td>
<td>Malek et al., 2002</td>
</tr>
<tr>
<td>STAT5(^{-/-}) (which mediates signaling from the IL-2R(\beta) chain)</td>
<td>Profound reduction Treg</td>
<td></td>
<td>Burchill et al., 2007</td>
</tr>
<tr>
<td>IL-2(^{-/-})</td>
<td>Slightly reduced Foxp3(^+) Treg</td>
<td></td>
<td>Antony et al.; 2006</td>
</tr>
<tr>
<td>BWF1 and SNF1</td>
<td>Reduced number of CD4(^+)CD25(^+) T cells compared to Balb/c and DBA/1</td>
<td>Lupus</td>
<td>Wu and Staines 2004</td>
</tr>
<tr>
<td>NZM2410</td>
<td>Reduced number of CD4(^+)CD25(^+) T cells</td>
<td>Lupus</td>
<td>Chen et al. 2005</td>
</tr>
<tr>
<td>(NZB x NZW) F1</td>
<td>Reduced number of CD4(^+)CD25(^+) T cells</td>
<td>Lupus</td>
<td>Hsu et al. 2006</td>
</tr>
<tr>
<td>(NZB x NZW) F1</td>
<td>Reduced number of CD4(^+)CD25(^+) T cells compared to Balb/c</td>
<td>Lupus</td>
<td>Scalapino et al. 2006</td>
</tr>
<tr>
<td>NOD</td>
<td>Reduced number of CD4(^+)CD25(^+) T cells compared to Balb/c</td>
<td>Diabetes</td>
<td>Wu et al. 2002</td>
</tr>
</tbody>
</table>

continues
Examples of AID caused or aggravated by depletion of Treg are summarized in Table 1.II. The initially reported failure to induce AID through CD25 depletion by mAb in wt mice (McHugh and Shevach, 2002) can be attributed to the fact that CD25 depletion resulting from mAb (PC61) administration is quite significant but not complete (Zelenay and Demengeot, 2006). When Treg are completely depleted in wt mice then fulminant lethal autoimmunity ensues (Kim et al., 2007; Lahl et al., 2007). These studies provide a strong link between the deficiency, absence or depletion of Treg and development of AID.

Table 1.II - AID caused or aggravated by Treg depletion or absence

<table>
<thead>
<tr>
<th>MURINE MODEL</th>
<th>TREG MANIPULATION</th>
<th>CONSEQUENCE</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBA/1 – collagen + CFA</td>
<td>CD25 depletion by mAb</td>
<td>Hastens arthritis onset</td>
<td>Morgan et al. 2003</td>
</tr>
<tr>
<td>BDC2.5 TCR Tg mouse crossed with scurfy</td>
<td>No Treg</td>
<td>Accelerated Diabetes</td>
<td>Chen et al. 2005</td>
</tr>
<tr>
<td>Anti-MBP TCR Tg on RAG⁻/⁻ background (TR⁻)</td>
<td>No Treg</td>
<td>Spontaneous EAE</td>
<td>Lafaille et al. 1994</td>
</tr>
<tr>
<td>Anti-MBP TCR Tg (TR⁺)</td>
<td>CD25 depletion by mAb and pertussis toxin</td>
<td>Induces EAE</td>
<td>Zelenay et al. 2005</td>
</tr>
<tr>
<td>B6 - MOG and B10.S – PLP</td>
<td>CD25 depletion by mAb</td>
<td>Increases EAE severity</td>
<td>Reddy et al. 2004; McGeachy et al. 2005</td>
</tr>
<tr>
<td>SJL/J – PLP</td>
<td>CD25 depletion by mAb</td>
<td>Increases EAE relapse</td>
<td>Zhang et al. 2004; Gartner et al. 2006</td>
</tr>
<tr>
<td>NZM</td>
<td>Day 3 thymectomy</td>
<td>Increase anti-ds DNA titres</td>
<td>Bagavant and Tung 2005</td>
</tr>
<tr>
<td>NOD-H2(h4) – iodide</td>
<td>CD25 depletion by mAb</td>
<td>Exacerbation thyroiditis</td>
<td>Nagayama et al. 2007</td>
</tr>
<tr>
<td>Diphtheria toxin receptor control of Foxp3 gene locus</td>
<td>Treg depletion by diphtheria toxin</td>
<td>Fulminant AID</td>
<td>Kim et al. 2007; Lahl et al. 2007</td>
</tr>
</tbody>
</table>
1.6. Regulatory T cells in humans and in human autoimmune diseases

Unlike murine cells, activation of human naïve CD4+ T cells was inconsistently reported to result in Foxp3 expression and development of suppressor activity (Walker et al., 2003; Yagi et al., 2004; Walker et al., 2005). It seems in fact, that unlike murine cells, some human CD4+ T cells are capable of de novo Foxp3 induction in vitro but in contrast to humans, this up-regulation is transient and does not promote immunosuppressive function (Gavin et al., 2006; Allan et al., 2007). Foxp3 promoter demethylation allowing for stable Foxp3 expression has been reported to reveal the committed Treg population in humans and for the best possible differentiation of recently activated Foxp3 expressing T cells from Treg (Janson et al., 2008).

As shown in Table 1.III, with few exceptions, AID coupled with immunodeficiency are thought to be associated with a defect in Treg generation of thymic origin, through altered apoptosis, thymic antigen presentation or lack of expression of factors, such as IL-2 and Foxp3, indispensable for Treg generation, expansion or function (Ulmanen et al., 2005; Carneiro-Sampaio and Coutinho, 2007). Foxp3 deficiency in humans results in IPEX (Immune dysfunction, Polyendocrinopathy, Enteropathy, and X-linked inheritance), an invariably fatal multi-organ AID (Bennett et al., 2001), equivalent to the pathology observed in Foxp3 deficient scurfy mice (Clark et al., 1999). IPEX actually represents one of the few known examples of an AID with a known aetiology. CD25 deficiency in humans causes an IPEX like disease (Caudy et al., 2007) where CD4+Foxp3+T cells are present in normal numbers, further substantiating the CD25 requirement for normal Treg function.

The transcription factor Autoimmune Regulator (AIRE) controls the expression of self Ag in the thymus. Mutations in AIRE have been associated with AID in humans, known as autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) or autoimmune polyendocrinopathy syndrome type 1 (APS 1) (Nagamine et al., 1997). It is thought that, as a result of failure of negative selection, auto-reactive lymphocytes escape to the periphery and become pathogenic. It is controversial if Treg are also affected but contrary to mice (Anderson et al., 2005) it seems that humans with the AIRE mutation have a Treg defect (Kriegel et al., 2004; Kekalainen et al., 2007). Treg have not been tested in the three forms of autoimmune lymphoproliferative syndrome (ALPS) characterized by defects in apoptosis due to mutations in FasL, caspase 10 and FasR (Ulmanen et al., 2005).
The evaluation of Treg in human AID does not provide an uniform answer as regards the existence of a peripheral blood Treg deficit in frequency, number or suppressor function. An important question which remains unanswered concerns the possibility of physiological fluctuations of Treg frequency and number in peripheral blood (PB) in health. Bearing in mind that the analysis of PB T cells may not mirror the situation in tissues, while Treg frequency in PB
reflects Treg frequencies in lymphoid organs, the total number of circulating Treg may be a more accurate measurement, especially in patients that are under IS therapy or with ongoing inflammatory immune responses. Moreover, studies in humans are complicated by the fact that patients with the same disease are naturally considered as a uniform group but may actually be very different in terms of phenotypic manifestations, stage of disease development, duration of disease, different immunosuppressive therapies, time after the start of therapy and periods of remission or relapse. In addition, studies are also performed in small groups compared to healthy controls exacerbating the heterogeneity of the cohorts. Furthermore, the methods employed to identify Treg have not been uniform. Some groups verify the frequency, others cell number and, in the vast majority, the studies have been performed only once. There is a wide variation in the reported percentage of CD4+ T cells co-expressing CD25 largely due to differences in the definition of positivity for CD25 expression since expression of CD25 does not define a distinct population of CD4+ T cells, but rather it is present as a continuum of expression levels between negative and high expression. Foxp3 has been evaluated by mRNA detection and only recently are there tools available for Foxp3 detection by flow cytometry, which have improved the reliability of data. Even more specifically Foxp3 promoter demethylation has been reported to reveal the committed Treg population in humans (Janson et al., 2008). None of these studies measure the number or function of antigen specific Treg. In vitro suppression assays have been performed in a very small number of patients and these points are presented in chronological order in Table 1.IV.

Table 1.IV - Treg in several human AID (not associated with immunodeficiency)

<table>
<thead>
<tr>
<th>NAME OF DISEASE</th>
<th>TARGET</th>
<th>Treg *</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autoimmune Hepatitis</td>
<td>Liver</td>
<td>% CD4+CD25+ ↓ (41)</td>
<td>Longhi et al., 2004</td>
</tr>
<tr>
<td>Kawasaki’s disease</td>
<td>Coronary arteries</td>
<td>% CD4+CD25+ ↓ (54) Foxp3 mRNA* ↓</td>
<td>Furuno et al., 2004</td>
</tr>
<tr>
<td>Psoriasis</td>
<td>Skin</td>
<td>% CD4+CD25high NC (not IS) Suppression ↓</td>
<td>Sugiyama et al., 2005</td>
</tr>
<tr>
<td>Primary Biliary Cirrhosis</td>
<td>Liver</td>
<td>% CD4+CD25high ↓ (91) Suppression NC</td>
<td>Lan et al., 2006</td>
</tr>
<tr>
<td>Graves disease</td>
<td>Thyroid</td>
<td>% CD4+CD25high NC (32) % Foxp3+ ↓</td>
<td>Wang et al., 2006</td>
</tr>
<tr>
<td>Mixed Connective Tissue Disease</td>
<td>Features of SLE, RA, scleroderma and polymyositis</td>
<td>Patients with active disease (31) &lt; % CD4+CD25high than in remission (17)</td>
<td>Barath et al., 2006</td>
</tr>
<tr>
<td>Henoch Schonlein purpura</td>
<td>Purpura and nephropathy</td>
<td>% CD4+CD25+ ↓ (20) Foxp3+ mRNA ↓</td>
<td>Yang et al., 2006</td>
</tr>
</tbody>
</table>
Table 1.IV - continued

<table>
<thead>
<tr>
<th>NAME OF DISEASE</th>
<th>TARGET</th>
<th>Treg *</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wegener’s granulomatosis</td>
<td>Any organ</td>
<td>CD4⁺Fopx3⁺ NC (Active 17)</td>
<td>Abdulahad et al., 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Remission 40)</td>
<td></td>
</tr>
<tr>
<td>Guillain-Barré syndrome</td>
<td>Peripheral nerve</td>
<td>% CD4⁺CD25⁺ ↓ (35)</td>
<td>Chi et al., 2007</td>
</tr>
<tr>
<td>Aplastic anemia</td>
<td>Haematopoietic stem cells</td>
<td>% and number of CD4⁺CD25⁺Foxp3⁺ ↓ (20)</td>
<td>Solomou et al., 2007</td>
</tr>
<tr>
<td>Autoimmune thrombocytopenic purpura</td>
<td>Megakaryocytes</td>
<td>Patients with active disease (19) &lt; % CD4⁺CD25⁺ than in remission (13)</td>
<td>Liu et al. 2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD4⁺CD25⁺ ↓ in severe disease (19)</td>
<td>Sakakura et al. 2007</td>
</tr>
<tr>
<td>Pemphigus</td>
<td>Skin</td>
<td>% CD4⁺CD25⁺ hi ↓</td>
<td>Sugiyama et al. 2007</td>
</tr>
<tr>
<td>Sjögren’s syndrome</td>
<td>Exocrine gland – tear and salivary</td>
<td>% CD4⁺CD25⁺ hi ↑ (21)</td>
<td>Gottenberg et al. 2005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD4⁺Fopx3⁺ ↓ (30)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Suppression NC</td>
<td>Li et al. 2007</td>
</tr>
<tr>
<td>Myasthenia gravis</td>
<td>Neuromuscular junction</td>
<td>% CD25⁺Foxp3⁺ ↓ (75)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(study did not discriminate between Tx/IS and non-Tx non IS patients)</td>
<td>Kosec et al. 2005</td>
</tr>
<tr>
<td>Sarcoïdosis</td>
<td>Lung, skin, eye, CNS</td>
<td>% CD4⁺Fopx3⁺ ↓ (14)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>mRNAFoxp3⁺ ↓ (24)</td>
<td>Idali et al. 2008</td>
</tr>
</tbody>
</table>

* Treg measurements were performed in peripheral blood and compared to healthy controls; %: frequency of CD4⁺ T cells that are CD25⁺; Suppression: in vitro suppressive function; ↑: increased, ↓: decreased; NC: no change; Tx: thymectomized; Where known, number of patients tested indicated in parenthesis.

With the exclusion of psoriasis, Wegener’s granulomatosis and Sjögren’s syndrome, there is a reduction in the frequency of circulating Treg and except for Sjögren’s syndrome and primary biliary cirrhosis, suppression function is generally reduced in all AID tested. Of note, even in Sjögren’s syndrome, one of the few diseases where more than one study was performed, results are not concordant on the number and function of Treg in specific AID. Results on MS, SLE, RA and DM, where most studies were performed, are presented separately, in Tables 1.V and 1.VI.

The only longitudinal study of untreated patients with MS, correlating serial frequency of CD4⁺CD25⁺ T cells in PB with disease activity and reporting that an increase in CD4⁺CD25⁺ T cells over the period of 1 week increased the odds of a relapse, may have simply been measuring activated effector CD25⁺ T cells because there was no selection for the CD4⁺ T cells that were high expressors of CD25 (Khoury et al., 2000), the subpopulation of CD25⁺ T cells known to have suppressor potential.
**Table 1.V - Treg in patients with MS**

<table>
<thead>
<tr>
<th>CLINICAL PHENOTYPE</th>
<th>IMMUNE-SUPPRESSIVE THERAPY</th>
<th>Treg DEFINITION</th>
<th>REGULATORY T CELLS*</th>
<th>REFERENCES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not specified (21)</td>
<td>No</td>
<td>CD4(^{+})CD25(^{high})</td>
<td>NC</td>
<td>Viglietta et al. 2004</td>
</tr>
<tr>
<td>High lesion load (9)</td>
<td>No</td>
<td>Suppressor Function</td>
<td>↓</td>
<td>Putheti et al. 2003</td>
</tr>
<tr>
<td>Low lesion load (19)</td>
<td>Not specified</td>
<td>% CD4(^{+})CD25(^{+})</td>
<td>NC</td>
<td>Putheti et al. 2004</td>
</tr>
<tr>
<td>Not specified (15)</td>
<td>No</td>
<td>% CD25(^{high})</td>
<td>NC</td>
<td>Putheti et al. 2004</td>
</tr>
<tr>
<td>Not specified (19)</td>
<td>No</td>
<td>Foxp3 mRNA in CD4(^{+})CD25(^{+}) T cells</td>
<td>↓</td>
<td>Huan et al. 2005</td>
</tr>
<tr>
<td>Not specified (4)</td>
<td>No</td>
<td>Suppressor Function</td>
<td>↓</td>
<td>Haas et al. 2005</td>
</tr>
<tr>
<td>Not specified (13)</td>
<td>No</td>
<td>Foxp3 mRNA in CD4(^{+})CD25(^{+}) T cells</td>
<td>NC</td>
<td>Haas et al. 2005</td>
</tr>
<tr>
<td>Not specified (73)</td>
<td>No</td>
<td>% CD25(^{high})</td>
<td>NC</td>
<td>Haas et al. 2005</td>
</tr>
<tr>
<td>Not specified (17)</td>
<td>No</td>
<td>Suppressor Function</td>
<td>↓</td>
<td>Haas et al. 2005</td>
</tr>
<tr>
<td>RR + SP (50)</td>
<td>Yes but not lymphopenic</td>
<td>% CD25(^{high})</td>
<td>NC</td>
<td>Venken et al. 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Suppressor Function</td>
<td>↓ in RR</td>
<td>Venken et al. 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NC in SP</td>
<td>Venken et al. 2006</td>
</tr>
<tr>
<td>RR + SP (35)</td>
<td>No</td>
<td>Foxp3 mRNA in CD4(^{+})CD25(^{+}) T cells</td>
<td>NC</td>
<td>Kumar et al. 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>% CD25(^{high})</td>
<td>↑</td>
<td>Kumar et al. 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD25(^{high}) + intermediate</td>
<td>↑</td>
<td>Kumar et al. 2006</td>
</tr>
<tr>
<td>All types (36)</td>
<td>No</td>
<td>% CD25(^{high})</td>
<td>NC</td>
<td>Feger et al. 2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>NC between types</td>
<td>Feger et al. 2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>MS and disease</td>
<td>Feger et al. 2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>activity Enriched in CSF</td>
<td>Feger et al. 2007</td>
</tr>
<tr>
<td>Not specified (4)</td>
<td>No</td>
<td>Suppressor Function</td>
<td>↓</td>
<td>Haas et al. 2007</td>
</tr>
<tr>
<td>RR (40)</td>
<td>No</td>
<td>% recent thymic</td>
<td>↓ &lt; 45 years of</td>
<td>Haas et al. 2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>emigrant Treg</td>
<td>age NC &gt; 45 years</td>
<td>Haas et al. 2007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(CD4(^{+})Foxp3(^{+})CD31(^{+}))</td>
<td>of age</td>
<td>Haas et al. 2007</td>
</tr>
<tr>
<td>RR (40)</td>
<td>No</td>
<td>% CD4(^{+})CD25(^{+})Foxp3(^{+})</td>
<td>↓ in RR</td>
<td>Venken et al. 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N in SP</td>
<td>Venken et al. 2008</td>
</tr>
<tr>
<td>RR (10)</td>
<td>No</td>
<td>Suppressor Function</td>
<td>↓</td>
<td>Venken et al. 2008</td>
</tr>
</tbody>
</table>

* Treg measurements were performed in peripheral blood and compared to healthy controls; %: frequency of CD4\(^{+}\) T cells that are CD25\(^{+}\); Suppression: *in vitro* suppressive function; ↑: increased, ↓: decreased; NC: no change; number of patients tested indicated in parenthesis; RR: relapsing remitting; SP: secondary progressive; CSF: cerebrospinal fluid.

In another study, the frequency of CD4\(^{+}\)CD25\(^{+}\) T cells in CSF was negatively correlated with the CSF concentration of myelin basic protein and the presence of IgG oligoclonal bands, measured at an early phase of disease (Jensen et al., 2004). The frequency of Treg, as defined by high CD25 expression, has also been found to be similar (Putheti et al., 2004; Haas et al., 2005) or higher (Kumar et al., 2006) than in healthy controls. When defined by Foxp3 positivity, Treg were found to be significantly elevated in the CSF from MS patients when compared with patients with other neurological disorders, but once again the frequency in the periphery did not differ between...
MS patients and healthy donors (Feger et al., 2007). One study found a decreased number of Foxp3+CD4+ T cells that express the CD31 marker, indicating recent thymic emigrants (Haas et al., 2007), and others reported a reduced number of PB CD4+ CD25high Foxp3+ T cells and lower FOXP3 protein expression per cell in relapsing remitting-MS patients when compared to the secondary progressive forms and control individuals, a finding correlated with a diminished suppressive capacity of Treg in these patients (Venken et al., 2006; Venken et al., 2008).

Table 1.VI - Treg in patients with SLE, RA and DM

<table>
<thead>
<tr>
<th>NUMBER OF PATIENTS</th>
<th>IMMUNE-SUPPRESSION</th>
<th>Treg DEFINITION</th>
<th>Treg NUMBER AND FUNCTION</th>
<th>REFERENCES</th>
</tr>
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<tr>
<th>NUMBER OF PATIENTS</th>
<th>IMMUNE-SUPPRESSION</th>
<th>Treg DEFINITION</th>
<th>Treg NUMBER AND FUNCTION</th>
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<td>27</td>
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<td>SF of inflamed joint &gt; PB</td>
<td>Cao et al. 2003</td>
</tr>
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<td>9</td>
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<td>Suppressor Function</td>
<td>NC</td>
<td></td>
</tr>
<tr>
<td>125</td>
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<td>% CD4+CD25+</td>
<td>PB &lt; SF</td>
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<td>8</td>
<td>Yes</td>
<td>Suppressor Function</td>
<td>NC</td>
<td></td>
</tr>
<tr>
<td>PB=79; SF=21</td>
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<td>↑ (SF &gt; PB &gt; Control)</td>
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<td>5</td>
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<td></td>
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<tr>
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<td></td>
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<td>CD4-Foxp3+</td>
<td>NC</td>
<td></td>
</tr>
<tr>
<td>9</td>
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<td>Suppression Function</td>
<td>NC</td>
<td></td>
</tr>
<tr>
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<td>Not known</td>
<td>% CD4-Foxp3+</td>
<td>SF &gt; PB</td>
<td></td>
</tr>
<tr>
<td>Not known</td>
<td>Not known</td>
<td>% CD4-Foxp3+</td>
<td>NC</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>No</td>
<td>% CD4+CD25+</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>54</td>
<td>No</td>
<td>% CD4-25+</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>21</td>
<td>No</td>
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<td>NC</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>No</td>
<td>Suppression Function</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>No</td>
<td>% CD4+CD25+</td>
<td>NC</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>No</td>
<td>Suppression Function</td>
<td>Longstanding diabetes - NC</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>No</td>
<td>% CD4+CD25+</td>
<td>NC</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>No</td>
<td>Suppression Function</td>
<td>↓</td>
<td></td>
</tr>
<tr>
<td>31</td>
<td>No</td>
<td>% CD4+CD25+Foxp3+</td>
<td>NC</td>
<td></td>
</tr>
</tbody>
</table>

*Unless otherwise mentioned Treg measurements performed in peripheral blood (PB) and compared to healthy controls; %: frequency of CD4+ T cells that are CD25+; SF: synovial fluid; Suppression: in vitro suppressive function; ↑: increased, ↓: decreased; NC: no change.
In summary, with only one exception, where the authors of the report themselves volunteer differences in CD25 expression threshold to account for a higher frequency of Treg in PB of MS patients (Kumar et al., 2006), half of the studies show decreased frequency of Treg, in direct opposition to the other half that reports no change. What seems to be common to all the studies is a diminished Treg suppressor function.

Likewise, in SLE, it is impossible to reach a firm conclusion upon analysis of the studies that have been published. In terms of the frequency of Treg defined by CD25\textsuperscript{high} expression, it is reported to be decreased in at least 3 different studies (Crispin et al., 2003; Liu et al., 2004; Miyara et al., 2005) but when Treg are defined as CD4\textsuperscript{+}Foxp3\textsuperscript{+} there seems to be no change (Alvarado-Sanchez et al., 2006; Zhang et al., 2008), with CD4\textsuperscript{+}CD25\textsuperscript{−}Foxp3\textsuperscript{+} T cells higher (Lin et al., 2007) or lower (Zhang et al., 2008) in active disease. While one study reported a decrease in suppressor function correlating with higher disease activity (Alvarado-Sanchez et al., 2006), another reported no change (Zhang et al., 2008), with the caveat, as already mentioned, that a change in the frequency of Foxp3\textsuperscript{+} T cells within the CD25\textsuperscript{+} subset may affect the results of suppression assays \textit{in vitro}. In family members there is a positive correlation between reactivities and Treg frequencies which is lost in patients suggesting a loss of regulation in the latter (C. Fesel, submitted for publication).

The study of SF in RA provided a rare opportunity to study Treg at the site of target organ damage in human disease. Treg were found to accumulate in SF with no evidence of a functional defect (Cao et al., 2003; Cao et al., 2004; van Amelsfort et al., 2004). Only one small study (out of a total of nine studies on patients with RA) revealed a reduction in suppression function (Ehrenstein et al., 2004). In one study there was a decrease in the PB frequency of CD4\textsuperscript{+}CD25\textsuperscript{high} defined Treg (Chen et al., 2007) while in two other studies the frequency of CD4\textsuperscript{+}Foxp3\textsuperscript{+} T cells was not different from controls (Jiao et al., 2007; Lin et al., 2007).

As regards DM, there was no change in the frequency of Treg as defined by CD25\textsuperscript{high} expression or Foxp3. With one exception (Putnam et al., 2005), all studies showed a decrease in suppressor Treg function (Brusko et al., 2005; Lindley et al., 2005; Brusko et al., 2007).

Genetic polymorphisms in the FOXP3 gene have been studied in several AID with disparate results and no such firm conclusion can be drawn. A functional polymorphism in the promoter/enhancer region was found to be associated with type 1 diabetes (T1D) in a Japanese population sample (Bassuny et al., 2003) but disappointingly, despite a strong male bias, no association between variation and T1D was found in the Sardinian population (Zavattari et al., 2004). Another study from Norway could not confirm any impact on T1D susceptibility for two microsatellites (Bjornvold et al., 2006). Certain FOXP3 polymorphisms were associated with
primary biliary cirrhosis but not significantly associated with Chron’s disease (Park et al., 2005), Graves and Addison’s disease (Owen et al., 2006). There was an association to thyroid disease in Caucasian but not Japanese patients (Ban et al., 2007) and no relation to juvenile idiopathic arthritis in a UK Caucasian population (Eastell et al., 2007).

Genetic polymorphisms of other functional markers of Treg may also be strong candidates for AID susceptibility. For instance, a recent meta-analysis of 33 studies of CTLA-4 gene polymorphisms suggest an involvement in the susceptibility to T1D (Kavvoura and Ioannidis, 2005). Polymorphisms in the human PD-1 gene have been reported to link with SLE, RA and T1D (Okazaki and Wang, 2005) and in the CD25 gene with T1D (Vella et al., 2005; Qu et al., 2007), Graves disease (Brand et al., 2007) and MS (Hafler et al., 2007).

1.7. Regulatory T cell immunotherapy

The use of Treg-based immunotherapy is currently intensively studied in a variety of animal models where natural Treg (nTreg), either freshly isolated or ex-vivo expanded, have been successfully used to prevent autoimmunity (Table 1.VII). Antigen-specific Treg, such as those derived from BDC2.5 TCR Tg mice can be expanded in sufficient quantities in vitro without loss of their characteristic phenotype and regulatory properties and are effective in diabetes prevention in contrast to polyclonal Treg derived from NOD mice which are not (Tang et al., 2004), illustrating the importance of Ag specificity for Treg function in the prevention of AID.

Table 1.VII - Effectiveness of nTreg in prevention AID

<table>
<thead>
<tr>
<th>MODEL</th>
<th>Treg DONOR</th>
<th>PHENOTYPE/EXPANSION PROTOCOL</th>
<th>Treg THERAPY RESULT</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adoptive transfer diabetes</td>
<td>Pre-diabetic NOD</td>
<td>Polyclonal CD4+ L-selectin&lt;sup&gt;high&lt;/sup&gt;</td>
<td>Delay in disease transfer</td>
<td>Herbelin et al. 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Polyclonal CD4+CD25+</td>
<td></td>
<td>Lepault and Gagnerault 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Polyclonal CD4+CD25+L-selectin&lt;sup&gt;high&lt;/sup&gt;</td>
<td></td>
<td>Salomon et al. 2000</td>
</tr>
<tr>
<td>Diabetes in NOD mice</td>
<td>Pre-diabetic NOD</td>
<td>Polyclonal CD4+CD25+ T cells</td>
<td>Diabetes not prevented</td>
<td>Szanya et al. 2002</td>
</tr>
<tr>
<td>BDC2.5 TCR Tg</td>
<td>Anti-CD28/CD3 and IL-2 expanded BDC2.5 TCR Tg CD4+CD25+</td>
<td>Diabetes prevented and cured</td>
<td>Tang et al. 2004.</td>
<td></td>
</tr>
<tr>
<td>Collagen induced arthritis</td>
<td>wt</td>
<td>Polyclonal CD4+CD25+</td>
<td>Slowed disease progression</td>
<td>Morgan et al. 2005</td>
</tr>
<tr>
<td>Lupus</td>
<td>(NZB/NZW)F1</td>
<td>Anti-CD28/CD3 and IL-2 polyclonal CD4+CD25+</td>
<td>Slowed disease progression</td>
<td>Scalapino et al. 2006</td>
</tr>
<tr>
<td>Induced EAE</td>
<td>wt</td>
<td>Polyclonal CD4+CD25+ T cells.</td>
<td>Inhibit disease severity</td>
<td>Kohm et al. 2002</td>
</tr>
<tr>
<td>Scurfy</td>
<td>wt</td>
<td>Polyclonal CD4+ T cells</td>
<td>Long term survival</td>
<td>Smyk-Pearson et al. 2003</td>
</tr>
</tbody>
</table>
Non-regulatory T cells may also up-regulate Foxp3 to levels observed in nTreg. These induced Treg (iTreg) can be generated by the stimulation of naïve T lymphocytes in the presence of IL-2 and TGF-β (Zheng et al., 2002; Chen et al., 2003; Zheng et al., 2007) and effectively prevent AID as shown in Table 1.VIII. After in vivo transfer, many iTreg down-regulate Foxp3, though a subset retains Foxp3 for a period of greater than 1 month (Davidson et al., 2007; Selvaraj and Geiger, 2007).

Table 1.VIII - Effectiveness of Treg in prevention AID

<table>
<thead>
<tr>
<th>MODEL</th>
<th>Treg DONOR</th>
<th>Treg ORIGIN / INDUCTION PROTOCOL</th>
<th>RESULT Treg THERAPY</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart Transplantation Rejection</td>
<td>wt</td>
<td>Polyclonal sorted Foxp3^- / IL-2 and TGF-β</td>
<td>Prevention</td>
<td>Zheng et al. 2006</td>
</tr>
<tr>
<td>Diabetes</td>
<td>BDC2.5 TCR Tg</td>
<td>TCR Tg Islet / IL-2 and TGF-β</td>
<td>Prevention</td>
<td>Weber et al. 2006</td>
</tr>
<tr>
<td>Gastritis</td>
<td>wt</td>
<td>Polyclonal sorted Foxp3^- / TGF-β</td>
<td>Prevention</td>
<td>DiPaolo et al. 2007</td>
</tr>
<tr>
<td>MOG-EAE</td>
<td>wt</td>
<td>IL-10 sufficient polyclonal sorted Foxp3^- / IL-2 and TGF-β</td>
<td>Prevention</td>
<td>Selvaraj and Geiger 2008</td>
</tr>
<tr>
<td>Myasthenia gravis</td>
<td>wt</td>
<td>Polyclonal CD4^+ / anti-CD3, anti-CD28, IL-2 wt 2, TGF-β</td>
<td>Inhibition</td>
<td>Aricha et al. 2008</td>
</tr>
</tbody>
</table>

In humans, due to the reduced number of Treg in circulation (estimated at between 50 to 100 Treg per μl of blood), ex vivo expansion without loss of suppressor function would be an important prerequisite for using regulatory T cells for immunotherapy. This requirement is fulfilled by all the protocols in Table 1.IX using lymphocytes obtained from healthy subjects.

Table 1.IX - Expansion and induction protocols in human Treg

<table>
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<tr>
<th>PNENOTYPE</th>
<th>EXPANSION / CONVERSION PROTOCOL</th>
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<tr>
<td>Polyclonal CD4^+ T cells</td>
<td>IL-10 induces IL-10 producing T cells</td>
<td>Groux et al. 1997</td>
</tr>
<tr>
<td>Polyclonal CD4^-T cells</td>
<td>CD86 / CD40 blockade and IL-15</td>
<td>Koenen and Joosten 2000; Koenen et al. 2003</td>
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<tr>
<td>Polyclonal CD4^-CD25^-</td>
<td>Anti-CD3 and IL-2</td>
<td>Levings et al. 2001</td>
</tr>
<tr>
<td>CD45RA^-RO^-T cells</td>
<td>TGF-β up-regulated CD25 and CTLA-4</td>
<td>Yamagiwa et al. 2001; Zheng et al. 2002</td>
</tr>
<tr>
<td>Polyclonal CD4^-CD25^-</td>
<td>Foxp3 up-regulated by anti-CD3/ anti-CD28^- IL-2</td>
<td>Longhi et al. 2008</td>
</tr>
</tbody>
</table>
Several protocols that involve an increase in Treg number in vivo, such as the use of an analog of 15-deoxypergualin (Duplan et al., 2006), estrogen (Polanczyk et al., 2005), transfer of dendritic-cell presenting myelin antigens (Hirata et al., 2005; Hirata et al., 2007), copolymer-I (Hong et al., 2005), salmonella vaccine (Ochoa-Reparaz et al., 2007), anti-thymocyte globulin (Chung et al., 2007), IFN-β (de Andres et al., 2007) and IVIG (Furuno et al., 2004; Chi et al., 2007; Ephrem et al., 2008) have shown some effectiveness in AID prevention.

Rapamycin stands out as the IS agent that inhibits T cell proliferation but selectively spares or de novo induces Treg (Battaglia et al., 2005; Battaglia et al., 2006; Keever-Taylor et al., 2007; Strauss et al., 2007; Kang et al., 2008). A subset of Foxp3+ T cells suppresses T cell immunity in spontaneously diabetic NOD mice in a TGF-β-dependent manner and can be induced from peripheral CD4+CD25− T lymphocytes by anti-CD3 immunotherapy (You et al., 2007), reducing insulin needs in a recent clinical trial in diabetic patients (Keymeulen et al., 2005).

1.8. Aims and scope of the thesis

Autoimmune diseases (AID) are a major challenge to the advancement of medical science in that its aetiology remains unknown, relapses are often unpredictable as trigger factors remain unidentified, prevention is therefore unattainable and diagnosis is only possible at a late stage of disease, after end-organ damage. In addition, drug-therapy while it controls relapses it is also associated with a high morbidity and mortality. Developments in the field of lymphocyte selection and tolerance mechanisms have brought Treg to the forefront of research in the pathogenesis of AID and allows for a fresh look in the search for new therapies.

Most current therapies consist of non-specific immunosuppression (IS) and are unable to cure human AID, raising the hypothesis that these therapies, because of their generalized effect, are harmful to Treg. The central question asked in this work was whether steroids, the most commonly used form of treatment in human AID, have a deleterious effect on Treg number or function. To determine the impact of steroids, hydrocortisone (HC), defined in a dose and duration of therapy designed to cause short-lived immunosuppression, is tested in a model of multiple sclerosis using an experimental model of EAE - the anti-myelin basic protein T cell receptor transgenic (anti-MBP TCR Tg) mouse - where the absence of disease is due to the presence of Treg and where, conversely, EAE develops in the absence of effective Treg, allowing for a clinical readout of Treg function. Testing the effect of HC upon Treg also involves in vitro and in vivo functional assays.
Susceptibility to AID may lie in the genetics of Treg and triggers of human AID may lie with those that cause lymphopenia and decrease Treg number or function below a certain threshold. The lymphopenia caused by GC is also to be compared to known AID triggers, such as cyclophosphamide, irradiation, pertussis and CD25 depletion that affect the thymus, the peripheral lymphoid compartment and the blood brain barrier. Once again, these stresses upon the immune system are to be evaluated in the anti-MBP TCR Tg mouse and an attempt to be made to correlate the outcome (capacity to trigger EAE) with the utilization of generalized IS versus Treg selective effects and with the degree of lymphopenia and thymic atrophy.

*Helicobacter* is a recognized facilitator of T cell-induced colitis and therefore making use of the fact that there was a colony of mice $H^+$ and another which was $H^-$, T cell-induced colitis is to be primarily induced in both colonies and cells transferred between colonies to check effector and protective responses in the recipients. Aspects of Treg physiology are to be approached through the biological effect of inflammation, determining Treg number at several time points during severe inflammation and ongoing colitis, testing the possibility of conversion of naïve cells to a Foxp3$^+$ phenotype and assessing a possible functional role.

In accordance with the hygiene hypothesis, the notion that organ-specific autoimmune disease is poorly induced in animal colonies of relaxed biocontainment rules inspired the study of the influence of T cell-induced colitis on the course of encephalomyelitis in a transgenic mouse model, aiming to understand whether an inflammatory immune response can favourably affect the course of an AID, and more specifically whether this protection is Treg mediated.

In order to contribute to the study of regulatory T cells in autoimmune disease we carried out three different experimental layouts, designed to appraise: (i) the effect of steroid treatment on the number and function of regulatory T cells in mice; (ii) the response of regulatory T cells to inflammation and; (iii) the effect of T cell-induced colitis on the repression of encephalomyelitis in transgenic mice. Even though the study of these three aspects required different experimental layouts - and was therefore included in separate chapters - an effort was made to coherently study a common objective namely the understanding of the effects of modulation of Treg in AID.

Included in the appendix are two published works with experimental results obtained in the course of the present thesis.
2. EFFECT OF CORTICOSTEROID TREATMENT ON THE NUMBER AND FUNCTION OF REGULATORY T CELLS IN MICE

Most non-specific immunosuppressive regimens are unable to cure human autoimmune disease (AID) raising the hypothesis that these therapies are harmful to CD4+Foxp3+ Regulatory T cells (Treg). To determine the impact of corticosteroids (CS) on Treg number and function, hydrocortisone (HC), defined in a dose and duration of therapy designed to cause short-lived significant immunosuppression, was tested in experimental autoimmune encephalomyelitis (EAE) and T cell-induced colitis, two models of AID that are dependent on Treg function for disease manifestation. In the EAE model, anti-myelin basic protein T cell receptor transgenic mice (called TR+) are naturally protected from developing EAE by Treg and the transfer of Treg prevents the EAE which develops when these mice are set in a RAG−/− background (called TR−). T cell-induced colitis is induced by the transfer of naïve CD4+ T lymphocytes to a lymphocyte depleted donor and prevented by Treg co-transfer. The effect of HC alone was compared to irradiation, cyclophosphamide (Cyp), pertussis toxin (Ptx) and CD25 depletion, each alone or in combination with HC. Frequencies of Treg (defined as CD4+Foxp3+) were obtained by flow cytometry and Treg function was determined by in vitro and in vivo functional assays. On the effect of HC: (i) the number of CD4+Foxp3+ T cells was markedly decreased but not selectively depleted in HC treated wild type (wt) or TR+ mice; (ii) HC or irradiation, matched to cause a similar degree of lymphopenia, were not capable of triggering EAE; (iii) Neither HC nor irradiation exacerbated Ptx induced EAE, the latter only potentiated by CD25 depletion; HC (iv) had no effect on the expansion of CD4+ T cells or Treg upon transfer.
to lymphocyte deficient animals; (v) did not affect Treg capacity to prevent naïve T cell-induced colitis; (vi) did not affect the capacity of donor CD4+ T cells to protect TR- mice from EAE and finally, (vii) did not change Treg suppressor function measured in vitro. On the effect of Cyp: (i) Cyp alone caused lymphopenia and selectively depleted CD4+Foxp3+ T cells in wt and TR+ mice, (ii) Cyp caused EAE in the TR+ mice; (iii) when administered together with Cyp, HC potentiated the effect of cyclophosphamide; (iv) Cyp, in contrast to HC, affected the capacity of donor CD4+ T cells to protect TR- from EAE. Taken together these observations lead us to conclude that the combination of HC and Cyp was particularly harmful to Treg number and function. The IS caused by a short course of steroids was not deleterious to Treg but the effect of chronic or higher dosage corticosteroid therapy remains to be studied. Finally these results also suggested that lymphopenia is a trigger of EAE in TR+ mice only when associated with selective Treg depletion.

2.1. Introduction

Self tolerance is the absence of immune responses to self and to commensals that are, by definition, not pathogenic. AID are caused by inappropriate immune responses to self when tolerance breaks down. Treg are key players in maintaining dominant tolerance, as they prevent the pathogenicity of auto-reactive cells in the periphery, playing an important role in the prevention and control of AID and transferring tolerance from one organism to another (Sakaguchi et al., 1995; Takahashi et al., 1998).

Lymphocyte homeostasis refers to the numerical stability of the mature T cell population in the normal adult animal and homeostatic proliferation has been found to be responsible for AID onset. Experimentally, AID may also be precipitated by generalized IS and may be caused by irradiation, drugs and specifically by the use of monoclonal antibodies.
Immunosuppressants have been used to treat AID but not surprisingly, as the cause of the vast majority of these diseases remains unknown, treatment with non-specific IS is usually not curative, has serious side effects and disease usually recurs upon drug withdrawal. Treg are cycling cells, naturally activated (Fisson et al., 2003), which expand in response to inflammatory signals (Caramalho et al., 2003; Dai et al., 2007). Because of these characteristics, Treg may actually be more susceptible than other T lymphocytes to the action of IS medication and therefore, the ineffectiveness of IS could theoretically be attributed to a deleterious effect upon Treg.

2.1.1. Regulatory T cell phenotype markers

Naturally arising Treg are CD4⁺ T lymphocytes that are thymus selected, expressing the X chromosome encoded transcription factor Foxp3, shown to be indispensable for natural Treg development and function (Fontenot et al., 2003; Hori et al., 2003) and the best marker of Treg. CD25 is the alpha chain of the interleukin 2 (IL-2) receptor and while IL-2 is not required for the development of Treg, it is essential to maintain their homeostasis in the periphery as tested in IL-2⁻/⁻ mice and by anti-IL-2 antibody administration (D'Cruz and Klein, 2005; Fontenot et al., 2005; Setoguchi et al., 2005). When a T cell becomes activated, it leaves, by definition, the naïve T cell pool. CD45RB is a surface marker that allows the identification of a CD4⁺ T lymphocyte population that has been previously engaged in immune responses, activated by TCR-cognate antigen encounter. This membrane marker is an alternatively spliced form of CD45, a protein tyrosine phosphatase involved in antigen-TCR mediated signal transduction (Volarevic et al., 1993). Its level of expression is up-regulated during thymic development (Wallace et al., 1992) and its expression on naïve CD4⁺ T cells decreases upon activation (Lee et al., 1990). According to this marker, approximately two-thirds of CD4⁺ T cells are CD45RB⁺⁻ and constitute the naïve T cell compartment in un-manipulated mice and are, in other words, cells that have not been engaged in immune responses. The other third are activated cells, corresponding to the CD45RB⁻⁻ compartment. Roughly one third of these CD45RB⁻⁻ CD4⁺ lymphocytes express CD25 and, as such, this is the subset enriched in Treg (Sakaguchi et al., 1995; Takahashi et al., 1998). Of note, the expression of the CD25 molecule on CD45RB⁻⁻ CD4⁺ T lymphocytes in vivo is not stable (Annacker et al., 2000; Zelenay et al., 2005). CD62L or L-selectin is an adhesion molecule whose expression on CD4⁺CD25⁻ T cells defines a naïve phenotype. L-selectin is required for entry into lymph nodes (Szanya et al., 2002) and has also been shown to be required on effector cells that cause myelin damage in EAE and in fact, CD62L⁻⁻ mice were found to be refractory to classical
EAE induction (Grewal et al., 2001). High CD62L expression on Treg is also correlated with Treg suppressive activity (Fernandez et al., 2007). Finally, the integrin αE (CD103), which recognizes epithelial cadherin, identifies a potent subpopulation of regulatory CD25+ T cells, specialized in cross-talk with epithelial environments (Lehmann et al., 2002).

2.1.2. T lymphocyte homeostasis

Homeostatic mechanisms control the overall size and composition of the mature T cell pool. The murine thymus produces around $10^6$ T cells per day that are released into the periphery as recent thymic emigrants (RTE) (Modigliani et al., 1994). In the neonatal period, once in the periphery, there is physiologic proliferation of RTE filling up the initial population of the peripheral T cell compartment (Min et al., 2003). Thereafter, homeostasis is maintained by ongoing peripheral cell death, spontaneous “physiological” proliferation and thymic output. Some CD4+ T cells survive for long periods of time as resting, non-dividing cells (Tough and Sprent, 1994) and homeostasis requires the maintenance of a diverse TCR repertoire to ensure protective immunity.

The TCR repertoire and relative proportions of various T cell subsets are established in the thymus, and continue to be shaped and regulated in the periphery. Neonatal but not adult experimental thymectomy is associated with several types of AID (Kojima and Prehn, 1981; Sakaguchi et al., 1995; Asano et al., 1996). As might be predicted, neonatal thymectomy is associated with a restriction in the peripheral TCR repertoire and oligoclonal expansion (La Gruta et al., 2000). Unlike humans, mice are lymphopenic at birth and therefore AID occurs as a result of peripheral T cell reactivity in the context of global T cell lymphopenia which includes Treg lymphopenia.

Following a lymphopenia-inducing insult, T cells undergo proliferation for restoration of peripheral population size - so-called lymphopenia induced proliferation (LIP) - a situation considered distinct from homeostatic basal proliferation expansion or from response to known antigenic stimulation (Surh et al., 2000). In response to lymphopenic episodes, total T cell number is restored by two main pathways: peripheral expansion and thymic export. Experimentally, in the absence of thymus, peripheral expansion participates in T cell restoration (Modigliani et al., 1994; Mackall et al., 1997). In humans, because of thymic involution in all but the youngest patients, T cell regeneration is thought to be largely reliant on peripheral expansion (Mackall et al., 1995). It has been suggested that the drive to maintain population size may also be accompanied by loss of TCR diversity and the peripheral emergence of auto-reactive effector T cells. As recently reviewed (Khoruts and Fraser, 2005), in conditions of severe lymphopenia, when reduced amounts of naïve T cells are transferred into hosts lacking lymphocytes and a functional thymus,
autoimmune phenomena set in as self-reactive clones proliferate preferentially. The proliferative capacity of individual T cells correlates with TCR avidity for self peptides/MHC ligands (Ge et al., 2001) but such experimental models may introduce the bias of an activation phenotype that effector cells acquire in proliferating conditions (Kieper and Jameson, 1999; Oehen and Brduscha-Riem, 1999). Nevertheless, naturally occurring lymphopenia induced proliferation has been correlated with the severity of diabetes in NOD mice (King et al., 2004) and with the onset of autoimmune gastritis (McHugh and Shevach, 2002).

In clinical practice, as the diagnosis of AID occurs at a late stage of disease, initial events remain unknown. CD4+ T cell lymphopenia in humans is well described after measles (Okada et al., 2000) and after influenza infection (Nichols et al., 2001) but the few epidemiological studies linking viral infections and autoimmune diabetes or multiple sclerosis bear no emphasis on the degree of lymphopenia and mechanisms of T cell restoration, but rather focus on possible direct effects of the virus on target organs. Lack of increased reported incidence of autoimmunity among cancer survivors suggests that transient lymphopenia caused by organ irradiation or chemotherapy alone is insufficient for autoimmune disease induction. In fact, immunosuppression and increased susceptibility to infections rather than autoimmune diseases are the expected consequence of lymphopenia. Of note, a third of patients with multiple sclerosis that received Campath-1H, an anti-CD52 monoclonal antibody which causes profound and prolonged depletion of T cells and other hematopoietic cells, developed autoimmune hyperthyroidism (Coles et al., 1999).

Treg control LIP as shown by their capacity to inhibit the expansion of a co-transferred naïve T cell population (Annacker et al., 2000; Almeida et al., 2002; Bourgeois and Stockinger, 2006). Importantly, in the presence of a normal thymus, it has been shown that Treg effectively prevent peripheral lymphocyte depletion induced proliferation and activation of transferred T cells, except when the degree of depletion exceeds 90% of the peripheral T cell pool (Bourgeois and Stockinger, 2006). Treg themselves absolutely require interaction with MHC-peptide complexes for expansion upon transfer to lymphopenic hosts (Seddon and Mason, 1999; Cozzo et al., 2003; Fisson et al., 2003; Klein et al., 2003). As their TCR have a higher avidity for self-antigens than the other CD4+ T lymphocytes (Hsieh et al., 2004), Treg may have a greater potential to undergo lymphopenia-induced proliferation than naïve T cells and the T cell population may be selectively enriched.

Lymphopenic insults due to drugs, irradiation or a viral infection may have completely different consequences in an immunocompetent host. These differences are related to specific and combined effects on lymphocyte precursors, thymus size and capacity for T lymphocyte
differentiation, cytokine expression, residual populations of T cells and differential effects on peripheral effector and Treg subset. Immunosuppressants may alter the Treg repertoire, Treg effector ratios or decrease the number of Treg below the minimum requirement necessary to prevent AIDs or precipitate a relapse in established disease. Therefore, paradoxically, IS may be a trigger for AID.

2.1.3. Triggers of auto-immune diseases
Any of the trigger factors of AID mentioned below could cause AID through an effect on a particular T cell population (such as Treg) or through the induction of lymphopenia–induced proliferation per se.

2.1.3.1. Irradiation
Ionizing radiation can functionally alter the immune system and break self-tolerance. High dose (42.5 Gy) fractionated (2.5 Gy 17 times) total lymphoid irradiation (TLI) on mice has been described to cause various organ-specific autoimmune diseases, such as gastritis, thyroiditis, and orchitis, diagnosed at 6 months post TLI (Sakaguchi et al., 1994). It takes 6 to 8 weeks for the number of T cells and composition of T cell subsets to recover to the control level after completion of TLI. In these experiments, transfer of naïve T cells within 2 months of TLI prevented AID, suggesting that TLI may potentially interfere with regulatory T cells. In these experiments, the T cell recovery was faster in the group with thymus shielding suggesting that thymic output contributed to restoration of the peripheral T cell population after irradiation. In humans, environmental and therapeutical irradiation have been associated to autoimmune thyroid disease (Hancock et al., 1991; Imaizumi et al., 2006).

2.1.3.2. Non-steroidal Immunosuppressants
Immunosuppressants, such as cyclosporin A (CsA) and Cyp have actually been shown to cause or precipitate AID in animal models.

Cyclosporin A inactivates the Ca/calmodulin dependent serine–threonine phosphatase calcineurin leading to the inactivation of the Nuclear Factor of Activated T cells (NFAT) and in its absence, T cells are unable to produce IL-2 and consequently do not proliferate (Morgan et al., 1976). It has been shown to cause a wasting disease resembling graft-versus-host disease in lethally irradiated rats reconstituted with syngeneic bone marrow (Sorokin et al., 1986) and autoimmune gastritis and oophoritis in newborns BALB/c mice (Sakaguchi and Sakaguchi, 1989)
which is most probably due to a subsequently demonstrated inhibitory effect on Treg function (Zeiser et al., 2006).

Cyclophosphamide (Cyp) is a nitrogen mustard compound traditionally utilized as a cytotoxic chemotherapeutic agent in the treatment of neoplasia but also used for the treatment of severe AID in humans. Cyp exhibits most cytotoxicity against cells actively replicating their DNA, as unpairing of DNA strands at this stage makes the nucleotide residues more susceptible to the alkylation mediated by Cyp (Moore, 1991). Paradoxically, Cyp can induce AID in the experimental animal, namely diabetes acceleration and increase in incidence in the NOD mouse (Harada and Makino, 1984; Yasunami and Bach, 1988) and autoimmune gastritis in thymectomized adult BALB/c mice (Barrett et al., 1995). The effect on diabetes was subsequently shown to be associated with a reduction in the number and function of CD4⁺CD25⁺Foxp3⁺ T cells within pancreatic infiltrates (Brode et al., 2006). In fact, Cyp fails to prevent clinical disability progression in MS, thus bringing into question its therapeutic value in this disease (La Mantia et al., 2007; Perini et al., 2007). Cyp is often used concomitantly with steroids in the treatment of AID and neoplastic disease. The lack of Cyp long term beneficial effect in AID may be explained by its effect on Treg number and function. Moreover, Cyp causes a significant decline in Treg number in rats with established tumours (Ghiringhelli et al., 2004) thus enhancing anti-tumour responses (Savani et al., 2005). Notably, Cyp has been shown to cause short lived lymphopenia, affect Treg function in vitro and cause a slight down-regulation of Foxp3 expression (Lutsiak et al., 2005). Cyp significantly decreases CD25⁺CD4⁺ and Foxp3⁺ T cells in the spleen (Sp) and lymph nodes (Ln) of B6 mice with a maximal effect 2 days post administration with partial recovery by day 6 and total recovery by day 9 (Brode et al., 2006).

Methotrexate modestly reduced human Treg suppression in vitro (Porter et al., 2006). Mycophenolate mofetil and leflunomide, both DNA synthesis inhibitors (Cherwinski et al., 1995; Allison and Eugui 2000) and rapamycin - an inhibitor of proliferative responses of lymphocytes to IL-2 (Abraham and Wiederrecht, 1996) - preserve Treg function and expansion in mice (Zeiser et al., 2006; Battaglia 2005) and that effect also occurs in humans with rapamycin (Battaglia et al., 2006).

2.1.3.3. Pertussis toxin

Experimental autoimmune encephalomyelitis (EAE) is an animal model that recapitulates many features of MS. It can be induced by immunization of susceptible animals with a number of myelin antigens including myelin basic protein (MBP) (Zamvil et al., 1986), proteolipid protein (PLP) (Tuohy et al., 1989) and myelin oligodendrocyte glycoprotein (MOG) (Mendel et al., 1995) or
occur spontaneously or with minimal induction, in genetically manipulated mice with T cell populations TCR specific for one of these antigens.

Following administration of Ptx, several mechanisms concur to exacerbate autoimmune diseases. Ptx affects dendritic cell maturation, cytokine production, T lymphocyte migration and promotes clonal expansion of auto-reactive T cells among many other immunological effects recently reviewed in Cassan et al. (2006). While this effect of Ptx in demyelination models has been principally attributed to the opening of the blood brain barrier based on evidence that Ptx increases vascular permeability through sensitization to vasoactive amines (Linthicum et al., 1982), the recently reported reduction in Treg cell number and function (Cassan et al., 2006; Chen et al., 2006) is thought to contribute to the immunological adjuvanticity of Ptx.

**Table 2.I – Pertussis toxin administration in EAE induction protocols in wt mice**

<table>
<thead>
<tr>
<th>Ptx brand</th>
<th>Dose (ng)</th>
<th>Frequency</th>
<th>Route</th>
<th>Strain/peptide</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. pertussis vaccine, Commonwealth Serum Laboratories, Australia</td>
<td>6 to 27 × 10^9 organisms</td>
<td>D0 and D2</td>
<td>i.v.</td>
<td>SJL – MBP</td>
<td>Bernard and Carnegie, 1975</td>
</tr>
<tr>
<td>Pertussis toxin (Ptx) List Biological Laboratories, Campbell, USA (LBL)</td>
<td>100</td>
<td>D1 and D3</td>
<td>i.v.</td>
<td>B10.PL / MBP</td>
<td>Kumar and Scercarz, 1993</td>
</tr>
<tr>
<td>Ptx LBL</td>
<td>200</td>
<td>D0 and D3</td>
<td>i.v.</td>
<td>SJL/PLP</td>
<td>Kuchroo et al., 1994</td>
</tr>
<tr>
<td>Ptx LBL</td>
<td>1000</td>
<td>D0, D3, D7</td>
<td>i.v.</td>
<td>Lewis/spinal cord</td>
<td>Arima et al., 1996</td>
</tr>
<tr>
<td>Ptx LBL</td>
<td>200</td>
<td>D1 and D3</td>
<td>i.v.</td>
<td>SJL/PLP</td>
<td>Falk et al., 2000</td>
</tr>
<tr>
<td>Ptx LBL</td>
<td>200</td>
<td>D0 and D1</td>
<td>i.v.</td>
<td>SJL/PLP</td>
<td>Hofstetter et al., 2002</td>
</tr>
<tr>
<td>Ptx LBL</td>
<td>200</td>
<td>D0 and D2</td>
<td>i.p.</td>
<td>C57BL/6 – MOG</td>
<td>Kohm et al., 2002</td>
</tr>
<tr>
<td>Ptx Sigma-Aldrich, St. Louis, Missouri, USA)</td>
<td>250</td>
<td>D1 and D3</td>
<td>i.p.</td>
<td>C57BL/6 – MOG</td>
<td>Becher et al., 2002</td>
</tr>
<tr>
<td>Ptx LBL</td>
<td>200</td>
<td>D0 and D3</td>
<td>i.v.</td>
<td>C57BL/6 – MOG</td>
<td>Montero et al., 2004</td>
</tr>
<tr>
<td>Ptx LBL</td>
<td>150</td>
<td>D0 and D2</td>
<td>i.v.</td>
<td>C57BL/6 – MOG</td>
<td>Zhang et al., 2004</td>
</tr>
<tr>
<td>Ptx Sigma-Aldrich</td>
<td>400</td>
<td>D-1 and D1</td>
<td>i.p.</td>
<td>C57BL/6 – MOG</td>
<td>Shao et al., 2004</td>
</tr>
<tr>
<td>Ptx Sigma, Deisenhofen, Germany</td>
<td>400</td>
<td>D1 and D3</td>
<td>i.p.</td>
<td>C57BL/6 – MBP</td>
<td>Linker et al., 2005</td>
</tr>
<tr>
<td>Ptx LBL</td>
<td>400</td>
<td>D0 and D2</td>
<td>i.p.</td>
<td>C57BL/6 – MOG</td>
<td>Chen et al., 2006</td>
</tr>
<tr>
<td>Ptx LBL</td>
<td>400</td>
<td>D0</td>
<td>i.v.</td>
<td>C57BL/6 – MOG</td>
<td>Cassan et al., 2006</td>
</tr>
<tr>
<td>Ptx LBL</td>
<td>200</td>
<td>D2</td>
<td>i.v.</td>
<td>C57BL/6 – MOG</td>
<td></td>
</tr>
</tbody>
</table>

The dose of Ptx utilized in EAE induction protocols varies considerably, as shown in Table 2.I.
Increasing the concentration of pertussis vaccine (Levine et al., 1966; Bernard and Carnegie, 1975; Linthicum et al., 1982) and in house preparations of Ptx (Munoz et al., 1984) have long been reported to increase the incidence of EAE. In 1981, Ptx was crystallized (Arai and Munoz, 1981) and, since the 90’s, is commercially available. Since then, the different toxin dosages which have been used probably represent adjustments for particular strains of mice, in order to achieve maximal EAE incidence in accordance with strain susceptibility to induction. However, no comparisons have been made between the different commercial preparations that are available.

Ptx is used to induce EAE in myelin-specific TCR transgenic mice, either alone or in combination with the nominate peptide; there is an enormous variability in dose, frequency of administration and EAE-induction rates in transgenic (Tg) mice, as shown in Table 2.II.

Table 2.II - Isolated Ptx administration in EAE induction protocols in TCR Tg mice

<table>
<thead>
<tr>
<th>Ptx brand</th>
<th>Dose (ng)</th>
<th>Frequency</th>
<th>Route</th>
<th>TCR transgene/strain</th>
<th>Incidence EAE after Ptx</th>
<th>Spontaneous Incidence EAE</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBL</td>
<td>75</td>
<td>D1</td>
<td>i.v.</td>
<td>Anti-MBP-1-11 (B10-H2)</td>
<td>75% (6/8)</td>
<td>43% Most at 5-11 wks of age</td>
<td>Goverman et al., 1993 – Conventional Facility</td>
</tr>
<tr>
<td>LBL</td>
<td>400</td>
<td>D0 and D2</td>
<td>N/A</td>
<td>Anti-MBP-1-11 (B10-H2)</td>
<td>100% (5/5)</td>
<td>15%</td>
<td>Goverman et al., 1993 – SPF Facility</td>
</tr>
<tr>
<td>LBL</td>
<td>100</td>
<td>D0</td>
<td>i.v.</td>
<td>Anti-MBP-1-17 (B10-H2)</td>
<td>(N/A) EAE is induced</td>
<td>0%</td>
<td>[Lafaille et al., 1994]</td>
</tr>
<tr>
<td>LBL</td>
<td>200</td>
<td>D1 and D3</td>
<td>i.p.</td>
<td>Anti-MBP-1-17 (B10-H2)</td>
<td>(N/A) EAE is induced</td>
<td>0%</td>
<td>Marusic and Tonegawa, 1997</td>
</tr>
<tr>
<td>LBL</td>
<td>75</td>
<td>D0 and D2</td>
<td>i.v.</td>
<td>Anti-PLP-139-151 (SJL-H2)</td>
<td>83 % (5/6)</td>
<td>Variable, at any age</td>
<td>Waldner et al., 2000</td>
</tr>
<tr>
<td>LBL</td>
<td>150</td>
<td>D0 and D2</td>
<td>i.v.</td>
<td>Anti-MOG 35-55 (C57Bl/6-H2)</td>
<td>56% (9/16)</td>
<td>35% optic neuritis</td>
<td>Bettelli et al., 2003</td>
</tr>
</tbody>
</table>

N/A= not available

2.1.3.4. Anti-CD25 monoclonal antibody

CD25 is a marker of both activated and regulatory T cells. Therapy with anti-CD25 monoclonal antibody could be beneficial if, on balance, most of the activated cells and the least Treg were affected. In humans, there are several anecdotal reports documenting a beneficial effect in diseases such as psoriasis vulgaris (Salim et al., 2000; Wohlrab et al., 2001), erosive lichen planus (Rebora et al., 2002), pemphigus vulgaris (Renkl et al., 2004), systemic sclerosis (Scherer et al., 2006), multiple sclerosis (Bielekova et al., 2004) and inflammatory bowel disease (Creed et al., 2003; Van Assche et al., 2003; Schwarzer et al., 2006; Van Assche et al., 2006).
On the other hand, anti-CD25 therapy in humans is utilized to decrease Treg in situations where they are known to contribute to disease. Examples of its beneficial use are the prevention of acute cellular rejection in renal and heart transplantation (Adu et al., 2003; Segovia et al., 2006) and the prevention and treatment of graft vs host disease (Chen et al., 2003; Ji et al., 2005). In clinical practice, targeting Treg to prevent tumor-related immunosuppression remains of potential benefit but research is much behind the field of transplantation and limited to experimental murine models (El Andaloussi et al., 2006; Fukuhara et al., 2006; Simova et al., 2006).

There are 2 clones of anti-CD25 antibodies used in animal models: PC61 and 7D4. The PC61, but not the 7D4 clone, results in the depletion of a large fraction of Foxp3+ CD4+ Treg (Zelenay and Demengeot, 2006). As PC61 does not appear to fix complement it has also been claimed that it leads to the downregulation of CD25 (Kohm et al., 2006). PC61 depletion remains the best way to deplete Treg as it is not possible to deplete Foxp3 due to its intra-nuclear localization. Of note, not all Treg are CD25+ and, in fact, wt mice remain healthy after anti-CD25 administration (McHugh and Shevach, 2002; Caton et al., 2004). Nevertheless, CD25 depletion abolishes the need for Ptx to induce EAE (Montero et al., 2004) and aggravates the severity of experimentally induced EAE as reviewed in Table 1.II.

2.1.4. Immunosuppression mediated by glucocorticoids

Since Edward Kendall isolated cortisone in the late 1930s and Philip Hench first used it to treat rheumatoid arthritis in 1948 (Hench, 1949), steroids have been routinely used therapeutically for over five decades. Steroids have become the cornerstone of therapy in human autoimmune disorders and allergic conditions, both acute and chronic. As an example of their widespread use, up to 0.9% of the adult population in the United Kingdom receives oral corticosteroid therapy at any given point (van Staa et al., 2000).

The genomic mechanism of action of glucocorticoids (GC) has been extensively reviewed (Boumpas et al., 1993). There are four major types of steroids: progestins, androgens, estrogens and corticosteroids. The latter are sub-divided into 2 groups according to biological activity: Mineralocorticoids (MC) regulate fluid and electrolyte balance and GC are involved in the regulation of carbohydrate metabolism and endowed with profound anti-inflammatory and immunosuppressive properties. Potencies based on effects on glucose metabolism (but not effects on sodium retention) closely parallel those for anti-inflammatory effects. This was the reason for using the term GC where anti-inflammation is the therapeutically desired effect. GC are lipophilic substances that easily pass into the cell, bind to the ubiquitously expressed cytosolic
glucocorticoid receptor (GR) and are translocated into the nucleus, where they regulate gene transcription as early as 30 minutes after GR binding. At the molecular level GC modulate genes involved in inflammatory and immune responses. At the cellular level, GC inhibit adhesion molecules and therefore the access of leucocytes to inflammatory sites; interfere with the functions of leucocytes, endothelial cells, and fibroblasts and suppress the production and the effects of humoural factors involved in the inflammatory response.

While the classical anti-inflammatory potency of GC reflects a genomic interaction, those immediate effects associated with high dose CS therapy occur within seconds and seem to result from direct contact with biological membranes. These so-called non-genomic effects are independent of the GR, result in inhibition of ion entry across plasma membranes, interfere with mitochondrial membrane and ATP availability and, in this way, prevent lymphocyte activation and cause apoptosis (Brann et al., 1995; Buttgereit et al., 1997; Buttgereit et al., 1999; Gold et al., 2001). In humans, the GC receptors are fully saturated at a daily dose of approximately 100 mg of prednisone equivalent (Buttgereit et al., 2002). In mice no data is available but a dose equivalent to HC above 20 mg/kg is likely to be saturating and above that non-genomic effects come into play (Schmidt et al., 2000).

2.1.4.1. Preparations in clinical use
Glucocorticoids in therapeutic use for anti-inflammatory and immunosuppressive effects are nowadays exclusively synthetic molecules; preparations are many and are used in various therapeutic regimens with choices based on MC and GC properties. Management strategies differ substantially depending on the underlying disease but it must be said that the basis for the use of different dosages is essentially empirical as the evidence to support preferences in specific clinical settings is very scarce. The following few examples illustrate the most common utilization of GC in human therapy:

- The MC effect of hydrocortisone (HC) is too strong for long-term treatment of AID and as such, HC is only used on a short term basis, by intravenous (i.v.) injection, for the emergency management of some conditions such as asthma, anaphylaxis and allergy.
- Prednisolone (PRED) has predominantly a GC activity and is the oral corticosteroid most commonly used for short courses of therapy and in long term treatment of AID.
- Prednisone has a similar potency to PRED. Prednisone is a prodrug that is converted by the liver into prednisolone, which is the active drug. For historical reasons, because prednisone was the first synthetic, pharmacologically relevant GC to be introduced into clinical medicine, GC can be expressed by converting them into mg of “prednisone equivalent”.

35
Dexamethasone (DEX) has a very high affinity for the GR, low level of binding to plasma proteins and insignificant MC activity. This makes it particularly suitable for high dose therapy in conditions such as cerebral oedema, where water retention would be a disadvantage.

- The non-genomic effect of methylprednisolone (MP) is more potent than DEX (2.5x) and as such is preferred to DEX in very high doses. For instance, a 5 day course of very high dose MP remains of unequivocal benefit in the immediate control of MS relapses (Kupersmith et al., 1994; Brusaferri and Candelise, 2000).

*In vitro* studies have shown dissociation between anti-inflammatory and immunosuppressive properties but studies vary in their reports. For instance, using a standardized assay based on the capacity of the substance to be tested to inhibit phytohaemagglutinin-induced lymphocyte proliferation in whole blood culture, HC was reported as being the weakest IS of all therapeutic GC with an IC50 approximately 10 times greater than DEX (Mager et al., 2003) while earlier studies described HC to have an intermediate immunosuppressive action comparable to DEX (Langhoff and Ladefoged, 1983; Langhoff et al., 1987).

The anti-inflammatory equivalencies and dosages most commonly used in human therapy (Buttgereit et al., 1999; Buttgereit et al., 2002; Lionakis and Kontoyiannis, 2003) are shown in Table 2.III.

**Table 2.III - Most commonly used GC: Anti-inflammatory equivalencies and dosages in human therapy.**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Equivalent potencies</th>
<th>Equivalent dosages</th>
<th>Disease</th>
<th>Daily dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrocortisone</td>
<td>1</td>
<td>20 mg</td>
<td>Allergy, Asthma</td>
<td>1.5 mg/kg</td>
</tr>
<tr>
<td>Prednisolone (Prednisone)</td>
<td>4</td>
<td>5 mg</td>
<td>Asthma, Autoimmune Disease Transplant</td>
<td>High: 0.5-1 mg/kg (30-100 mg) Low: 0.25 mg/kg (&lt;7.5 mg)</td>
</tr>
<tr>
<td>Methylprednisolone</td>
<td>5</td>
<td>4 mg</td>
<td>Exacerbation of MS Cancer</td>
<td>1 g/day</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>25</td>
<td>0.75 mg</td>
<td>Cerebral oedema Cancer</td>
<td>0.1-0.3 mg/kg</td>
</tr>
</tbody>
</table>

**2.1.4.2. Effect on the immune system**

GC have been clearly demonstrated to cause several immunosuppressive effects in man, including decreased migration of cells into inflammatory sites (Rebuck and Mellinger, 1953;
Boggs et al., 1964), peripheral blood lymphocytopenia (Coburg et al., 1970; Fauci and Dale, 1974; Yu et al., 1974; Fauci and Dale, 1975), lymphocyte re-distribution to the bone marrow (Cohen, 1972), inhibitory effects on adhesion molecules (Perretti et al., 1996) and decreased immunoglobulin levels (Butler and Rossen, 1973). Thymic atrophy in human babies has long been an acknowledged side effect (Caffey and Silbey, 1960) but CS-induced lymphopenia was then thought to be due mainly to re-distribution.

The original paper describing DNA laddering during apoptosis by gel electrophoresis (Wyllie, 1980) took advantage of the susceptibility of thymocytes to the genomic action of CS hormones. Double positive (DP) thymocytes, that express both CD4 and CD8, are much more sensitive to CS induced apoptosis than single positive (SP) CD8+ or CD4+ thymocytes (Compton et al., 1987; Compton et al., 1990; Cohen, 1992). These effects of steroids on lymphoid cells are mediated through the GC receptor (Tsai and O'Malley, 1994; Tronche et al., 1998). The variation in sensitivity to glucocorticoid-induced apoptosis observed as a function of T cell differentiation is associated with changes in the levels of Notch and Bcl-2 expression. Bcl-2, an antiapoptotic factor, is preferentially expressed by CD4+ and CD8+ thymocytes and peripheral T cells and not by the CD4+CD8+ thymocytes. Notch is highly expressed in the progenitor subpopulation of CD4-CD8 thymocytes, absent in CD4+CD8+ DP thymocytes, and re-expressed at intermediate levels in SP thymocytes. This coordinate pattern of expression is due to the fact that activation of the Notch signalling pathway results in the up-regulation of Bcl-2 expression, thereby conferring resistance to glucocorticoids in the CD4+ and CD8+ single-positive thymocytes (Sentman et al., 1991; Hasserjian et al., 1996; Deftos et al., 1998).

It is not known how much thymic output contributes to peripheral re-population after CS induced lymphopenia in humans but in an avian model, thymectomy does not affect the number of circulating CD4+ T lymphocytes in the recovery period after CS induced lymphopenia (Kong et al., 2002). In humans, with increasing age, it has been reasoned that recovery from CS-induced involution of the human thymus would likely be limited by a reduction in thymic regenerative capacity (Mackall et al., 1997; Haynes et al., 2000).

In very high dosages GC cause structural disorganization of the thymic stroma, abnormal MHC expression and ultimately defective T-cell differentiation (van Vliet et al., 1986). GC reportedly prevent T cell division by inducing cell cycle arrest (Harmon et al., 1979), suppress cytokine gene transcription (Almawi et al., 1996) and repress signalling pathways involved in regulation of cytokines such as those mediated by AP-1 and NF-κB (Planey and Litwack, 2000). GC affect dendritic cell differentiation and maturation (Piemonti et al., 1999) and inhibit the antigen presentation of dendritic cells in MHC class II pathway (Pan et al., 2001) therefore
inhibiting TCR mediated lymphocyte activation. Furthermore, DEX has been reported to attenuate T cell receptor signalling through the inhibition of phosphorylation (Van Laethem et al., 2001).

Different dosages and preparations of GC may therefore potentially interfere with development, number and function of all cellular components of the innate and adaptive immune systems.

2.1.4.3 Effect on regulatory T cells

In both animal models and human studies there are opposing evidences on the effect of GC on Treg:

**In support of beneficial effects:**
- CD25 expression increases in CD4+ T lymphocytes cultured *in vitro* with DEX (Wiegers et al., 1995).
- A subset of so-called adaptive regulatory IL-10 producing CD4+ Foxp3+ T lymphocytes has been described as being induced, *in vivo*, from naïve T cells, in animals treated with IL-10 (Groux et al., 1997; Asseman et al., 1999), under the control of IL-10 conditioned DC (Wakkach et al., 2003) and *in vitro*, by culture of CD4+ T cells with DEX and vitamin D3 (Barrat et al., 2002).
- Myelin Basic Protein (MBP)-specific Th2 cell lines generated *in vitro* in the presence of DEX plus IL-4 were shown to protect rats from EAE (Ramirez and Mason, 2000).
- Murine CD4+CD25+ T cells express a higher level of glucocorticoid receptor (GR) and Bcl-2 and have been claimed to be more resistant to DEX-induced cell death than their CD4+CD25- counterparts. A single injection of DEX 5 mg/kg has been reported to increase the frequency of CD25 expressing SP CD4+ thymocytes (Chen et al., 2004).
- Steroids have also been claimed to promote IL-10 expressing Foxp3+Treg development in patients with asthma (Karagiannidis et al., 2004) but for obvious ethical reasons, the study was unable to study a control population of patients with asthma of a similar severity not under CS therapy.
- A recent study found that while MP did not affect Treg suppressor function it decreased Treg expansion in response to alloantigen (Lim et al., 2007).
- Human therapy with high dose MP increased circulating Treg in MS (Navarro et al., 2006) and idiopathic thrombocytopenic purpura (Ling et al., 2007).

**In support of deleterious effects:**
- In previous work with animal models of EAE, disease was induced by immunization of susceptible animals with myelin-derived Ags emulsified in CFA, or following adoptive
transfer of T cell lines or clones specific for various myelin proteins (Swanborg, 1995; Steinman, 1996; Storch et al., 1998). The clinical course of EAE varies according to the animal model and to the protocol used to induce the disease, varying from an acute monophasic illness which fully recovers, to a transient remission followed by relapses. In Lewis rats and in B6 mice, recovery from the first episode is followed by long-term resistance to further induction of disease (Hinrichs et al., 1981; Fillatreau et al., 2002). In B6 mice this refractoriness to disease induction has subsequently been attributed to Treg as, post recovery, CD25 depletion abrogates resistance to re-induction (McGeachy et al., 2005). In Lewis rats, where EAE may be induced by immunization with spinal cord extract emulsified in CFA, earlier studies determined that DEX 0.1 mg/kg, for 5 days, delays disease development for 1 week while DEX 1 mg/kg abrogates disease development (Bolton and Flower, 1989). Paradoxically, in this model, a 5 day course of 200 μg DEX daily (equivalent to HC5 mg/kg/day) post recovery, caused severe EAE to appear 7 days after the last DEX administration (Reder et al., 1994). Even though, in the Lewis rat model there is, to date, no demonstration that Treg are responsible for this refractory phase, it is possible that this represents a deleterious effect of steroids on Treg function.

- Injection of GC in the perinatal period led to a reduction in suppressor function as measured by antibody response post immunization (Ezine and Papiernik, 1981) and neonatal administration of dexamethasone increased susceptibility to EAE in adult rats (Bakker et al., 2000). Early neonatal GC therapy in humans has been used successfully for the treatment of neonatal respiratory distress syndrome in newborn infants (Mammel et al., 1983) but little is known about the long term side effects of GC and whether these children are at an increased risk of AID.

- DEX 5 mg/kg – 2 administrations - has been claimed to limit the development of Foxp3+ T cells and subsequent tolerance to allergens in experimental asthma (Stock et al., 2005). Consistent with this idea, treatment of young children with corticosteroids was shown to paradoxically increase the likelihood of later developing asthma and allergy (Fox et al., 1999), but no study has, so far, shown that steroids increase the incidence of AID.

2.1.5. Objectives

It is a very difficult task to dissociate the effect of long term GC therapy from the natural course of disease and thus to evaluate whether GC adversely affect AID. At present, there is in fact little hope of ever finding out the real value of chronic steroids in human therapy as patent rights have expired and the drug industry is unlikely to be interested in controlled clinical trials.
The effect of GC upon the immune system depends on the type of preparation and dosage used. Short courses of steroids such as hydrocortisone have been very useful for control of respiratory and allergic disorders in humans. The specific questions asked in the present study were whether in susceptible individuals, steroids themselves may potentially harm Treg homeostasis and in that way paradoxically induce AID. Steroids were to be utilized in a manner as clinically relevant as possible and compared to known experimental triggers of AID. It was attempted to delineate critical cofactors which result in AID during lymphopenia in the hope this can provide insight into the pathophysiology of naturally occurring autoimmune diseases.

2.2. Material and methods

2.2.1. Experimental model

Anti-Myelin Basic Protein TCR transgenic mice develop EAE and are considered to be a suitable experimental model of human MS. These mice are transgenic for the $\alpha$ and $\beta$-chains of a TCR specific for the NH2-terminal Ac1-17 peptide of MBP associated with the I-A$^\text{u}$ molecule, thus presenting a peripheral repertoire dominated by the MBP-specific CD4$^+$ T cells. They remain healthy on a RAG sufficient background and are called TR$^+$ mice. When the transgenes are introduced in a RAG deficient background the T cell repertoire is strictly monoclonal and all mice (called TR$^-$) develop EAE spontaneously at 2-3 months of age, characterized by meningeal infiltration and patchy demyelination predominantly in the spinal cord, resulting in ascending paralysis affecting the tail and then the limbs. While all lymphocytes in TR$^-$ mice are CD4 TCR Tg, T/R$^+$ mice have, in addition, $\gamma\delta$, CD8, NK-T cells and B cells as well as CD4$^+$ T cells which express endogenously encoded TCR in combination or not with the Tg TCR $\alpha$ or $\beta$ chain. Whenever both TCR chains are transgenic, this can be detected by an anti-clonotype monoclonal antibody called 3H12 (Olivares-Villagomez et al., 1998), which also stains T cells that are double expressors (cells that contain 2 types of TCR receptors, usually a Tg $\alpha$ and $\beta$ chain and a Tg $\beta$ chain with a non-Tg $\alpha$ chain) (Lafaille et al., 1994; Marusic and Tonegawa 1997). These mice show inefficient allelic exclusion of the $\alpha$ chain because cells that express the MBP specific $\alpha$ TCR chain co-express non-Tg $\alpha$ chains. The same does not occur for the $\beta$ chain and, therefore, the non-Tg $\alpha$ chain requirement for Treg is more important. About 2% of the T cells
express non-Tg $\alpha$ and $\beta$ chains while 10% of the T cells are double expressors (Olivares-Villagomez et al., 1998). It was established that the reason why TR$^-$ mice become ill is because they lack Treg as they are protected by a CD4$^+$ Treg population (Olivares-Villagomez et al., 1998; Van de Keere and Tonegawa, 1998; Olivares-Villagomez et al., 2000) and it was subsequently shown that Treg contained in the double expressor subpopulation are more potent than those exclusively encoding non-Tg TCR chains (Hori et al., 2002). This indicated that while the non-Tg chain is important for Treg selection in the thymus, it is the Tg TCR which confers the specificity through which Treg exert their protective function.

This transgenic model is ideal to study Treg because it has been shown that EAE only develops in their absence and provides an easy clinical readout. TR$^+$ mice are healthy and can be used to test AID triggers. TR$^-$ mice develop spontaneous meningeal inflammation and demyelination and are protected by a CD4$^+$ T cell transfer containing Treg. Therefore, if Treg function is affected by steroids/immunosuppression, a CD4$^+$ T transfer from a donor treated with HC may fail to protect from EAE. Treg generation in this model could well be affected by steroids which target the immature CD4$^+$CD8$^+$ T cell population in the thymus, at a step when the already re-arranged $\beta$ chains have to find matching $\alpha$ chains and where the pressure for diversity of the non-Tg population is extreme. Experimental autoimmune encephalomyelitis was monitored at least every 3 days and attributed the following scores: 1, limp tail; 2, weak or partial hind leg paralysis; 3, total hind leg paralysis; 4, hind leg paralysis and weak or partial front leg paralysis; 5, moribund and 6, dead (Lafaille et al., 1994). EAE was only considered when the score was $\geq 2$.

T cell-induced colitis model involves the adoptive transfer of wt naïve CD4$^+$ T cells into SCID or RAG$^{-/-}$ mice resulting in bowel inflammation and epithelial damage due to an immune response and resulting immunopathology (Morrissey et al., 1993; Powrie et al., 1993). The co-administration of Treg leads to complete inhibition of colitis development (Powrie et al., 1993; Powrie et al., 1994; Aranda et al., 1997) and a CD4$^+$CD25$^+$ T cell transfer actually cures already established colitis (Mottet et al., 2003). HC treatment of donor mice from which the Treg are obtained and subsequent co-transfer with naïve T cells allows for a measure of Treg function after HC treatment.

### 2.2.2. Mice

The following strains were used in the present study: B10.PL-H2u obtained from Jackson Laboratory; B10.PL-RAG-1$^{-/-}$, B10.PL-MBP-TCR-Tg, and B10.PL-RAG-1$^{-/-}$ MBP-TCR-Tg mice developed by J Lafaille; B10.PL-H2u-Thy1a,Igha,Gpi1a obtained from breeding B10.PL-H2u mice with C57BL/6-Thy1a,Igha,Gpi1a, selecting the Thy1a,Igha,Gpi1a+H2u+ offspring to start a new
Material and methods

colony. B10.PL were kept in specific pathogen-free conditions in the production room and subjected to periodic control of Helicobacter infection. B10.PL-RAG-1−/−, B10.PL-MBP-TCR-Tg and B10.PL-RAG1−/− MBP-TCR-Tg mice were known to be Helicobacter positive for at least 6 months prior to the start of the experiments. Re-derivation of the RAG-1−/− colony was performed by embryo transfer as reported in Labosky et al. (1994).

For all experiments, donor and recipient mice were bred and maintained in the animal house and used between 3.5 and 10 weeks of age. All the animals were weighed regularly and the presence of diarrhoea was noted in each cage. Mice experimental protocols were approved by the institutional ethical committee as well as the Portuguese veterinary general division.

2.2.3. Experimental agents

2.2.3.1. Hydrocortisone

In accordance with the objective of this work, to study whether a period of CS-induced lymphopenia could lead to AID in the context of a Treg aggression, the type of GC formulation, dose and duration of therapy had to be defined. These choices were made in an attempt to both approximate the experimental background as near as possible to the clinical setting and suit the experimental model chosen, taking into account the following:

a) The patient that suffers a viral infection, undergoes a short course of GC or is under maintenance GC therapy is not severely lymphopenic as opposed to patients that are under high dose steroids (Lionakis and Kontoyiannis, 2003) implying that the degree of IS with these forms of intercurrent illness or therapy is mild.

b) Studies such as those by Chen et al. (2004) and Chen et al. (2006), that report that steroids increase Treg number in vivo, utilize DEX in dosages far superior to those that are used in maintenance therapeutic courses in humans and act within the boundaries of non-genomic effects. Of note, the increase in Treg attributed to high dosages of potent steroids is a theoretical disadvantage in cancer treatment.

c) An immunomodulatory effect on Treg may be masked by drugs with a high anti-inflammatory effect such as DEX or MP, precluding the utilization of encephalomyelitis as a clinical readout of AID.

Hydrocortisone was chosen as the GC to be studied. It is one of the least potent GC and frequently used for human treatment. A study on the effect of GC administration abrogating the refractory phase of EAE induction (Reder et al., 1994) provided the rational for the 5 day duration of therapy and for the chosen titration dosages of HC. It should be noted that the MC side effects of HC are irrelevant in short term therapy and that after dose adjustment, HC has a very similar
anti-inflammatory potency to prednisolone. In order to find a critical dose to be used in this study, the dose of HC was titrated to verify its effect on lymphocyte number, naïve and Treg subsets in wt mice. HC 0.5 mg/kg/day, HC 5 mg/kg/day (HC5) or HC 50 mg/kg/day (HC50) were injected intra-peritoneally (i.p.), in a final concentration of 10, 100 or 1000 μg /100 μl, for 5 days (D-5 to D-1) respectively and compared to controls injected with PBS. Mice were analyzed 12 to 24 hours post last administration (D0). During the time period that these titrations were undertaken, Foxp3 antibody staining was not commercially available and as such, CD25 expression was used to define the Treg subset. The results of this titration are shown in point 2.3.1.1. of the present study. The dose of 5 mg/kg/day of HC was used in further assays (injected i.p.) in a final concentration of 100 μg/100 μl for 5 days.

Hydrocortisone ampoules, commercialized for intra-venous (i.v.) use, contained 100 mg of hydrocortisone sodium succinate in powder form and were reconstituted with sterile water (Rapicort, Pharmis Biofarmaceutica, Portugal). The integrity of the HC preparation was evaluated through comparison with Dexamethasone (DEX). In order to compare the effect of HC with previously reported protocols, DEX 5 mg/kg was tested (point 2.3.1.1. of the present study) and found to have a similar effect to HC in a dose of 125 mg/kg – an equivalent GC potency to DEX 5 mg/kg - on D-1. DEX ampoules, commercialized for i.v. use, contained 5 mg/ml of dexamethasone sodium phosphate in liquid form (Oradexon Organon, Holland).

In order to check for a possible interaction between HC and Treg mediated control of physiological auto-antibody production, wt Balb/c female mice (eight weeks old) were subjected to HC5x5 and compared to controls (n=5 per group). The mice remained healthy throughout. Sera collected prior to HC5x5 and serially every two weeks for three months was tested for anti-thyroglobulin, anti-DNA, anti-histone and anti-myosin antibodies by ELISA. One out of five mice in the HC5x5 treated group, transiently developed a high titre of anti-thyroglobulin antibody which became undetectable after 3 months, compared to none in control group. However these results could not be reproduced in a further group of 10 mice, tested over a six month period (results not shown).

To test whether HC had an effect on Treg mediated control of antibody production in NZM mice, that spontaneously develop a disease similar to human lupus, six-week old mice were treated with HC5x5 once and compared to a PBS treated control group (n=5 in each group). These mice were monitored every week for proteinuria and bled every two weeks for anti-DNA Ab, anti-myosin Ab, anti-Histone Ab, anti-Scl-70 Ab and Substance P Ab as well as IgG determinations by ELISA. No significant differences were found in antibody titres between the different groups. However, at 40 weeks of age, the group of mice to which the 5 day course of HC
had been administered 34 weeks previously had a 20 % mortality compared to 80% in the PBS treated group and upon confirmation, the explication for this phenomenon should be pursued.

2.2.3.2. Cyclophosphamide
Cyclophosphamide was obtained from Sigma, prepared in 0.9% normal saline at 33 mg/ml and administered i.p. once, at a concentration of 200 mg/kg.

2.2.3.3. Pertussis toxin
Experimental autoimmune encephalomyelitis (EAE) is an animal model that recapitulates many features of MS. It can be induced by immunization of susceptible animals with a number of myelin antigens including myelin basic protein (MBP) (Zamvil et al., 1986), proteolipid protein (PLP) (Tuohy et al., 1989) and myelin oligodendrocyte glycoprotein (MOG) (Mendel et al., 1995) or occur spontaneously or with minimal induction, in genetically manipulated mice with T cell populations with a TCR specific for one of these antigens. Ptx is produced by Bordetella pertussis, the bacterium responsible for whooping cough in humans. For long it has been known to facilitate the immunization-induced EAE in wild type (wt) mice (Lee and Olitsky, 1955) and to induce encephalomyelitis in TCR transgenic mice (Governan et al., 1993; Lafaille et al., 1994). Its disease-enhancing properties are not restricted to CNS autoimmunity, but also apply to other animal models of autoimmunity such as orchitis (Kohno et al., 1983) or inflammatory myopathy (Hart et al., 1987).

In the present work, the choice of the correct dose of Ptx to establish whether different forms of immunosuppression enhanced a Ptx effect proved to be fraught with difficulties. Environmental changes related to the animal house and a technical problem with Ptx toxin had far reaching consequences. These are herewith explained in order to justify why some of the experimental results obtained with the use of Ptx (Zelenay et al., 2005) differ substantially from the present results.

Experimental autoimmune encephalomyelitis induction in anti-MBP TCR Tg mice was first attempted with Ptx alone from Sigma–Aldrich, injected i.v. (200 ng per mouse) on D0 and 48 hours later (D2). EAE induction in anti-MBP TCR Tg mice (TR+) with Ptx (Sigma Aldrich), compared to PC61 alone or in combination with PC61 or HC is detailed in Table 2.IV.
Table 2.IV - EAE induction in anti-MBP TCR Tg mice (TR+) with Ptx (Sigma-Aldrich), compared with PC61 alone, or in combination with PC61 or HC.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Helicobacter positive - TR+</th>
<th>Helicobacter negative - TR+</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>% EAE</td>
<td>% Diarrhoea</td>
</tr>
<tr>
<td>Ptx alone</td>
<td>6 (n=2/37)</td>
<td>14 (n=5/37)</td>
</tr>
<tr>
<td>PC61</td>
<td>10 (n=2/19)</td>
<td>0 (n=0/19)</td>
</tr>
<tr>
<td>Ptx + PC61</td>
<td>50 (n=15/30)</td>
<td>46 (n=14/30)</td>
</tr>
<tr>
<td>Ptx + HC</td>
<td>14 (n=6/49)</td>
<td>8 (n=4/49)</td>
</tr>
</tbody>
</table>

Ptx also induced diarrhoea (a previously unreported effect). As part of the animal house surveillance program, faecal matter was collected and analyzed by PCR for *Helicobacter* species, as described by Bourgade *et al.* (2004). Healthy TR+ mice were found to be colonized with *Helicobacter* in the gut, a bacteria known to facilitate the induction of Inflammatory Bowel Disease (IBD) in experimental models. The cause of diarrhoea was not investigated through histological analysis but the presence of *Helicobacter* suggests the possibility that the diarrhoea was due to IBD. In these circumstances, Ptx alone induced more diarrhoea than EAE.

Similarly to what is already reported (Laflaile *et al.*, 1994), immunization with a CFA-peptide mixture and Ptx (Sigma-Aldrich), was found to induce EAE in 80% of TR+ mice (Fig. 2.1). Immunization consisted of 100μl (50 μl in each flank) of a mixture containing 100μg of N-terminal Ac1-17 MBP14 (MedProbe) emulsified in CFA and containing 400μg of *Mycobacterium tuberculosis* H37Ra (both from Difco). Pertussis toxin (Sigma-Aldrich) was administrated i.v. in two doses of 200 ng with a 2-day interval. The fact that the Ptx when administered in conjunction with MBP and CFA effectively induced EAE demonstrates that this lot of toxin from Sigma behaved as expected and is reassuring of the legitimacy of the results. Immunization of TR+ mice with CFA and peptide without Ptx does not trigger EAE (Zelenay, personal communication).

HC had a very minor effect on baseline Ptx-induced EAE in *Helicobacter*+ TR+ mice. Upon re-derivation of the TR+ mice no further diarrhoea was observed in *Helicobacter* free TR+ mice and the effect of HC upon the incidence of Ptx-induced EAE rose from 17 to 60%. This represents a single experiment and is not reproducible, as presented in the results point 2.3.3.3.2. of the present work. Soon afterwards, TR+ mice were again found to be *Helicobacter* positive as a result of which routine treatment with an antibiotic (bactrim 10 mg/L) in the drinking water was instituted, and no further diarrhoea was observed. The continuous administration, during a 3 month period, of bacon flavoured food mixed with amoxicillin (3 mg), metronidazol (0.69 mg) and bismuth (0.185 mg) per food tablet failed to clear *Helicobacter* infection.
Material and methods

Importantly, the data presented in the results section, pertaining to the use of Ptx alone or in combination with other agents on TR+ mice, was obtained under bactrim antibiotic therapy in the water. The experimental use of steroids may have contributed to the unusual susceptibility of the rodent B10.PL wt colony to Helicobacter infection.

Cumulative EAE score

<table>
<thead>
<tr>
<th>Weeks post MBP + CFA + Ptx (EAE = 16/20)</th>
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<td>0</td>
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Mean EAE score

<table>
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<th>Weeks post MBP + CFA + Ptx (EAE = 16/20)</th>
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<td>0</td>
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<td>0</td>
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</table>

Figure 2.1

EAE incidence and mean EAE score after MBP, CFA and Ptx immunization in TR+ mice

Immunization consisted of 100 μl (50 μl in each flank) of a mixture containing 100μg of N-terminal Ac1-17 MBP14 (MedProbe) emulsified in CFA and containing 400μg of Mycobacterium tuberculosis H37Ra (both from Difco). Pertussis toxin (Sigma-Aldrich) was administrated i.v. in two doses of 200 ng with a 2-day interval (n=20).

The experimental set up was subsequently complicated by the fact that a batch of Sigma Ptx consistently failed to induce EAE in TR+ mice and this failure of a particular batch of Ptx occurred even in the classical EAE induction model (Chora, personal communication). As a result, Ptx was acquired from another source namely LBL (List Biological Laboratories, Campbell, USA). LBL Ptx was more potent than Ptx from Sigma-Aldrich and was therefore titrated (results presented in point 2.3.1.2. of the present work). Ptx (LBL) in a dose of 200 ng, administered once, was chosen as the dose that induced EAE in 50% of the mice and therefore against which the effect of other interventions was to be measured. This dose was used in all further experiments presented in points 2.3.3.2. and 2.3.3.3 of the present work.

2.2.3.4. Anti-CD25 monoclonal antibody

CD25 cell depletion was performed with 200 μg of anti-CD25 monoclonal antibody (mAb) (clone PC61) injected i.p. This antibody has been shown to result in the depletion of a large fraction of
Foxp3+ CD4+ Treg (Stephens and Anderton, 2006; Zelenay and Demengeot, 2006). A single injection of CD25 depleting mAb in healthy TR+ mice did not lead to significant disease; however, when combined with Ptx administration, the incidence of Ptx induced EAE increased from 6 to 50%. As EAE did not progress beyond level 2 this was interpreted to represent the regulatory presence of CD25- T cells in TR+ mice, preventing EAE progression (Zelenay et al., 2005).

2.2.3.5. Irradiation
Gamma irradiation was obtained from a Cesium-137 source (GAMMACELL 2000 irradiator; Molsgaard Medical, Denmark). The dose of irradiation was titrated (point 2.3.1.3. of the present work) and a single dose of 50 RAD was considered to have a similar effect to HC5x5 on the basis that it caused similar T lymphocyte depletion and did not selectively reduce CD25 expression in the CD4+ pool neither in the thymus nor in the spleen.

2.2.4. Cell recovery, antibodies and flow cytometric analysis
Cell suspensions from spleen and pooled inguinal, axillary, brachial and mesenteric lymph nodes were prepared, stained and washed in PBS containing 2% FCS and 0.01% sodium azide. Analyses were performed inside a live lymphocyte gate on a FACSCalibur (Becton Dickinson) by using CELLLQUEST software. Live lymphocyte counts were deduced from the acquisition of a fixed number of 10-μm latex beads (Coulter) mixed with a known volume of unstained cell suspension.

Allophycocyanin (APh), CyChrome-, Phycoerythrin (PE)- and PercP- conjugated anti-CD4 mAb (clone RM4-5), APh and PE- anti-CD8 mAb (YTS169.4), CD45RB-PE (clone 16A), Thy1.2-biotin (clone 53-2.1), Thy1.1-PE (clone OX-7) were obtained from BD Biosciences. Anti-MBP TCR clonotype (3H12)-biotin, Thy1.1 (CD90.1) biotin (clone 19E12) and Alexa Fluor TM 488-CD25 (clone PC61) were in-house produced. Biotinylated antibodies were revealed with streptavidin–APh (BD). Anti-CD103-biotin (2E7), Fluorescein (FITC)-conjugated anti-CD62L-(MEL-14), anti-Foxp3-FITC and -PE mAb (FJK-16) were purchased from eBbioscience and intracellular Foxp3 staining was performed according to the manufacturer’s instructions. The anti-MBP TCR clonotypic mAb (3H12) stains MBP-specific T cells from the TCR transgenic mice. It only recognizes the TCR when a transgenic encoded α chain is paired with transgenic-encoded β chain and not when a Tg chain is paired with a non-Tg chain or vice-versa (Olivares-Villagomez et al., 1998).
2.2.5. Cell purification and transfer

Total pooled inguinal, axillary, brachial and mesenteric lymph nodes and erythrocyte-lysed splenocytes were stained with anti-CD4 (L3T4) microbeads and positively separated by magnetic activation cell sorter (MACS) on LS columns (beads and columns from Miltenyi Biotec). Purity was routinely over 94% for MACS sorted CD4+ T cells. Pooled lymph nodes (Ln) were stained with a mixture of anti-CD4-APh or anti-CD4-CyChrome, CD45RB-PE and CD25-Alexa mAbs or anti-CD4-APh, Thy1.1 PE and CD25-Alexa mAbs and sorted on a MoFlo High Speed Cell Sorter (Cytomation, Fort Collins, CO). Purity was routinely 98-100% for CD4+CD45RB<sup>high</sup>CD25<sup>−</sup> and CD4+CD45RB<sup>high</sup> T cells. Purified cells were suspended in PBS (100 μl per mouse) and injected in the retro-orbital plexus.

2.2.6. Cell cultures and suppression assay

All cultures were set in RPMI-1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 g/ml streptomycin, 50 g/ml gentamycin, 50 μm 2β-ME, 10 mM Heps, and 1 mM sodium pyruvate (all from Life Technologies). IL-2 from X63-IL2 cell supernatant (~10 U/ml) was diluted at 1/500. Erythrocyte-depleted splenocytes were irradiated at 30 Gy and used as a source of APC. CD4+CD25<sup>−</sup> cells (target cells) were plated at 2.5x10^4 /well in U-shape 96-well plates together with 10^5 APC and 0.5 g/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 days. All proliferations were monitored by addition of [³H] thymidine (1 Ci/well; Amersham Biosciences) for the last 6 h of culture.

2.2.7. Histological evaluation

The colon was removed from mice six to eight weeks after T cell reconstitution and fixed in 10% formalin. Paraffin-embedded sections were cut and stained with hematoxylin and eosin (H&E) for assessment of morphology. Inflammation was scored in a blinded fashion, on a scale of 0–9 as described (Liu et al., 2000). Mice were perfused with PBS followed by 10% formalin after which the brain and spinal cord were removed, embedded in paraffin and prepared with hematoxylin and eosin or with luxol fast blue (LFB) stains.
2.2.8. Statistical methods

Statistical analysis was performed with GraphPad prism 4.0 (GraphPad Software Inc, San Diego, California). Statistical significance was determined using the one-tailed Student’s t test, the Mann Whitney test and the Chi-square exact test. P<0.05 was considered significant (*, P<0.05; **, P<0.01; ***, P<0.001). Results were expressed as mean ± SD in all figures.

2.3. Results

2.3.1. Titrations

2.3.1.1. Hydrocortisone

We first performed a titration of hydrocortisone in order to establish a dose which would: (i) cause significant thymic atrophy and peripheral Ln lymphopenia but allow for the preservation of lymphocytes for functional studies and cell transfers; (ii) act within the boundaries of genomic effects of steroids and (iii) approximate the type of CS and dosages that are used in low dose chronic maintenance or short term human therapies.

The higher the dose of HC, the more thymic atrophy was observed (Fig 2.2 A) and, as expected, the major effect of HC was a decrease in the frequency and number of DP CD4+CD8+ thymocytes, accompanied by a relative increase in the frequency of SP CD4+ thymocytes which were nevertheless significantly reduced (Fig. 2.2 B, C and D). While the frequency of SP CD4+CD25+ thymocytes was maintained with increasing doses of HC, their number decreased significantly (Fig. 2.2 E). After HC, there was no selective decrease in Treg, on the contrary, the ratio between SPCD4+CD25- and SPCD4+CD25+ was slightly lower (Fig. 2.2 F), indicating that Treg in the thymus were, in fact, relatively spared. In the Ln, HC caused peripheral CD4+ T cell lymphopenia, however, no significant effect on splenic lymphocytes could be evidenced (Fig. 2.2 G and H).
Results

Fig. 2.2 (A-H)

**Thymocytes (x 10^-7)**

![Graph showing thymocyte counts for C, HC5, and HC50](image)

**Figure 2.2 A - Number of thymocytes**

![FACS plots of CD4 and CD8 populations](image)

**Figure 2.2 B - FACS plots of CD4 and CD8 populations in the thymus indicating frequencies (mean and SD) of DP and SP CD4+ thymocytes**

**Figure 2.2 C - Frequency and number of DP thymocytes**

continues
Effect of steroid treatment on the number and function of regulatory T cells in mice

Fig. 2.2 continued

**Figure 2.2 D** - Frequency and number of SP CD4⁺ thymocytes

**Figure 2.2 E** - Frequency and number of SP CD4⁺CD25⁺ thymocytes

**Figure 2.2 F** – Number of SP CD4⁺CD25⁻ and SP CD4⁺CD25⁺ thymocytes with fold difference indicated in the graph
**Fig. 2.2 continued**

**Figure 2.2 G** - Number of lymphocytes in Ln and Sp

**Figure 2.2 H** - Number of CD4⁺ T lymphocytes in Ln and Sp

**Figure 2.2**

**Hydrocortisone titration: effect on thymus, lymph nodes and spleen**

B10.PL wt mice were subjected to 5 daily i.p. injections of HC5 (n=3) and HC50 (n=3) from days D-5 to D-1, analyzed one day post last injection (D0) and compared to PBS injected controls (C) (n=6). Graphs indicate means and bars are SD; unpaired t test (Mann Whitey): * < 0.05; ** < 0.01; *** < 0.001. Shown are results representative of 2 separate experiments.

When comparing HC5x5 with HC50x5 in the Ln or Sp there was no selective effect over the ratios of (i) CD4⁺CD25⁻ to CD4⁺CD25⁺ T cells (Fig. 2.3 A), (ii) CD25 expression in the CD45RBlow and RBhigh sub-populations (Fig. 2.3 B) and (iii) CD25 expression in the CD62Lhigh subset (Fig. 2.3 C) indicating no selective effect over activated T cells or Treg. Shown are the frequencies of CD25⁺ CD4⁺ T cells in the CD45RBlow and CD45RBhigh subsets (Fig. 2.3 D) and CD103 expression in CD4⁺CD25⁻ and CD4⁺CD25⁺ T cells (Fig. 2.3 E). Based on these results, HC5x5 was chosen for this study.
Effect of steroid treatment on the number and function of regulatory T cells in mice

Figure 2.3 A - Fold differences between the number of CD25 negative and positive populations

Figure 2.3 B - Fold differences between number of CD45RB high and low expressors

Figure 2.3 C - Fold differences between number of CD62L high expressors in the CD25 negative and positive populations

continues
Figure 2.3 D- Representative FACS plots indicate CD45RB and CD25 expression (mean and SD) in CD4+ T cell pool and CD25 expression in the CD45RB high and low populations in pooled Ln from HC5 (D0) vs control (C)
Effect of steroid treatment on the number and function of regulatory T cells in mice

Figure 2.3

Hydrocortisone titration
Effect on naturally activated and naïve T lymphocytes in Ln and Sp. B10.PL wt mice were subjected to 5 daily i.p. injections of HC5 (n=2) and HC50 (n=1) from D-5 to D-1, analyzed on D0 and compared to a PBS injected control (C) (n=1), Fig. A-D.

In humans, Dexamethasone (DEX) is not used in short treatment courses but in mice it is the CS preferred for experimentation on the basis that it has a high affinity for the GC receptor and low plasma binding. However, it has a powerful anti-inflammatory and IS effect, as a result of which it was considered to be too potent and out of clinical context as previously explained. It is however in studies that use DEX in a dose of five mg/kg, administered once, in alternate days or consecutively over the course of five days that, in the thymus and in the spleen, a relative sparing of Treg by steroids has previously been claimed (Chen et al., 2004; Chen et al., 2006).

Testing the effect of DEX (5 mg/kg) upon Treg confirmed that it had an effect similar to HC 125 mg/kg (an equivalent dose) and, indeed, while decreasing the number of CD25 expressing CD4+ T cells, both increased their frequency and had a mild sparing effect on CD4+CD25+ in the periphery and in the thymus as reflected by a decrease in ratio between the number of CD4+ CD25- and CD25+ CD4+ T cells (Fig. 2.4 A and B). In this titration, once again, the lower dosages of HC had a sparing effect on thymic CD4+CD25+ T cells. In a separate experiment, DEX (5 mg/kg) administered once on D-1 or 3 times, on D-5, D-3 and D-1, increased
the frequency of SP CD4⁺CD25⁺ thymocytes from 6 ± 0.1 (control n=4) to 9 ± 1% (n=3) and 11 ± 2% respectively. The fact that equivalent potencies of HC and DEX are comparable in what concerns their immunosuppressive effect is reassuring of the quality of the HC preparation. The effect of higher doses of steroids on Treg supports the importance of finding a critical dose in this study protocol.

2.3.1.2. Pertussis toxin

There is no uniformity in the dosages and brands of Ptx that are used for EAE induction in the TR⁺ mice. Even though EAE has actually been shown to be induced by Ptx in the TR⁺ mice used in this study (Lafaille et al., 1994; Marusic and Tonegawa, 1997), there is no information available as regards a minimal or standard dose of Ptx that induces EAE.

Figure 2.4 A – Representative FACS plots indicating CD4 and CD8 populations showing frequencies of DP and SP CD4⁺ thymocytes. CD25 expression in SPCD4⁺ T cells in the thymus and CD4⁺ T cells in the Ln and Sp are indicated (mean and SD).

continues
Effect of steroid treatment on the number and function of regulatory T cells in mice

Figure 2.4 continued

**SPCD4+ thymocytes (x 10^-6)**

wt mice

**CD4+ (x 10^-6)**

**CD4+ (x 10^-7)**

Figure 2.4 B - Graphs represent numbers of SP CD4+ T cells in the thymus and CD4+ and CD4+CD25+ T cells in the Ln and Sp, with fold differences indicated.

Figure 2.4

Comparison of hydrocortisone with dexamethasone

B10.PL wt mice, were subjected to 5 daily i.p. injections of PBS (n=2), HC5 from D-5 to D-1 (n=3), HC50 from D-5 to D-1 (n=3), HC 125 mg/kg once (HC 125) on D-1 (n=3) or DEX 5 mg/kg once D-1 (n=3) and analyzed on D0. Graphs indicate means and bars are SD; Inguinal, axillary, brachial and mesenteric Ln were pooled.

LBL Ptx was found to be more potent than Sigma Ptx as the same dosages of toxin, 200 ng two days apart, increased EAE induction in TR+ from 6% (point 2.2.3.3) to 52% (Fig. 2.5 A). The incidence of EAE was proportional to the dose of Ptx (Fig. 2.5 B). Ptx (LBL) in a dose of 200 ng, administered once, was chosen as the dose that induced EAE in 50% of the mice as it allows
monitoring of the beneficial or deleterious effects of other interventions. A higher incidence of Ptx induced EAE in TR+ mice would likely mask any synergistic effect upon various interventions. This dose of Ptx was used in all further experiments presented in points 2.3.3.2 and 2.3.3.3 of the present study.

---

**Figure 2.5 A** - Cumulative incidence of EAE, defined as EAE level > 1, plotted according to the dosages and number of administrations of LBL Ptx

**Figure 2.5 B** – A snapshot of EAE incidence at 4.5 weeks after the first Ptx injection

**Figure 2.5**

**Titration of LBL pertussis toxin**

TR+ mice were injected with increasing dosages of Ptx (LBL). Dosages and number of injections indicated in the graphs.
2.3.1.3. Irradiation

Irradiation causes lymphopenia and the dose of irradiation was titrated in wt mice to verify its effect on lymphocyte number, naïve and Treg subsets, in order to choose a single dose which would cause a similar effect to HC5x5. Just like for the HC titration, CD25 expression was used to define the Treg subset. Mice were irradiated with 25, 50, 100, 200 or 300 RAD once on D-2, analyzed on D0 and compared to sham irradiated controls. As shown in Fig. 2.6 A, B and C, a single dose of 50 RAD was considered to have a similar effect to HC5x5 and did not selectively reduce CD25 expression in the CD4+ pool neither in the thymus nor in the Sp.

![Fig. 2.6 (A – C)](image)

**Figure 2.6 A** – Representative FACS plots of CD8 and CD4 populations in the thymus indicating respective frequencies of DP and SP CD4+ T cells (mean and SD)
Results

Fig. 2.6 continued

**Figure 2.6 B** – Representative FACS plots indicating frequency of CD25 expression in SP CD4+ thymocytes and in CD4+ T cells in the Sp (mean and SD)
Effect of steroid treatment on the number and function of regulatory T cells in mice

Figure 2.6 continued

wt mice

**Thy**

**SPCD4**\(^+\) (x 10\(^{-6}\))

![Graph of SPCD4\(^+\)](image)

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**SPCD4**\(^+\) CD25\(^+\) (x 10\(^{-6}\))

![Graph of SPCD4\(^+\) CD25\(^+\)](image)

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**Sp**

**CD4**\(^+\) (x 10\(^{-6}\))

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**CD4**\(^+\) CD25\(^+\) (x 10\(^{-6}\))

![Graph of CD4\(^+\) CD25\(^+\)](image)

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Figure 2.6 C - Number of SP CD4\(^+\) and SP CD4\(^+\)CD25\(^+\) thymocytes and CD4\(^+\) and CD4\(^+\)CD25\(^+\) T cells in the Sp

**Figure 2.6**

**Titration of the irradiation dose**

A-C: B10.PL wt mice were subjected to HC5x5 on D-5 to D-1 (n=4) and progressively increasing dosages of irradiation on D-2 from 25 to 200 RAD, 2 mice per group.
2.3.2. Comparison of the immunosuppressive effect on regulatory T cell number.

2.3.2.1. Hydrocortisone

Effect of HC on wt mice:

To evaluate whether HC had an effect on Treg in the recovery period from HC induced lymphopenia, wt mice were treated with HC5x5 daily for five days after which the recovery of thymocyte and peripheral lymphocyte populations was examined at D0 and D7 after HC discontinuation. To confirm the “true nature” of the CD4+CD25+ T cell population, intracellular staining with the Ab against the Treg-specific marker Foxp3 was performed. In the present study HC caused short-lived lymphopenia with almost 100% reconstitution in Ln, Sp and thymus by 7 days post therapy (Fig 2.7 A). As shown in Fig. 2.7 B, the frequency of CD4+Foxp3+ T cells was maintained after HC5x5 at D0 and upon reconstitution (D7) in the thymus, Ln and Sp. There was no selective decrease in the number of Treg in the Ln or Sp or thymus. At D0, the ratio between SP CD4+ Foxp3− T cells and SP CD4+ Foxp3+ T cells is slightly reduced, indicating a relative sparing effect of the Foxp3+ population in the thymus as observed during the HC titration, the ratio remaining unaltered in Ln and Sp. At D7, the ratio between SP CD4+ Foxp3− T cells and SP CD4+ Foxp3+ T cells was higher than the control, indicating that, at this time point, Treg are selectively decreased in the thymus during regeneration but at this time point, the ratio between Foxp3− and Foxp3+ T cells was not altered in the Ln or Sp (Fig. 2.7 C).

Fig. 2.7 (A-C)

wt mice

Lymphocytes (x 10^-7)

Thy

Ln

Sp

Figure 2.7 A – Number of lymphocytes in Thy, Ln and Sp

continues
Fig. 2.7 continued

**Figure 2.7 B** – Representative FACS plots depicting Foxp3 frequency in SP CD4+ thymocytes and CD4+ T cells in thymus, Ln and Sp, and CD25 with Foxp3 co-expression in CD4+ T cells of Ln and Sp after HC5x5 at D0 and D7
**Fig. 2.7 continued**

**Figure 2.7 C** - Number of CD4\(^+\) T cells and CD4\(^+\)Foxp3\(^+\) T cells in thy, Ln and Sp are plotted. Ratios between these 2 populations are shown in graphs at D0 and D7.

**Figure 2.7**

**The effect of hydrocortisone on regulatory T cells in wt mice**

B10.PL wt mice, were subjected to injections of PBS (n=5) or HC5x5 from D-5 to D-1 and analyzed on D0 (n=3) and D7 (n=3) post administration. Graphs indicate means and bars are SD; unpaired t test (Mann Whitey): * < 0.05; ** < 0.01; *** < 0.001. Shown are results representative of 2 separate experiments.
Effect of HC on TR+ mice:
A HC dose titration indicated that thymic atrophy is dose dependant and mostly due to a major decrease in the frequency and number of DP thymocytes, as described for wt mice (Fig. 2.8).

![Graph of Thymocyte and DP counts for different HC doses](image)

**Figure 2.8**
Hydrocortisone titration on TR+ mice
TR+ mice were treated with PBS (n=12), HC 0.5x5 (n=2), HC5x5 (n=8) or HC50x5 (n=2) from D-5 to D-1 and analyzed on D0. Thymocyte count, frequency of DP and DP count in Thy are shown according to treatment group. Controls were pooled from 4 experiments. Graphs indicate means and bars are SD; unpaired t test (Mann Whitey): * < 0.05; ** < 0.01; *** < 0.001. Shown are results representative of 2 separate experiments.

To evaluate whether HC had an effect on Treg, TR+ mice were treated with HC5x5. At D0, there was a significant reduction in DP frequency and cell number and, as expected, there was a corresponding increase in SP CD4+ frequency, the number of SP CD4+ thymocytes being nevertheless reduced. On D7 after HC discontinuation, the recovery of thymocytes was almost complete. The maintenance in frequency and number of SPCD4+ Foxp3+ thymocytes and the decrease in the ratio between Foxp3- and Foxp3+ SPCD4+ suggest a sparing effect on the SP CD4+ Foxp3 population on D0 as for wt mice (Fig. 2.9 A and B). TR+ mice have a smaller frequency of DP thymocytes and a smaller frequency of SP CD4+ Foxp3+ T cells (Fig. 2.9 A) than wt mice (represented in Fig. 2.6A and 2.7 B).
Results

Figure 2.9 A – Representative FACS plots of the thymus showing frequencies of DP (CD4+CD8+), SP CD4+ T cells and Foxp3 expression in SP CD4+ T cells

Figure 2.9 B - Plots of cell number, and ratio between Foxp3⁻ and Foxp3⁺ SP CD4+ T cells at D0 and D7

Thymic effects of hydrocortisone in TR⁺ mice

HC5x5 was administered to TR⁺ mice from D-5 to D-1 and compared to PBS injected control (n=3) on D0 (n=4) and D7 (n=5). Graphs indicate means and bars are SD; unpaired t test (Mann Whitey): * <0.05; **<0.01; ***< 0.001.
In TR+ mice both the non-Tg and Tg T cells contribute to the Treg pool, as CD4+Foxp3+ Treg are encompassed in these lymphocyte populations. As previously discussed, Treg in the Tg population are more potent suppressors than those in the non-Tg pool (Hori et al., 2002a) but the latter are more numerous. It is therefore important to look at Treg in both non-Tg and Tg populations because there remains the possibility that a mild reduction in Foxp3 in the former group could impair tolerance by as much as a large reduction in the latter.

At D0, despite a reduction in the frequency and number of non-Tg SP CD4+ thymocytes with only partial recovery at 7 days post HC (Fig. 2.10 A), there was an increase in the frequency of non-Tg SP CD4+ thymocytes that express Foxp3+ (Fig. 2.10 B). As such, the number of Foxp3+ T cells is maintained in the thymus (Fig. 2.9 B), contrasting with the selective increase at D0 observed in wt mice (Fig. 2.7 C). The predominant effect of HC5x5 was on non-Tg thymocytes.

In the periphery HC does not affect the frequency, the number or ratio between non-Tg and Tg T cells (Fig. 2.10 C) or the frequency and number of Foxp3+ T cells in the Tg and non-Tg pools (Fig. 2.10 D). Furthermore, HC does not affect the ratio of activated to naïve T cells in the non-Tg and Tg T cell pools as determined by L-selectin (Fig. 2.10E) and CD45RB expression (Fig. 2.10 F). Foxp3 expression is also not altered in CD45RBlow non-Tg or Tg T cell pools (Fig. 2.10 G).

2.3.2.2. Cyclophosphamide alone or in combination with hydrocortisone

Effect on wt mice:

Before testing the effect of Cyp in TR+ mice, its effect on Treg defined by Foxp3 expression was established in wt mice. In clinical therapy, HC and Cyp are often used together and therefore it was considered meaningful to study the effect of Cyp alone or in combination with HC. Cyp 200 mg/kg given once, 5 days before analysis, caused significant thymic atrophy in wt mice due to DP and SP CD4+ thymocyte loss (Fig. 2.11 A and B). This is the dose used by Brode et al. (2006) in their demonstration that Cyp selectively reduces Treg. Despite a significant reduction in the number of Foxp3 expressing SP CD4+ thymocytes, the ratio of Foxp3− to Foxp3+ SP CD4+ thymocytes actually decreased implying that Treg were relatively spared (Fig. 2.11 C). In other words, Foxp3+ SP CD4+ thymocytes were proportionally less reduced than the total SP CD4+ population, showing the same tendency as in the HC treatment group.
**Results**

Fig. 2.10 (A-G)

**Figure 2.10 A** – Representative FACS plots of non-Tg (3H12⁻) and Tg (3H12⁺) SPCD4⁺ thymocytes indicating frequencies of non-Tg SPCD4⁺ T cells (mean and SD). Number of non-Tg and Tg SPCD4⁺ thymocytes are plotted.

**Figure 2.10 B** – Representative FACS plots showing the frequencies of Foxp3⁺SPCD4⁺ thymocytes in the non-Tg and Tg pools.

continues
Fig. 2.10 continued

**Figure 2.10 C** – Representative FACS plots showing frequencies and plots of numbers of Tg and non-Tg T cells and ratio between Tg and non-Tg T cells in Ln and Sp.


**Results**

Fig. 2.10 continued

![Graph showing the number of CD4+Foxp3+ and CD4+CD25+Foxp3+ T cells in non-Tg and Tg populations](image)

2.10 D – Representative FACS plots and number of CD4+Foxp3+ T cells and CD4+CD25+Foxp3+ T cells in the non-Tg and Tg populations
Fig. 2.10 continued

**Figure 2.10 E** - FACS histograms showing overlapping recovery frequencies of L selectin in Foxp3 positive and negative population in the Ln and Sp

Fig. 2.10F - Representative FACS plots of the CD45RB expression in CD4+ T cells in the Ln and Sp with numbers of each subset in the non-Tg and Tg CD4+ T cells pools.

**TR+ mice**

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**D0**

**D7**

CD62L

**CD45RB**

**CD4**

**C**

**D0**

**D7**

**3H12-**

**3H12+**

**High**

**low**

**High**

**low**

**High**

**low**

**C**

**D0**

**D7**

**3H12-CD4+ (x 10^-5)**

**3H12+CD4+ (x 10^-5)**

**3H12-CD4+ (x 10^-5)**

**3H12+CD4+ (x 10^-5)**
**Results**

**Fig. 2.10 continued**

The effect of hydrocortisone on the cellular composition of TR⁺ mice

HC5 was administered to TR⁺ mice from D-5 to D-1 and analyzed on D0 (n=2) and D7 (n=2) compared to PBS injected TR⁺ controls (n=2). Graphs indicate means and bars are SD; unpaired t test (Mann Whitey): * <0.05; **<0.01; ***< 0.001.
Despite the fact that Cyp caused a rise in CD4\(^+\) T cell frequency in Ln and Sp, due to a selective decrease in B lymphocytes - an effect of Cyp previously known (Turk et al., 1972) – the number of CD4\(^+\) T cell in the Ln and in the Sp were significantly reduced compared to controls (Fig. 2.11 D). In contrast to the effect on SP CD4\(^+\) T cells in the thymus, Cyp significantly and selectively decreased the frequency and the number of CD4\(^+\)Foxp3\(^+\) (Fig. 2.11 E) and CD4\(^+\)Foxp3\(^+\)CD25\(^+\) T cells with no effect on CD25 expression in Foxp3\(^+\) T cells (Fig. 2.11 F) in the Ln and Sp. This resulted in a near duplication of the ratio between Foxp3\(^-\) and Foxp3\(^+\) T cells in relation to the control (Fig. 2.11 G). The combination of Cyp and HC results in changes similar to Cyp alone, in the thymus and in the periphery but further exacerbates the increase in the ratio between the Foxp3 negative and positive populations in the periphery.

**Figure 2.11 (A – G)**

![FACS plots](image)

*Figure 2.11 A - FACS plots of the CD4 and CD8 populations in the Thy showing the frequency of DP and SP CD4\(^+\) T cells and the frequency of SP CD4\(^+\)Foxp3\(^+\) thymocytes*
**Results**

**Figure 2.11 continued**

wt mice

**Thymocytes (x 10^-7)**

**DP (x 10^-7)**

**SP CD4+ (x 10^-6)**

![Graphs](image-url)

**Figure 2.11 B** – Number of thymocytes, DP and SP CD4+ thymocytes

**SP CD4+ Foxp3+ (x 10^-5)**

**SP CD4+ (x 10^-6)**

![Graphs](image-url)

**Figure 2.11 C** - SP CD4+Foxp3+ counts and ratios between Foxp3- and Foxp3+ SP CD4+ T cells

continues
Effect of steroid treatment on the number and function of regulatory T cells in mice

Figure 2.11 continued

**Figure 2.11 D** - Frequency and number of CD4+ T cells in Ln and Sp

**Figure 2.11 E** - Number of CD4+Foxp3+ T cells in the Ln and Sp continues
Figure 2.11 continued

**Figure 2.11 F** - FACS plots indicating Foxp3 expression in CD4+ T cells, co-expression of Foxp3 and CD25 and the frequency of CD25+ T cells within the Foxp3+ T cell population in Ln and Sp.
**Figure 2.11 continued**

![Graph](image-url)

**Figure 2.11 G - Ratio between Foxp3⁻ and Foxp3⁺ T cells in the Ln and Sp**

**The effect of cyclophosphamide on regulatory T cells in wt mice**

Cyclophosphamide 200 mg/kg was administered on D-5 to B10.PL wt mice (n=3), alone or in combination with HC5x5 D-5 to D-1 (n=3) and compared to HC5x5 (n=3) or PBS injected controls (n=3). Graphs indicate means and bars are SD; Unpaired t test (Mann Whitey): *<0.05; **<0.01; ***<0.001.

**Effect on TR⁺ mice:**

In TR⁺ mice, Cyp 200 mg/kg given once, 5 days before analysis caused significant thymic atrophy predominantly in DP thymocytes, with no selective reduction in the frequency or number of Foxp3 expressing SP CD4⁺ thymocytes (Fig. 2.12 A and B). Of note, the addition of HC accentuates the effect of Cyp on the thymic cellularity and effectively reduces SP CD4⁺ Foxp3⁺ T cells (Fig. 2.12 A and B).

In the periphery, as expected, there is a significant increase in CD4⁺ T lymphocytes frequency as a result of a selective reduction of B lymphocytes. Consequently the number of CD4 was not significantly reduced, despite a significant reduction of spleen cellularity (Fig. 2.12 C). Splenic CD4⁺Foxp3⁺ T cells frequency and number are significantly decreased, and selectively reduced in relation to the CD4⁺ population, by a factor of 6 (Fig. 2.12 D). The significant increase in CD4⁺T cell frequency is reflected in a less severe decrease of the Tg T cells (Fig. 2.12 E). Foxp3 frequency and cell number decreases selectively in both Tg and non-Tg compartments, slightly more so in the latter than in the former (Fig. 2.12 F). The combination of Cyp and HC
Results

results in changes not different from Cyp alone. In conclusion, Cyp alone and in combination with HC selectively reduces the non-Tg population and Foxp3 expression in the TR⁺ mouse.

Figure 2.12 (A – F)

**Figure 2.12 A** – Representative FACS plots of the CD4 and CD8 populations in Thy, showing the frequency of DP, SP CD4⁺ and SP CD4⁺ Foxp3⁺

**Figure 2.12 B** - Thymocyte, DP, SP CD4⁺, SPCD4⁺Foxp3⁺ cell counts, frequency and number of Foxp3 expressors in SPCD4⁺ thymocytes showing fold differences from control

continues
Effect of steroid treatment on the number and function of regulatory T cells in mice

**Figure 2.12 continued**

<table>
<thead>
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<th>TR+ mice</th>
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<tbody>
<tr>
<td>Sp</td>
</tr>
<tr>
<td>Lymphocytes (x 10^6)</td>
</tr>
<tr>
<td>CD4+ (%)</td>
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<td>CD4+ (x 10^6)</td>
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<td>C</td>
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**Figure 2.12 C** - Splenocyte and CD4+ T lymphocyte frequency and number showing fold differences from control

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<tr>
<td>Sp</td>
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<td>CD4</td>
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<td>Foxp3</td>
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<tr>
<td>C</td>
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<td>3 ± 0.5</td>
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**Figure 2.12 D** – Representative FACS plots showing Foxp3 frequency in CD4+ T population in the Ln and Sp and plots of CD4+Foxp3+ T cell frequency and number in the Sp showing fold reduction from control

continues
**Figure 2.12** continued

TR+ mice

CD4+ gate

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<tr>
<td></td>
<td>2 ± 0.5</td>
<td>1 ± 0.03</td>
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<td>22 ± 1</td>
<td>42 ± 1</td>
<td>45 ± 3</td>
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Figure 2.12 E – Representative FACS plots showing the frequency of non-Tg (3H12-) and Tg (3H12+) CD4+ T lymphocytes in the Ln and Sp showing number and fold reductions from control, in the Sp

continues
Effect of steroid treatment on the number and function of regulatory T cells in mice

Figure 2.12 continued

Figure 2.12 F – Representative FACS plots showing the frequency of Foxp3+ T cells within the Tg (3H12+) and non-Tg (3H12-) populations in the Ln and Sp. Plotted are the numbers of Foxp3+ CD4+ T cells in non-Tg and Tg populations with fold reduction from control in the Sp.

Figure 2.12

The effect of cyclophosphamide alone or in combination with hydrocortisone on the cellular composition of TR+ mice

Cyclophosphamide 200 mg/kg was administered on D-5 to TR+ mice, alone (n=2) or in combination with HC5 mg/kg/day D-5 to D-1 (n=3) and compared to PBS injected controls (n=2). Graphs indicate means and bars are standard deviation. Results are representative of 2 separate experiments apart from Foxp3 which was only determined once. Unpaired t test (Mann Whitey) * <0.05; **<0.01; ***< 0.001.
2.3.2.3. Pertussis toxin alone or in combination with hydrocortisone

Ptx does not: (i) cause significant lymphopenia; (ii) alter the frequency, number or ratio between RBl^high and RBl^low T cells; (iii) reduce CD25 expression in CD4, CD45RB high and low subsets in the Ln or Sp (Fig 2.13 A and B). Ptx does not decrease thy or Ln Foxp3^+Treg (Fig 2.13 C). Ptx reduces Sp CD25^+ T cells in the CD4^+ and CD45RB^low populations shown to correspond to a reduction in CD4^+Foxp3^+ T cell frequency and cell number (Fig 2.13 A, B and C). The addition of HC to Ptx had no additional effect upon cellularity.

Figure 2.13 A – FACS plots of CD45RB expression of CD4^+ T lymphocytes and CD25 expression within the CD45RB low and high populations (mean and SD)

continues
Effect of steroid treatment on the number and function of regulatory T cells in mice

**Fig. 2.13 continued**

*Ln* wt mice

**CD4⁺ (x 10⁶)**

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<td>Ptx+HC</td>
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**CD4⁺ (x 10⁶)**

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**CD45RB³⁺ (x 10⁶)**

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**CD45RB⁺⁺⁺ (x 10⁶)**

<table>
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**CD45RB⁺⁺⁺ (x 10⁶)**

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**Sp**

*Figure 2.13 B* - FACS plots of distribution of CD45RB and CD25 expression in CD4⁺ T lymphocytes

continues
**Fig. 2.13 continued**

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<th>Gate</th>
<th>Thy</th>
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<th>Sp</th>
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<td>C</td>
<td>82 ± 1</td>
<td>4 ± 0.4</td>
<td>3 ± 0.1</td>
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<td>HC</td>
<td>32</td>
<td>7</td>
<td>4</td>
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<tr>
<td>Ptx</td>
<td>80 ± 1</td>
<td>4 ± 0.2</td>
<td>3 ± 0.2</td>
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<td>Ptx + HC</td>
<td>53 ± 2</td>
<td>5 ± 0.04</td>
<td>15 ± 1</td>
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**Figure 2.13 C** – Representative FACS plots of Foxp3+ expression in the CD4+ population in the Ln and Sp with cell counts showing fold difference between Foxp3 positive and negative population subsets

**Figure 2.13 (A - C)**

**Effect of Ptx on T lymphocyte subpopulations in wt mice**

A-B: B10.PL wt mice were subjected to HC5 from D-5 to D-1 (n=1), Ptx (LBL) 200 ng, on D-5 and D-3 (n=2), Ptx (LBL) 200 ng on D-5 and D-3 combined with HC5 from D-5 to D-1 (n=2) and compared to PBS treated control (n=2). The Thy, Ln and Sp were analysed on D0.
2.3.2.4. Irradiation alone or in combination with hydrocortisone

In TR+ just as for wt mice, irradiation with 50 RAD has an equivalent effect to HC with no selective effect on frequency (Fig 2.14 A) and number (Fig. 2.14 B) of CD25+ T cells or in CD45RB expression in Tg and non-Tg pools in Ln and Sp of TR+ mice (Fig. 2.14 C and D). This dose of irradiation does not selectively reduce SP CD4+CD25+ thymocytes causing significant thymic atrophy in TR+ mice (not shown).

Figure 2.14 A - FACS plots showing distribution of non-Tg (3H12-) and Tg (3H12+) T lymphocytes and CD25 expression within each cell pool in Ln and Sp
Figure 2.14 B - Plots of non-Tg and Tg and CD25 expressing Tg and non-Tg T cells showing ratio between Tg and non-Tg and CD25 negative and positive populations within each pool continues
**Fig. 2.14 continued**

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<tr>
<td>3H12</td>
<td>72 ± 4</td>
<td>73 ± 4</td>
<td>69 ± 6</td>
</tr>
<tr>
<td>3H12^-</td>
<td>20 ± 5</td>
<td>44 ± 20</td>
<td>27 ± 10</td>
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**Sp  | TR^+ mice**

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<tr>
<th>C</th>
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<th>Irradiated</th>
</tr>
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<tbody>
<tr>
<td>3H12</td>
<td>68 ± 8</td>
<td>68 ± 6</td>
</tr>
<tr>
<td>3H12^-</td>
<td>16 ± 1</td>
<td>34 ± 4</td>
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**Figure 2.14 C - FACS plots of CD45RB expression in non-Tg and Tg pools**

**Figure 2.14 D - Number of CD45RB low and high expressors within the non-Tg and Tg T cells showing ratios between subpopulations**

**Figure 2.14**

**Comparison of hydrocortisone and irradiation in TR^+ mice**

TR^+ mice were subjected to HC5x5, irradiation (50 RAD) or PBS injections, 2 mice per group. The Sp and pooled Ln were analyzed.
2.3.3. *In vivo* effect of immunosuppressants on regulation:

2.3.3.1. Regulatory T cell mediated protection of TR\(^{-}\) mice

As already mentioned, TR\(^{-}\) mice that are younger than 4 weeks of age are reported to be protected by a CD4\(^{+}\) transfer containing enough T lymphocytes with regulatory function (Furtado *et al.*, 2001). The smallest number of CD4\(^{+}\) T lymphocytes that has been reported to confer full protection is 3x10\(^{5}\) but after 7 weeks of follow-up, when reported data is analysed carefully, at a time when the recipients are approximately 11 weeks of age, about 20% of the recipients show signs of mild EAE (Furtado *et al.*, 2002).

In order to facilitate the detection of a change in Treg cell function from HC treated donors, 3x10\(^{5}\) MACS sorted CD4\(^{+}\) T cells were transferred to TR\(^{-}\) recipients. A high failure rate of protection was similar in both groups and independent of whether the transfer came from untreated or HC treated donors (Fig. 2.15 A).

![Figure 2.15 A](image)

**Figure 2.15 A** - MACS-purified CD4\(^{+}\) T cells were transferred to less than 4 weeks of age TR\(^{-}\) mice (3x10\(^{5}\) T cells per mouse, purity always over 94%). Plots represent outcome of transfers in both groups, namely health, EAE, diarrhoea and weight loss or a combination of the latter two, at 15 weeks post transfer. Results pooled from separate transfers in the control (untreated donors, number of transfers=6) and HC (HC treated donors, number of transfers=8) groups.
Effect of steroid treatment on the number and function of regulatory T cells in mice

Fig. 2.15 continued

**Figure 2.15 B** - Plots represent cumulative incidence and mortality and mean EAE scores along the age of TR- recipients of 3x10^5 MACS purified CD4+ T cells (purity > 96%) from Cyp 200 mg/kg once, HC5x5 and HC5x5+Cyp treated donors in comparison with recipients of CD4+ T cells from untreated donors (C) and TR- mice that did not receive a cell transfer. Numbers in legend indicate the final number of mice that developed EAE per number of animals analyzed. Results in Cyp and Cyp + HC groups pooled from 2 experiments.

**Figure 2.15 C** - Plots represent cumulative percent EAE incidence in TR- recipients of transfers of sorted 2x10^5 pooled from five experiments. Control (untreated donors); HC (HC treated donors). Sort purity of the CD25+ population was between 93% and 98%.

2x10^5 CD4+CD25+

| No transfer | EAE=38/38 |
| Cyp+HC      | EAE=6/6   |
| Cyp         | EAE=7/9   |
| C           | EAE=13/34 |
Results

Fig. 2.15 continued

**Figure 2.15 D** - Plots represent cumulative percent EAE incidence in TR⁺ recipients of transfers of 3x10⁵ sorted CD4⁺CD25⁺ T cells (one experiment, sort purity 98%). Control (untreated donors); HC (HC treated donors).

**Figure 2.15 E** – Representative FACS plot indicating the frequency of donor T cells (CD4⁺3H12⁻) in recipients from transfer in (D). Control (untreated donors); HC (HC treated donors).
Fig. 2.15 continued

![Graph](image_url)

**Figure 2.15 F** - EAE incidence in TR− mice recipients of 1x10^6 unfractioned MACS purified CD4+ T cells (purity 98.5%) isolated from donors subjected to HC5x5 (HC) or untreated (C), compared to TR− that did not receive a cellular transfer (one experiment).

**Figure 2.15**
Adoptive transfer of CD4+ and CD4+CD25+ T cells from hydrocortisone, cyclophosphamide or untreated donors to TR− mice

After a 3 month follow-up, chronic diarrhoea and visible weight loss occurred more frequently than EAE. The wasting disease was interpreted to be a form of T-cell induced colitis caused by the transferred CD4+ T cells to *Helicobacter* colonized recipients and was never present in TR− mice that did not receive a T cell transfer.

As shown in Fig. 2.15 B, when the donor was treated with either Cyp or Cyp + HC, there was a significant loss of EAE protection in the TR− mice, the score in the Cyp and HC donor treatment group resembling the incidence and score of TR− that received no transfer. Transfer with CD4+ T cells from Cyp alone treated donors induced less severe EAE and less mortality than when the course of disease was left unperturbed. The incidence and score of EAE in the Cyp alone recipient group remained stable over the next 22 weeks (follow-up not shown). The addition of HC to Cyp donor treatment increased the incidence, severity and EAE mortality in the recipient TR− mice, in comparison to Cyp alone and to the un-manipulated transfer. Transfer of ten times more cells from Cyp treated donors was performed and still failed protection from EAE (data not shown, n=3) suggesting that Cyp severely affects Treg function.
With such a high failure rate of protection with total CD4⁺ T cell transfer, it was decided to test the effect of CD4⁺CD25⁺ T cells. Previous work in our lab has shown that at 6 weeks post transfer of 2x10⁵ sorted CD4⁺CD25⁺ T cells from wt donors, 37% of TR⁺ recipients develop mild EAE (Hori et al., 2002), a failure rate slightly higher than the 20 % previously reported by Furtado et al. (2001). Similar results with 2x10⁵ CD4⁺ T cells were obtained in this time frame (Fig 2.15 C). Transfer of 3x10⁵ sorted CD4⁺CD25⁺ T cells delayed EAE onset when compared to 2x10⁵, but there was no difference between HC and the control group (Fig. 2.15 D).

There was also no difference between both groups in the frequency of recipient donor cells in the spleen at 10 weeks post transfer (Fig. 2.15 E). Significantly, the mice that received the CD25 transfer and were ill with EAE, did not progress beyond EAE score 2 in any of the groups. Transfer of 1x10⁶ MACS purified CD4⁺ T cells afforded better protection than CD4⁺CD25⁺ T cell alone but again failed to reveal any significant differences between the recipients of the HC and control groups (Fig. 2.15 F).

When TCR Tg T cells are transferred to MHC compatible RAG⁻/⁻ recipients, severe EAE is induced after 3 weeks. Co-transfer of sorted CD4⁺CD25⁺ T cells obtained from PBS or HC treated donors affords partial EAE protection but there was no statistically significant difference between HC and control at 1/1 ratio (Fig. 2.16 A). There seems to be less late protection in the HC group, when the TCRtg:CD25⁺ ratio in the transfer is increased by a factor of 3, where some mice developed mild EAE (Fig. 2.16 B).

Figure 2.16 (A-B)

![Cumulative EAE incidence graph](image)

**Figure 2.16 A** - Transfer of Tg T cells (1x10⁵), from a four week old TR⁻ mouse compared to the co-transfer with CD4⁺CD25⁺ T in a ratio of 1:1 from PBS or HC treated wt donors.
Effect of steroid treatment on the number and function of regulatory T cells in mice

2.3.3.2. Induction of encephalomyelitis in TR⁺ mice

HC and Cyp are IS drugs that are used to treat patients with multiple sclerosis and therefore EAE induction may be prevented or masked by these drugs in the anti-MBP TCR Tg mouse model. In fact, neither HC alone (Fig. 2.17 A) nor Cyp alone or in combination with HC (Fig. 2.17 B) prevent EAE in TR⁻ mice, suggesting lack of therapeutic effect in this disease model.

2.3.3.2.1. Hydrocortisone

In order to test the functional implications of the contrasting effects of Cyp and HC on Foxp3⁺ T cells, namely that Cyp causes selective Treg depletion but HC does not, TR⁺ mice were treated and observed for signs of EAE. Increasing dosages of HC5 (n=30), HC50 (n=5) or HC500 (n=5) mg/kg/day during five days do not cause EAE in TR⁺ mice (data not shown). In order to determine an effect of chronic CS therapy upon Treg three healthy TR⁺ mice were treated with HC5 everyday, for 2 months. Twelve days after HC withdrawal, 1 out of 3 mice developed EAE level 2 (data not shown). It is very likely that chronic CS therapy suppressed the hypothalamic-pituitary-adrenal axis and therefore the interpretation of this result must take into account a possible effect.
Results

of CS withdrawal, described to abrogate spontaneous EAE recovery in Lewis rats (MacPhee et al., 1989).

**Figure 2.17 A** - TR- mice treated with HC50 mg/kg every 3 days from 4.5 to 10 weeks of age compared to untouched TR- mice. The cumulative EAE incidence is plotted vs the age of the mice in weeks.

**Figure 2.17 B** - TR- mice treated with Cyp 200 mg/kg once followed by HC50 mg/kg every 3 days from 4.5 to 10 weeks of age compared to untouched TR- mice. The cumulative EAE incidence is plotted vs the age of the mice in weeks.

Figure 2.17

The effect, in TR- mice, of hydrocortisone or cyclophosphamide, alone or in combination with hydrocortisone.
2.3.3.2. Cyclophosphamide alone or in combination with hydrocortisone

Cyp alone induces EAE in TR+ mice and HC potentiates EAE induction from Cyp (Fig. 2.18 A). While the EAE induction frequency with Cyp alone is small (18%) and resulted in death (Fig. 2.18 B), this becomes significant in the light that untreated TR+ mice never developed EAE. Non-EAE related deaths in this group and in the Cyp and HC group may have prevented more mice from developing EAE (Fig. 2.18 C). It should be noted that these mice were colonized by *Helicobacter*, an intestinal pathogen that facilitates colitis and weight loss in experimental mice and which may have contributed to the wasting disease observed in the combined Cyp and HC treatment group (Fig. 2.18 D).

Fig. 2.18 (A-D)
Results

Fig. 2.18 continued

**Cumulative EAE mortality from unknown cause (%)**

- **Cyp** EAE=5/11
- **Cyp+HC** EAE=4/23

*Figure 2.18 C* - Mortality from an unknown cause plotted versus weeks of follow-up.

**Cyp + HC (%)**

- **EAE** n=9/23
- **EAE+Wasting disease** n=1/23
- **Wasting disease** n=4/23

*Figure 2.18 D* – Outcomes in TR⁺ mice subjected to Cyp and HC combination

**Figure 2.18**

The effect, in TR⁺ mice, of hydrocortisone, cyclophosphamide or the combination of both HC5 mg/kg/day X 5 days, Cyp 200 mg/kg once or Cyp+HC were administered to TR⁺ mice.
2.3.3.2.3. Pertussis toxin

We find that in TR⁻ mice, Ptx, 200 ng, 2 days apart, at 3.5 weeks of age, has no effect upon EAE incidence, onset or severity (Fig. 2.19).

![Cumulative EAE incidence (%) and Mean EAE score](image)

**Figure 2.19**

Cumulative incidence of EAE in TR⁻ mice treated with Ptx

Cumulative EAE incidence and mean EAE score of 3.5 week-old TR⁻ mice after the administration of Ptx (LBL) 200 ng (2 days apart) compared to untreated TR⁻ controls. Chi square at several points in the graph does not reveal statistically significant differences.

In TR⁻ mice, it is thought that EAE develops following transgenic cell activation at approximately 4 weeks of age, when myelin is expressed and presented and activated T cells are spontaneously capable of migrating through the meninges. As the time of spontaneous activation corresponded to the time of Ptx administration, there was no effect on the natural course of disease. If Ptx accelerated Tg cells activation one would have expected acceleration or higher scores of disease. In contrast, and as already mentioned, Ptx (LBL) in a dose of 200 ng, administered once, induced non-progressive EAE that remained at level 2 for up to 10 weeks after the onset of disease in 50% of TR⁺ mice (Fig. 2.5 A and B). Taken together, these results in Tg mice indicate that Ptx disrupt active mechanisms of tolerance (in TR⁺ mice) rather than directly promote effector cell activation or migration (in TR⁻ mice).

2.3.3.2.4. CD25 depletion

In order to test the effect of Treg depletion alone upon EAE induction, CD25 depletion was used. Neither PC61 (200 µg) alone (n=5) nor in combination with HC5x5 (n=5) led to EAE in TR⁺ mice. One-month-old TR⁻ animals (n = 7) received five weekly injections of PC61 (200 µg) and were followed for 4.5 months resulting in a 5 week delay in EAE onset, attributed to an effect on
transgenic T cells. These results have been published by our lab (Zelenay et al., 2005) - in appendix.

2.3.3.2.5 Irradiation

Irradiation with a single dose of 50 RAD did not induced EAE in the TR+ mice (n=5).

2.3.3.3. Modulation of Ptx-induced encephalomyelitis in TR+ mice

2.3.3.3.1. CD25 depletion

A synergistic effect on EAE severity was exerted by Ptx and PC61 treatment in TR+ mice, as published by our lab and shown by Zelenay et al (2005) - in appendix. This phenomenon has also been described by others, in an active model of EAE induction (Cassan et al., 2006).

2.3.3.3.2. Hydrocortisone

The demonstration that Treg depletion increased the susceptibility to induced EAE suggests that the Ptx effect can also be enhanced. In order to test whether HC increases the incidence of Ptx induced EAE in TR+ mice, these were treated with HC5x5 (D-5 to D-1) and injected with Ptx 200 ng on D0 and observed in comparison to Ptx alone. There was no difference in EAE incidence between the 2 groups (Fig. 2.20 A). The type of EAE which is induced in these mice is mild (L2) and non-progressive.

![Figure 2.20 A](#) - Cumulative EAE incidence and mean EAE scores in mice injected with Ptx (LBL) and HC in comparison with another group of mice injected with Ptx (LBL) alone in the same dose
Effect of steroid treatment on the number and function of regulatory T cells in mice

Fig. 2.20 continued

**Figure 2.20 B** - Representative haematoxylin and eosin (H&E) and luxol fast blue (LFB) tranverse spinal cord staining of healthy untreated TR⁺ mice, untreated sick TR⁻ (L3 EAE) and TR⁺ subjected to HC and Ptx that developed L2 EAE. Meningeal and parenchymal cellular infiltrates are present in HC and Ptx treated TR⁺ mice but areas of demyelination are only present in TR⁻ mice that developed spontaneous EAE. Original amplification - x40

**Figure 2.20 C** - Higher amplification of transverse spinal cord staining of a sick TR⁺ mouse with meningeal and parenchymal infiltrates after HC and Ptx. Original amplification - x100

continues
Results

Fig. 2.20 continued

Figure 2.20 D - Higher amplification of a longitudinal section of a sick TR+ mouse with meningeal and parenchymal infiltrates after HC and Ptx compared to a healthy control. Original amplification - x100

Figure 2.20

The effect of hydrocortisone on Ptx - EAE induction in TR+ mice
TR+ mice were subjected to HC5x5 from D-5 to D-1 and Ptx (LBS) 200 ng once, on Day 0.

Combined treatment with HC and Ptx induced EAE, at 2 months post administration, is characterized by cellular meningeal and parenchymal infiltration with no demyelination (Fig. 2.20 B, C and D). Demyelination may have developed with a longer follow-up.

2.3.3.3. Irradiation
A single dose of 50 RAD, judged to be equivalent to HC5x5, in the thymus and in the periphery, failed to increase the incidence of Ptx induced EAE (Fig. 2.21). In contrast, the administration of Ptx once on D0 (200 ng – LBL) to TR+ mice subjected to 3 sessions of irradiation (50 RAD) on alternate days (D0, D2, D4) induced EAE in 100% of the mice (n=5).

2.3.4. The effect of hydrocortisone on regulatory T cell expansion
In order to test whether HC5x5 donor treatment affects T cell homeostasis, transfer of total 3x10^5 CD4+ T cells from HC or control treated donors into Helicobacter positive B10.PL RAG−/− mice was performed. Severe colitis developed in all groups, preventing cellular analysis (data not shown). The next transfer of 3x10^5 CD4+ T cells to Helicobacter negative B10.PL RAG−/− recipients also resulted in T cell-induced colitis, presumably due to the presence of other gut...
bacteria. In order to avoid T cell-induced colitis, *Helicobacter* negative B6 recipients, housed in different quarters of the animal house were used instead.

![Graph showing cumulative EAE incidence and mean EAE scores](image)

**Figure 2.21**

**The effect of irradiation on Ptx - EAE induction in TR⁺ mice**

TR⁺ mice were subjected to Ptx (LBL) 200 ng and a single dose of irradiation (50 RAD) on Day 0 (n=8). Plotted are cumulative EAE incidence and mean EAE scores in comparison with the group of mice injected with Ptx alone in the same dose.

Total MACS purified 3x10⁶ CD4⁺ T cells from HC treated donors or controls, were thus transferred to *Helicobacter* negative B6 RAG⁻/⁻ recipients. No weight loss or diarrhoea was noted in either group (Fig. 2.22 A). Analyses at 2 months post transfer revealed that frequency and number of CD4⁺ and CD4⁺CD25⁺ T cells recovered in the Sp and Ln were similar in the HC transfer and control (Fig. 2.22 A). These data indicate that HC treatment did not interfere with the proliferating and migrating capacity of naïve T cells. The simultaneous transfer of CD4⁺CD25⁺ T cells into *Helicobacter* negative B6 RAG⁻/⁻ mice prevented T cell-induced colitis by naïve T cells at a 1/1 ratio (1x10⁵ per mouse). Of note, the mice in the single transfer group were starting to have diarrhoea and to lose weight at time of analysis, corresponding to an early phase of T cell-induced colitis at 7 weeks post transfer (Fig. 2.22 B). CD4⁺CD25⁺ T cells from HC treated donors expand and control the proliferation of naïve T cells as well as CD4⁺CD25⁺ T cells from untreated controls (Fig. 2.22 C and D).

In another experiment, upon transfer, CD25⁺ T cells from HC treated or control donors expand and limit naïve T cells proliferation even at an effector/Treg ratio of 10/1, similarly to control (Fig. 2.22 F). There was no T cell-induced colitis in the co-transfer but, unusually, 1 out of
Results

2 mice in the single transfer group lost and spontaneously re-gained weight (Fig. 2.22 E). These findings suggest that CD25+ T cells from HC treated donors are as effective as controls in the prevention of T cell-induced colitis.

Fig. 2.22 (A-F)

Figure 2.22 A - Donor mice were treated with HC5x5, D-5 to D-1 and sacrificed on D0. Donor MACS-purified CD4+ T cells (3x10^6 per mouse), from HC (purity 96%) or control (purity 94%) donors were transferred to B6 Helicobacter negative RAG–/– mice. Recipient serial weights plotted as % of initial weight over time post transfer. At 8.4 weeks post transfer the mice were sacrificed and the frequency and number of CD4+ and CD4+CD25+ T cells in the Sp, mLn and nmLn are plotted for each group. Results are pooled from 3 separate experiments

continues
Effect of steroid treatment on the number and function of regulatory T cells in mice

Fig. 2.22 continued

Figure 2.22 B - Transfer of naïve CD45RB<sup>high</sup> Thy1.1<sup>+</sup> T cells alone (purity 99.1%, 1x10<sup>5</sup> per mouse, n=3) is compared to the co-transfer of naïve T cells with sort-purified CD4<sup>+</sup>CD25<sup>+</sup> T cells from HC (purity 98.2%, n=4) or PBS (purity 99%, n=4) treated donors, on a 1:1 ratio. Serial weights plotted as % of initial weight over time post transfer in Helicobacter negative B6 RAG<sup>-/-</sup> recipients.

Figure 2.22 C – Representative FACS plots show the frequency of Thy1.1<sup>+</sup> T cells in mLn, nmLn and Sp at seven weeks post transfer (from Fig. 2.22 B). The complementary plots show similar frequencies of Thy1.2<sup>+</sup> T cells.
**Results**

Fig. 2.22 continued

**Figure 2.22 D** – Number of Thy1.1+ (originally CD45RB\(^{high}\)) and Thy1.1- (originally CD25\(^{+}\)) recovered from single and co-transfer (tf and Co-tf) groups in Fig. 2.22 B and C

**Figure 2.22 E** - Transfer (tf) of naïve CD45RB\(^{high}\)Thy1.1+ T cells alone (purity 98.3 %, 2.5x10\(^5\) per mouse), compared to recipients of co-transfer (Co-tf) of naïve T cells (2,5x10\(^5\) per mouse) with sort-purified CD4\(^{+}\)CD25\(^{+}\) T cells from HC (purity 99 %) or PBS (purity 99%) treated donors, on a 5:1 (5x10\(^4\) CD25\(^{+}\)) or on a 10:1 (2,5x10\(^4\) CD25\(^{+}\)) ratio. Serial weights plotted as % of initial weight over time post transfer in *Helicobacter* negative B6 RAG\(^{−/−}\) recipients

continues
HC treatment of recipients pre CD4+ T cell transfer was performed in order to verify indirect effects of APC upon Treg expansion or function. Some recipients also received HC post-transfer in order to check the effect of HC on expanding T cells. CD4+ T lymphocytes (1.5x10^5 per mouse) isolated from untreated wt donors were thus transferred to recipient RAG–/– mice that received HC5x5 before or after the cell transfer. HC treatment did not affect CD4+ or CD25+ T cell frequency or number at 2 or 7 weeks post transfer. As expected, the number of lymphocytes...
Results

collected from each recipient was higher at 7 than at 2 weeks post transfer, indicating that expansion took place (Fig. 2.23 A). The higher number of MLN CD4\(^+\) and CD4\(^+\)CD25\(^+\) T cells in the group that was not treated with HC probably reflects a higher number of activated T cells in the mLn of the group where there was no weight increase, corresponding to the initial phase of T cell-induced colitis (Fig. 2.23 A and B). The differences in weight loss between the treated and non-treated groups may also reflect an anti-inflammatory action of HC on conventional T cells.

Fig. 2.23 (A-B)

![Graph](image)

Figure 2.23 A - Serial weight plotted as % of original weight over time post transfer.

continues
Comparison between weight and recovery of T lymphocytes from recipients of control donor cells, treated before or after transfer with HC5x5

Transfer of MACS - sorted CD4+ T lymphocytes (1.5x10^6) from untreated WT donors (purity 92%) to Helicobacter negative B6 RAG-/- recipients that either received HC5x5, before or after the cell transfer.
CD4+ T cells recovered at 12 weeks post transfer from TR- mice that receive a protective 1x10^6 CD4+ T cell transfer, before they are 4 weeks old, show a high frequency of Foxp3+ T cells which is similar whether the T cells come from a PBS or a HC treated donor (Fig. 2.24). This indicates that HC has no effect on the accumulation of Treg originally present in the cell transfer.

![Figure 2.24](image)

**Figure 2.24**

**Comparison of CD4+Foxp3+ T cell accumulation between TR- mice protected from EAE by either a hydrocortisone or control CD4+ T cell transfer**

CD4+ T cells recovered at 12 weeks post transfer, from TR- mice that receive a protective 1x10^6 CD4+ T cell transfer at 3.5 weeks of age, show a high frequency of Foxp3+ T cells which is similar whether the T cells come from a PBS or a HC treated donor.

### 2.3.5 The effect of hydrocortisone on regulatory T cell function *in vitro*

In order to check if HC or DEX affected Treg *in vitro* function, Treg were isolated from DEX treated donors, compared to HC treated and control donors. HC5x5 did not affect the function of Treg *in vitro* as evaluated by standard suppression assay with target cells from untreated donors (Fig. 2.25 A) or in the presence of target cells (Fig. 2.25 B) or APC (Fig. 2.25 C) isolated from HC treated donors. The response of CD25+ T cells to IL-2 is also not altered by HC (Fig. 2.25 D). Treg isolated from DEX treated donors display an *in vitro* suppressive function similar to Treg from control donors (Fig. 2.25 E) even though the response of Treg to IL-2 is decreased by almost half (Fig. 2.25 F). The results of the *in vitro* functional assays parallel the *in vivo* results obtained described in the present study.
Figure 2.25 (A-F)

Inhibition index

A

Target - C  
APC - C  

Target:Treg

B

Target - HC  
APC - C  

Target:Treg

Figure 2.25 A - Treg from HC treated donor vs control, target cells and APC from control mice. Plot is representative of 3 separate experiments

Figure 2.25 B - Treg from HC treated donor vs control, target cells from HC treated mice and APC from control mice.

Inhibition index

Target - C  
APC - C  

Target:Treg

Figure 2.25 C - Treg from HC treated donor vs control, target cells and APC from HC treated mice. Plot representative of 2 separate experiments
Results

Figure 2.25 continued

Figure 2.25 D - Sort-purified CD4+CD25+ (2.5x10^4) from HC treated or control mice cultured with IL-2 in the presence of APC

Figure 2.25 E - Suppression assay with CD4+CD25+ T cells from donor mice treated with DEX 5 mg/kg once on D-1

Figure 2.25 F - Sort-purified CD4+CD25+ (2.5x10^4) from DEX or control mice cultured with IL-2 in the presence of APC from control mice

Effect of hydrocortisone and dexamethasone on the function of Treg in vitro
Sort-purified CD4+CD25+ T cells (Treg, purity > 97%), from Ln of HC5x5 or PBS treated mice were co-cultured with sort purified CD4+CD25- T cells (target, purity 99%) at different ratios and stimulated in culture with anti-CD3 in the presence of irradiated splenocytes acting as APC. Target cells were used at a fixed number of 2.5 x 10^4 /well and APCs at 1x10^5/well. Cultures were performed in triplicate. Purities of CD25+ and CD25- were always above 98% and 99% respectively. Proliferation was measured by radioactive thymidine uptake on day 3. Plots indicate inhibition index which is maximal at a 1:1 ratio and represents the percent of inhibition ((cpm in control - cpm in experiment)/cpm in control) plotted vs the ratio of CD4+CD25+/CD4+CD25- cell number at the origin of the culture.
A summary of the results obtained is detailed in Table 2.V.

**Table 2.V** – Selective effects upon the number of Foxp3 expressing CD4+ subpopulations of T cells in the Thy, Ln and Sp of wt and TR+ mice and capacity for AID induction. The presence of significant thymic atrophy and CD4+ lymphopenia is indicated.

<table>
<thead>
<tr>
<th>Population</th>
<th>HCx5x5</th>
<th>Cyp</th>
<th>HC + Cyp</th>
<th>Ptx ± HC</th>
<th>50 RAD</th>
</tr>
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<tr>
<td>Thy atrophy</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Thy SPCD4+ Foxp3+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D0: ↑ D7: ↓</td>
<td>↑</td>
<td>↑</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Ln CD4+ lymphopenia</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ln CD4+ Foxp3+</td>
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<td>↓↓</td>
<td>-</td>
<td>- (CD25)</td>
</tr>
<tr>
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<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sp CD4+ Foxp3+</td>
<td>-</td>
<td>↓</td>
<td>↓↓</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>Thy SPCD4+ Foxp3+</td>
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<tr>
<td>D0: ↑ D7: ↓</td>
<td>-</td>
<td>↓</td>
<td>ND</td>
<td>- (CD25)</td>
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<tr>
<td>Thy SPCD4+ 3H12+</td>
<td></td>
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<td></td>
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<td>D0: ↓ D7: ↓</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
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<td>ND</td>
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<tr>
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<td>ND</td>
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<tr>
<td>Ln CD4+ 3H12+ Foxp3+</td>
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<td>ND</td>
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<td>- (CD25)</td>
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<tr>
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<td>↓</td>
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<td>- (CD25)</td>
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<td>↓↓</td>
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<td>++</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

An effect is considered to be selective upon a subpopulation of cells if it is significantly greater than the effect on the parent population. ↓ = selective decrease; ↑ = selective increase; − = no effect. (where not discriminated, it signifies no effect at both time points; += effect; ND=not done. All results pertain to D0 except for the HCx5x5 where studies were performed in the recovery period.
2.4. Discussion

The principal question addressed in this study was whether GC, the most anciently and commonly used IS can have a deleterious effect upon Treg number or function and as such paradoxically contribute to precipitating or furthering autoimmunity. The main findings were that steroids do not selectively deplete Treg nor alter their suppressive function or capacity for expansion upon transfer. Hydrocortisone was chosen as the GC to be studied on the basis that it has an immunosuppressive and anti-inflammatory profile similar to the formulations that are used in the maintenance treatment of AID (Buttgereit et al., 1999; Buttgereit et al., 2002; Lionakis et al., 2003). In a first set of experiments, HC administration was titrated in normal, adult mice and a 5 days course of HC 5mg /kg/day (HC5x5) was then chosen as the standard treatment in all further experiments, as it scored as the lowest dose causing significant lymphocyte depletion in thymus and lymph nodes. This dose is equivalent to the doses used in maintenance therapy of patients with AID and avoids non genomic effects (Schmidt et al., 2000). Furthermore, HC 5x5 is actually the dose of HC equivalent in anti-inflammatory potency to the dose of DEX, (200 µg/day for 5 days), able to break refractoriness to EAE re-induction in Lewis rats (Reder et al., 1994). Hydrocortisone seems, therefore, preferable the usual choice of corticosteroids in experimental studies namely DEX 5 mg/kg administered once, a schedule with more potent anti-inflammatory and non-genomic effects than HC.

2.4.1. A short course of steroids, as opposed to cyclophosphamide, did not selectively deplete regulatory T cells

The different characteristics of the T lymphocyte subpopulations may dictate the way they respond to immunosuppressive agents with a distinct mechanism of action. As recently reviewed in Rothenberg et al. (2008), T cells originate from pluripotent precursors in the bone marrow or fetal liver, which migrate to the thymus to initiate and sustain T-cell differentiation. Relatively few T-cell progenitors migrate into the thymus per day and therefore T-cell differentiation in the thymus is characterized by extensive rounds of proliferation which correlate inversely with stages of RAG gene mediated TCR re-arrangement. Large immature double negative thymocytes proliferate vigorously and once a cell achieves a successful β chain gene re-arrangement, it expresses CD4 and CD8 and gives rise to many double positive (DP) cells, which are small quiescent cells, from where only those CD4+ T cells that eventually acquire cell-surface TCRαβ complexes survive and from this point, the fate of the lymphocyte is determined by the specificity
of the TCR. The ability of corticosteroids to induce T lymphocyte apoptosis depends on sufficient levels of the GR and several corticosteroid regulated genes, including members of the Bcl-2 family, are responsible for apoptosis induction (Ploner et al., 2005; Herr et al., 2007). Not surprisingly, DP are more vulnerable to corticosteroid induced apoptosis than SP CD4+ thymocytes or mature peripheral CD4+ T cells (Reichert et al., 1986; Screpanti et al., 1989; Sentman et al., 1991), the latter’s resistance to apoptosis occurring through more Bcl-2 expression (Sentman et al. 1991; Hasserjian et al. 1996; Deftos et al., 1998). In the present study the titration of HC confirmed a reduction of thymocytes in a dose dependent manner, mostly affecting DP thymocytes. The observed reduction in peripheral T cells due to HC, rather than a direct effect of HC, may be due to a decreased thymic output, a previously described effect of CS (Kong, Chen et al. 2002). In TR+ mice, the decrease in the number of Treg in the Ln, Sp or thymus after HC5x5 was not selective and there was actually a Treg sparing effect in the thymus of TR+ similar to wt mice. In this context, “selective” means that the drug has an effect on a cell population that differs from that on the population from which it derives.

Cyclophosphamide, on the other hand, exhibits most cytotoxicity against cells actively replicating their DNA, as unpairing of DNA strands at this stage makes the nucleotide residues more susceptible to the alkylation mediated by Cyp and is a myelossuppressant (Moore et al., 1991). Thymic atrophy due to Cyp administration may have occurred due to its effect on the rapidly dividing thymic populations at the pre-DP stage, not surprisingly resulting in a decrease of the DP population, in association with a possibly diminished amount of T cell precursors entering the thymus. The lack of effect of both HC and Cyp on SP CD4+ Foxp3+ thymocytes may be indicative that newly formed thymic Treg are not cycling in the case of Cyp or express apoptosis resistance factors, in the case of HC. The most severe reduction of Treg was caused by the addition of HC to the Cyp treatment, as precursors in the DP population may have been below the minimum threshold necessary to ensure Treg differentiation. Cyp selectively reduced CD4+ Foxp3+ T cells in the periphery, as expected from the knowledge that Treg are naturally activated and cycling T cells (Fisson et al., 2003; Hori et al., 2002) which expand in response to inflammatory signals (Caramalho et al., 2003; Dai et al., 2007) and are therefore more susceptible than other T lymphocytes to the action of Cyp. The TCR-non-Tg population in the TR+ mice is a preferential target for Cyp action, probably due to the large fraction of Treg it contains (over 30%). Contrarily to Cyp, HC has no selective effect upon Treg, suggesting that it has no particular effect upon cycling T cells. Apart from selective reduction, Cyp also affects Treg function as evidenced by the failure EAE protection in TR+ mice using the same number of T cells as in the control or HC transfer groups. This occurred even when ten times more cells from Cyp
treated donors were transferred.

HC in a dose of 5 mg/kg/day for 5 days that seems not to affect Treg in a wt mouse might, in the context of autoimmunity, have disrupted regulation and provoked EAE. In TR+ mice, positive selection of MBP specific T cells perturbs further TCR rearrangement and generates a highly restrictive environment for the generation and selection of edg TCR $\alpha$ encoded chains, more important than edg TCR $\beta$ chains for Treg, as with the former there is incomplete allelic exclusion (Olivares-Villagomez et al., 1998; Hori et al., 2002). In other words, the process of Treg generation in TR+ is probably more fragile than in wt mice thus explaining the smaller number of Treg in the thymus of TR+ when compared to wt mice. Furthermore, as in TR+ mice most of the DP thymocytes are tg and the edg sub-population is a much less abundant group of cells, the major disrupting effect of HC upon the DP population is likely to be upon edg SP CD4+ thymocytes. The edg SP CD4+ thymocytes are in turn enriched in Treg but, surprisingly, the total number of Treg was not reduced despite a selective reduction in thymic edg T cells after HC5x5, prolonged for at least seven days after HC. This is probably due to the mild sparing effect of HC exerts upon thymic Treg which is also seen in wt mice. Complete re-population of the thymus was observed one week after HC5x5 with no further selective effects. HC did not affect the frequency, the number or ratio between edg and tg T cells in the periphery of TR+ mice nor the frequency and number of activated CD4+ T cells or Foxp3+ T cells in the tg and edg pools. The reduction in edg T cells resisted the window of opportunity for a break in tolerance and EAE development. Indeed, peripheral expansion of thymically committed cells has been shown to ensure the maintenance of the peripheral Treg pool in the adult TR+ mouse (Hori, Haury et al. 2002) and may contribute to the lack of autoimmune manifestations after the severe thymic atrophy induced by HC therapy in TR+ mice.

2.4.2. In the transgenic mouse, lymphopenia only triggered auto-immune disease when regulatory T cells were selectively decreased

HC or irradiation provoked (i) significant thymic atrophy, (ii) little peripheral lymphopenia, (iii) did not selectively affect decrease Treg upon treatment or recovery, (iv) did not cause AID in wild type animals, (v) did not induce EAE in TR+ mice and (vi) did not increase the incidence of Ptx induced EAE. In contrast, Cyp produced as severe thymic atrophy but a more accentuated degree of peripheral lymphopenia than HC, selectively decreasing peripheral Treg upon administration and inducing EAE in TR+ mice. The addition of HC accentuated the effect of Cyp on the reduction of SP CD4+ Foxp3+ T cells thereby increasing the incidence of EAE even further.
These results confirm that in adult autoimmune prone individuals, temporary severe thymic atrophy and mild peripheral lymphopenia is unlikely to trigger AID unless it is accompanied by selective Treg depletion. This may explain why mild viral infections or any triggers that don’t selectively affect Treg in humans do not usually lead to AID, while the reverse may be true.

In contrast to conditions of mild lymphopenia, in the present work, severe lymphopenia acted as a trigger of encephalomyelitis in RAG–/- mice. When one million unsorted cells pooled from Ln and Sp of a healthy TR+ were transferred to RAG–/- mice, recipients developed EAE, detected at 5 weeks post transfer (data not shown). The effect of Ptx with repeated irradiation exposures causing EAE in 100% of the TR+ mice requires confirmation and population analysis after treatment but may be a reflection of very severe lymphopenia. It is possible that in severe lymphopenia other mechanisms affect Foxp3 expression, Treg function or that a minimum amount of Treg is necessary for control of autoimmunity as AID developed with Treg: effector ratios that normally prevent disease in TR+ mice.

It has been convincingly proven that the absence of Treg or complete Treg depletion of a lymphocyte sufficient animal causes AID (Kim et al., 2007; Lahl et al., 2007). But before the tools were available for complete depletion, it was thought that Treg depletion alone could not lead to AID, that it required the presence of lymphopenia. The failure to induce EAE in TR+ by CD25 depletion was not surprising in the light of the fact that a significant proportion of the CD4+Foxp3+ Treg in TR+ mice are CD25 negative and therefore not amenable to CD25 depletion (Fig. 10D). Nevertheless, the reduction of Treg caused by PC61 treatment is a likely explanation for its effect on increasing the incidence of Ptx-induced EAE and contrasts with the effect of HC, which did not decrease Treg or increase the incidence of Ptx induced EAE.

A selective decrease of Treg could contribute to the many possible effects of Ptx in EAE induction (Cassan, Piaggio et al. 2006; Chen, Winkler-Pickett et al. 2006). The lack of 100% effect of Ptx and the other IS agents may reflect important variations between genetically identical TR+ mice which determine EAE outcome. Those TR+ mice that are resistant to EAE induction may be protected by a particular repertoire of Treg which is stochastically generated or by an increased number of Treg, in a manner similar to a variable frequency of NOD mice that never get diabetes. Of note, it has long been known that anti-MBP TCR tg mice are extremely sensitive to EAE induction with Ptx but the EAE induction is generally less than 100%. Similarly, in this work, the EAE induction rate with CFA, MBP and Ptx was less than 100%.
2.4.3. A short course of steroids did not affect Treg function or expansion upon transfer

Treg function was tested as regards capacity to (i) confer EAE protection in TR\(^-\) mice, (ii) control conventional T cell expansion and (iii) prevent T cell induced colitis development, upon transfer to lymphopenic hosts.

The higher failure rate of protection than expected (approximately 30% showed signs of EAE) was similar in both groups independently of donor treatment. In both groups, however, there was partial EAE protection upon transfer, as the disease rarely progressed beyond L3 and did not usually lead to death. Similar capacity for partial protection was observed across the several type of transfers which were performed: (i) 1x10\(^6\) CD4\(^+\) T cells (corresponding to ~ three times the minimum number of un-fractioned CD4 T cells reported to afford EAE protection), (ii) sort-purified CD25 T cells from control and HC treated donors (iii) CD25\(^+\) T cells and transgenic T cell co-transfers into RAG\(^{-/-}\) recipients. In the co-transfer group (iii), a subtle decrease in protection from EAE in the HC donor group should be validated in a strict SPF environment. Cyp or Cyp + HC treatment of donor mice abrogated the partial EAE protective function of the CD4 cell transfer to TR\(^-\) mice. As the same number of T cells was used as in the HC transfer group, this suggests that Cyp affects Treg function as well as selectively decreasing Treg number.

It has been suggested that the initial ratio of CD4\(^+\)CD25\(^+\) T cells to naïve T cells present in the inoculum, rather than the absolute number of cells is important for Treg control of lymphopenia induced proliferation (Almeida et al., 2002). Furthermore, it appears that, because of the lack of T cells, Rag-deficient recipient mice abnormally present Ag from normal commensal bacteria and therefore, polyclonal T cells transferred into T cell-deficient hosts mainly react to the high load of enteric bacteria in the recipients rather than to self-peptides (Kieper et al., 2005; Min et al., 2005), frequently resulting in T cell induced colitis. In our work, HC treatment did not alter Treg expansion, the capacity for Treg control of the expansion of a co-transferred conventional CD4\(^+\) population, or Treg control of T cell induced colitis, not even after a co-transfer with naïve T cells at a reduced Treg/T-effector ratio.

In conclusion, a short course of steroids does not damage Treg number or function as established by the absence of an effect upon (i) Foxp3 expression; (ii) in vivo function (adequate expansion and capacity for control of T cell induced colitis in RAG\(^{-/-}\) recipients) and (iii) in vitro function (normal suppression assay). In combination with other IS treatments such as Cyp, HC may worsen disease outcome.
3. RESPONSE OF REGULATORY T CELLS TO INFLAMMATION

The present experimental layout was proposed to evaluate the impact of *Helicobacter* load, effector T cell specificity and presence of Treg on resultant immunopathology in the experimental model of T cell-induced colitis. It was designed to study the dynamics of inoculated Treg expansion and test the possibility of conversion of naïve donor T cells to Foxp3+CD4+ Treg during colitis induction. Besides an original description of the characteristic pattern of the T cell-induced model of colitis in B10.PL mice, with a faster onset and greater severity in the presence of *Helicobacter* colonization, evidence was provided for the facts that: (i) the T cell-induced model of colitis in mice may have a non-autoimmune basis, since colitogenic T cells in this system are *Helicobacter* and not tissue specific; (ii) during severe inflammation and ongoing colitis, Foxp3+ CD4+ T cells accumulate selectively; (iii) conversion of naïve cells to a Foxp3+ phenotype occurs, and; (iv) these converted cells play an important role in regulation by decreasing mortality in the T cell-induced colitis model. As a corollary of this work, we propose that a major effort should be made to find and eradicate individual gut micro-organisms which elicit specific immune responses and to consider the value of Treg supplementation in disease prevention and therapy.

3.1. Introduction

3.1.1. The aetiology of inflammatory bowel disease

In humans, Ulcerative Colitis (UC) and Crohn’s Disease (CD) are the main types of inflammatory bowel disease (IBD). Both forms present with bloody diarrhoea, abdominal pain, and malnutrition
but are distinguished on the basis of clinical, anatomical and histological features. UC affects the colon and rectum alone but CD can affect the whole length of the digestive tract from the mouth to the anus. These are severe idiopathic relapsing inflammatory diseases found to occur in genetically susceptible individuals and are thought to result from a complex interaction between environmental factors, infectious microbes, genetic susceptibility and a deregulated immune system. Several, not mutually exclusive pathways of oral tolerance, epithelial barrier function, antigen (Ag) recognition, and immunoregulation by the innate and adaptive immune system are thought to play a role in the initiation and perpetuating events of mucosal inflammation (Mowat, 2003; Baumgart and Carding, 2007). An enormous potential for immune reactivity exists in the gut and therefore in health, tolerance to commensals must be in place. This is supported by the observation that, in the healthy colon, T lymphocytes isolated from the intestinal lamina propria are tolerant to the individual’s own enteric bacteria but not to that of others (Groux and Powrie, 1999).

Based on circumstantial evidence, IBD has been considered to be an autoimmune disease. This concept started to be challenged in the 90’s and, in fact, no self-Ag has ever been described in IBD. As recently reviewed by Christen and von Herrath (2005), microbes are likely candidates for triggers of AID since, during an acute infection, inflammation can uncover self Ag, pathogen epitopes may have structural or sequential similarity to host epitopes (molecular mimicry) and bystander or specific activation of autoreactive lymphocytes may occur through epitope spreading to self Ag. In animal models most of these mechanisms have been shown to take place, however it is not known if they occur in human disease. In fact, in experimental models, defects in adaptive immunity do not lead to spontaneous colitis in a germ free (GF) environment, unless these defects affect the generation and function of Treg (Elson et al., 2005). Germ-free IL-2–/– mice, originally thought not to contract colitis (Sadlack et al., 1993), have actually been shown to develop mild late disease (Schultz et al., 1999) and TGF-β–/– mice also develop colitis in the absence of bacterial colonization (Boivin et al., 1997). Moreover, a severe enteropathy characterizes IPEX (Immune dysregulation, polyendocrinopathy, enteropathy, X-linked inheritance), a human disease caused by the lack of Foxp3, a transcription factor indispensable for the development and function of Treg in mice and humans (Brunkow et al., 2001; Wildin et al., 2002; Hori et al., 2003; Fontenot et al., 2003). In contrast, GF IL-10–/– mice do not develop colitis (Sellon et al., 1998) consistent with the fact that IL-10 is not absolutely essential for Treg function (Lee et al., 2007), contrary to IL-2, TGF-β and Foxp3 which are (recently reviewed in Sakaguchi et al., 2008). These findings suggest that Treg deficits are associated with a true autoimmune enteropathy and, therefore, in humans some forms of IBD
may be autoimmune. Perhaps one of the most important findings supporting the theory that T cell-induced colitis is an AID is the finding that naïve T cells from germ-free donors are capable of causing colitis upon transfer, suggesting an autoimmune process (Annacker et al., 2000) but in these experiments the recipients were not GF and there may have been cross-reactivity with a bacterial Ag such as *Helicobacter*.

In the T cell-induced colitis model, the transfer of naïve CD4+ T lymphocytes to alymphoid recipients causes colitis. At the time this model was developed, it reflected current thinking that IBD is a T lymphocyte mediated immune disease, based on the finding of reactive oligoclonal CD4+ T lymphocyte infiltrates, in biopsy specimens (Kaulfersch et al., 1988; Gulwani-Akolkar et al., 1996), considered to prevail over signs of antibody mediated pathology. Dendritic cells (DC) are the main APC regulating T cell responses and, in the absence of external stimuli, DC exist in a resting state in which they have a limited ability to prime naïve T cells. DC are differentially activated, promoting T helper responses in response to pathogens, and supporting CD4 clonal expansion upon exposure to inflammatory signals (Spörri and Sousa, 2005). In the T cell-induced colitis model, intestinal flora is thought to drive the intestinal immune response and inflammation but it remains to be established whether reactivity to self contributes to the disease process as the presence of pathogens may be required for DC to present self Ag.

### 3.1.2 Influence of bacterial luminal content on the outcome of colitis

In the T cell-induced colitis model, after adoptive transfer, oral antibiotics (Morrissey and Charrier, 1994) as well as a reduction in the recipient flora (Aranda et al., 1997) have been reported to ameliorate symptoms. In addition, the transfer of naïve T cells to germ-free recipients does not induce colitis (Powrie et al., 1997). No specific bacterial species have been implicated in the pathogenesis (Coombes et al., 2005) but there seems to be a requirement for filamentous bacteria and specific pathogen free (SPF) flora for colitis induction (Stepankova et al., 2007). In contrast to CD4+ T cells from normal mice, CD4+ T cells derived from SCID mice with T cell-induced colitis in SPF conditions, proliferate strongly in response to antigen presenting cells (APC) pulsed with faecal extracts but do not respond to either extracts from food Ag or faecal extracts from germ-free mice (Brimnes et al., 2001). All these observations strongly support an involvement of bacteria in the pathogenesis of T cell-induced colitis.

Colonization by *Helicobacter hepaticus* deserves a special mention as it is not just a harmless commensal, rather an invasive pathogen which, even though not pathological in C57BL/6 mice, leads to the development of significant intestinal inflammation, even in the absence of T cell reconstitution in RAG−/− mice (Ward et al., 1994; Ward et al., 1996).
H. hepaticus is a Gram negative spiral bacterium with bipolar single sheathed flagella, identified in the liver and intestinal crypts of cecum and colon of laboratory mice with hepatitis (Fox et al., 1994), and found to be prevalent in production and research mouse colonies (Shames et al., 1995). It does not cause colitis in wt mice. Direct infection with H. hepaticus severely aggravates colitis in SCID or RAG-2−/− mice reconstituted with CD45RBhigh T cells (Cahill et al., 1997) and in Interleukin (IL-10)−/− mice that spontaneously develop colitis (Kullberg et al., 1998).

In humans, even though no specific micro-organism has been directly associated with the pathogenesis of IBD, analysis of the luminal enteric flora has revealed differences in its composition compared to healthy controls (Linskens et al., 2001). However, it remains unknown whether these differences are causal or consequential and, at the present moment, there is no evidence that antibiotics should be used in clinical practice for prevention, induction of remission or maintenance therapy in IBD (Sutherland et al., 1991; Steinhart et al., 2002; Goossens et al., 2003).

3.1.3. The protection afforded by regulatory T cells in the T cell-induced colitis model

In the T cell-induced colitis model, adoptive transfer of naïve CD4+ T cells from wild type (wt) donors (characterized by CD45RBhigh expression) into SCID or RAG−/− mice results in colitis and wasting disease (Morrissey et al., 1993; Powrie et al., 1993). In contrast RAG−/− mice remain healthy after the transfer of CD4+CD45RBlow T cells. Colitis is actually abrogated by co-transfer of this cell subset with CD4+CD45RBhigh T cells (Morrissey et al., 1993; Powrie et al., 1993), and more recently the transfer of CD4+CD25+ T cells was actually shown to cure already established colitis (Mottet et al., 2003). The CD4+CD45RBlow and CD4+CD25+ T cell compartments are enriched in Foxp3+CD4+ T lymphocytes, providing convincing evidence that bowel inflammation can be controlled or suppressed by Treg. The presence of Treg has actually been documented in the lamina propria of the colon of wt mice (Uhlig et al 2006), shown to exert suppressive activity and prevent colitis when co-transferred with naïve T cells (Makita et al., 2007). In addition, Treg have also been shown to act independently of Treg–Th interactions in suppressing macrophage-mediated immunity to H. hepaticus in the absence of conventional T cells (Coombes et al., 2005). Anti-IL-10 monoclonal antibody administration can abrogate CD45RBlow mediated protection in the T cell-induced colitis model (Powrie et al., 1996) and a subset of regulatory IL-10 producing Foxp3+CD4+ T lymphocytes induced, in vivo, from naïve T cells, has been shown to be protective (Groux et al., 1997; Asseman et al., 1999).

It remains to be determined if T cell receptor (TCR) specificity is a strict requirement of Treg effectiveness. In diabetes, Ag-specific Treg have been reported far more effective in
controlling autoimmune disease than polyclonal Treg (Tang et al., 2004). In contrast, *ex-vivo* stimulated non-Ag specific Treg can exhibit *in vitro* suppression as well as Ag-specific Treg (Li et al., 2006). Polyclonal CD25⁺CD4⁺ T cells from wt donors suppress CD4⁺ T cell-mediated pulmonary hyper-inflammation driven by *Pneumocystis carinii* in immunodeficient mice (Hori et al., 2002) but in these lymphopenic hosts, a sufficient number of the relevant antigen-specific Treg may expand out of the inoculated polyclonal population and therefore these findings do not exclude a Treg requirement for Ag-specificity. More specifically, it has not been determined if the protective CD45RB<sub>low</sub> or CD25⁺ Treg have to interact with effector T cells with the same TCR specificity in order to control the colitogenic potential of the CD45RB<sub>hi</sub> transfer in the T-cell induced model of colitis. In other words, it remains an open question whether the control of colitis by Treg in the T cell-induced colitis model occurs independently of specificity, triggered by a non-specific TCR signal or indeed, dependent on Ag specific clones of Treg present in the polyclonal Treg inoculum.

### 3.1.4. The dynamics of regulatory T cells during acute inflammation

Foxp3 expressing Treg have been found to arise from peripheral polyclonal or Tg naïve CD4⁺CD25⁻ T cells (adaptive or induced Treg). Adaptive Treg exhibit the same suppressive and phenotypic characteristics as thymically derived, natural Treg. This phenomenon is called conversion, thought to be promoted by combined signals from the TCR, CD28, IL-2R, and TGF-β (Chen et al., 2003; Curotto de Lafaille et al., 2004; Kretschmer et al., 2005; Liang et al., 2005) and enhanced by retinoic acid (Benson et al., 2007; Kang et al., 2007; Mucida et al., 2007; Sun et al., 2007; Coombes et al., 2007), as described in Table 3.I. The possibility has been raised that this conversion phenomenon may also concern the selective survival of some preformed Treg included in the CD4⁺CD25⁻ T cell subset (Zelenay et al., 2005).

Increased frequency of CD4⁺ T cells expressing Foxp3 in the intestine in double transgenic mice, where T lymphocytes react with an Ag engineered to be expressed exclusively by epithelial cells, supports the idea that chronic mucosal Ag exposure may lead to the development of Treg *in situ* (Westendorf et al., 2005). Non-self-Ag specific Treg have been reported for Leishmania (Belkaid et al., 2002; Suffia et al., 2006) and Candida infections (Montagnoli et al., 2002). Adaptive conversion from naïve T cells to Foxp3⁻ (Vieira et al., 2004) T cells with suppressor function has also been reported.

Inflammation has been shown to promote the expansion of Treg (Caramalho et al., 2003) but, on the other hand, acute phase reactants, such as IL-6, have been claimed to render CD4⁺ T
cells refractory to Treg cell-mediated suppression (Pasare and Medzhitov, 2003) and to promote effector rather than Treg generation facilitating T cell differentiation to Th17 cells in mice (Veldhoen et al., 2006; Bettelli et al., 2006). IL-2 actually facilitates the differentiation of naive CD4+ T cells into Foxp3+ Treg but inhibits their differentiation into Th17 cells (Laurence et al., 2007). Inflammation may actually promote effector and Treg expansion, Treg conversion from naïve T cells in the periphery and de novo thymic selection of Treg depending on the type of immune response and cognate Ag exposure (Zelenay, S manuscript submitted). Conversion may represent an important tool in the induction of antigen-specific tolerance in the adult.

Table 3.1 - De novo generation of “adaptive” Treg by Foxp3 induction in non-regulatory T cells.

<table>
<thead>
<tr>
<th>Frequency Foxp3+ T cells</th>
<th>Origin / naïve T cells</th>
<th>Protocol</th>
<th>Recipient Inflammation</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not tested</td>
<td>Wt / CD25-</td>
<td>TCR stimulation and TGF-β</td>
<td>In vitro</td>
<td>Chen et al., 2003</td>
</tr>
<tr>
<td>Not tested</td>
<td>Wt / CD25-</td>
<td>Transfer to lymphopenic recipient</td>
<td>None</td>
<td>Curotto de Lafaille et al., 2004</td>
</tr>
<tr>
<td>15-25%</td>
<td>RAG-2-‡ HA TCR-Tg mice / CD25-</td>
<td>Transfer to recipient treated with agonist peptide ± TGF-β</td>
<td>None</td>
<td>Apostolou and Von Boehmer, 2004, Kretschmer et al., 2005</td>
</tr>
<tr>
<td>Not tested</td>
<td>Wt- – CD25-</td>
<td>Transfer to lymphopenic or lymphocyte replete recipients</td>
<td>None</td>
<td>Liang et al., 2005</td>
</tr>
<tr>
<td>IL-2 and TGF-β: 20%</td>
<td>Foxp3GFP reporter mice (GFP-) or wt - CD25-</td>
<td>Retinoic acid synergizes with TGF-β</td>
<td>In vitro</td>
<td>Benson et al., 2007, Kang et al., 2007, Mucida et al., 2007</td>
</tr>
<tr>
<td>IL-2 and TGF-β + retinoic acid: 91%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40% in intestine</td>
<td>Foxp3GFP reporter mice (GFP-) + CD25-</td>
<td>Transfer to lymphopenic recipient (via retinoic acid)</td>
<td>None</td>
<td>Sun et al., 2007</td>
</tr>
<tr>
<td>1.3%</td>
<td>OVA SCID Tg</td>
<td>Ova feeding</td>
<td>None</td>
<td>Coombes et al., 2007</td>
</tr>
</tbody>
</table>

3.1.5. Objectives
The established fact that a natural polyclonal Treg population controls and cures T cell-induced colitis is paradoxical in the light that Treg are traditionally thought to have specificity for self-Ag and it remains to be demonstrated that T cell-induced colitis has an autoimmune aetiology. In fact, T cell-induced colitis is more severe in the presence than in the absence of one specific microbe, H. hepaticus, which is found in the large intestine of recipient mice. This suggests that T cell-induced colitis involves an immune response to luminal antigens and may even be
Response of regulatory T cells to inflammation

considered an infectious disease. At the present moment, what is known about the behaviour of Treg, in the presence of inflammation, remains controversial and Treg have been “dismissed” from the evaluation of ongoing fully flared T cell-induced colitis in experimental models. Particularly in an environment where there is frequent chronic antigenic exposure, such as the gut lumen, conversion to Treg from naïve T cells remains likely.

More specifically, the present work proposed to evaluate the impact of *Helicobacter* load, effector T cell specificity and presence of Treg on resultant immunopathology in the experimental model of T cell-induced colitis. It was designed to study the dynamics of innoculated Treg expansion and test the possibility of conversion of naïve donor T cells to Foxp3+CD4+ T cells during colitis induction.

### 3.2. Materials and methods

#### 3.2.1. Mice

B10.PL-H2u wt and C57BL/6-Thy1a,Igha,Gpi1a (Thy1.1+) mice were obtained from the Jackson Laboratory. B10.PL-H2u-RAG-1−/− were provided by J Lafaille and Foxp3GFP reporter knockin mice FoxP3/GFP reporter mice described in Fontenot *et al.* (2005) and provided by A. Rudensky. B10.PL-H2u-Thy1a,Igha,Gpi1a were obtained from breeding B10.PL-H2u mice with C57BL/6-Thy1a,Igha,Gpi1a, selecting the Thy1a,Igha,Gpi1a+ H2u+ offspring to start a new colony. Mice were subjected to periodic control of *Helicobacter* (H) infection. Only the B10.PL-H2u-RAG-1−/− mouse colony was known to be H+ for 6 months prior to the start of the experiments and re-derivation of the RAG-1−/− colony was performed by embryo transfer (Hogan, 1994). For all experiments, donor and recipient mice were between six and ten weeks of age. All the animals were weighed regularly and the presence of diarrhoea was noted in each cage. Mice were killed by CO2 overdose and experimental protocols were approved by the institutional ethical committee as well as by the Portuguese Veterinary General Division.

#### 3.2.2. Antibodies

Allophycocyanin (Aph), CyChrome- and PercP- conjugated anti-CD4 mAb (clone RM4-5), CD45RB- Phycoerythrin (PE) (clone 16A), Thy1.2-biotin (clone 53-2.1), Thy1.1-PE (clone OX-7) anti-IFN-γ -FITC were obtained from BD Biosciences. Anti-Foxp3-FITC, -PE mAb (FJK-16s) and
anti-IL-17-AlexaFluor™ 647 were purchased from eBiosciences. Thy1.1 (CD90.1) biotin (clone 19E12) and AlexaFluor™ 488-CD25 (clone PC61) were in-house produced. Biotinylated antibodies were revealed with streptavidin–Aph (BD Biosciences). For intracellular cytokine staining, cells were first stimulated for 4 hours with ionomycin (500 ng/ml, Calbiochem) and brefeldin A (10 μg/ml, Sigma) was added for the final 2 hours of stimulation.

3.2.3. Cell purification and transfer
Pooled inguinal, axillary, brachial and mesenteric Ln from syngeneic donors were stained with non-overlapping mixtures of anti-CD4-Aph, anti-CD4-CyChrome, CD45RB-PE, CD25-Alexa and Thy1.1 PE mAb and purified on a MoFlo High Speed Cell Sorter (Cytomation, Fort Collins, CO). Purity was routinely 98-100% for CD4+CD45RBhighCD25− and CD4+CD45RBhigh T cells. Cells were suspended in PBS (100 ml per mouse) and injected in the retro-orbital plexus.

3.2.4. Cell recovery and flow cytometric analysis
Cell suspensions from Sp, mLn or nmLn were prepared, stained and washed in PBS containing 2% FCS and 0.01% sodium azide. Analyses were performed inside a live lymphocyte gate on a FACSCalibur (Becton Dickinson) by using CELLQUEST software. Live lymphocyte counts were deduced from the acquisition of a fixed number of 10μm latex beads (Coulter) mixed with a known volume of unstained cell suspension.

3.2.5. Histological evaluation
The colon was removed from mice after T cell reconstitution and fixed in 10% formalin. Paraffin-embedded sections were cut and stained with hematoxylin and eosin (H&E) for assessment of morphology. Inflammation was scored in a blinded fashion, on a scale of 0–9 as previously described by Liu et al., (2000).

3.2.6. Helicobacter PCR
Faecal matter was collected at necropsy and analyzed by PCR as previously described by Bourgade et al., (2004). The assay was normalized according to the Lactobacillus species band found in the gut of each individual mouse.
3.3. Results

3.3.1. Effect of recipient Helicobacter status upon the rapidity of onset and severity of T cell-induced colitis

To determine whether T cell-induced colitis is experimentally reproducible in B10.PL H2u RAG1−/− mice, 3x10^5 CD4^+ CD45RB^{high} T cells from H^+ syngeneic Thy1.1^+ donors were transferred to recipients naturally colonized with Helicobacter spp. (H^+). Remarkably, one week after cell transfer, recipient mice developed severe diarrhoea accompanied by weight loss (Fig. 3.1 A). CD4^+ T lymphocytes were found to express interferon-γ (IFN-γ) in the spleen and colon and IL-17 in the colon (Fig. 3.1 B) as previously described for this model of colitis (Kullberg et al., 2006).

After naïve T cell transfer, in order to assess the presence and severity of colitis, histological evaluation was performed every week showing that colitis severity increased with the duration of disease (Fig. 3.1 C). At six weeks post transfer, the colon was grossly enlarged, thickened and often adherent to adjacent viscera. Histologically, as indicated by the colitis score, there was severe pathology with extensive severe chronic active transmural inflammation and marked distortion and replacement of the normal architecture. The thickened mucosa was characterized by elongated irregularly shaped glands, and the lamina propria, sub-mucosa and serosa were expanded by dense inflammatory infiltrates.

Fig. 3.1 (A-C)

Figure 3.1 A - Serial weights plotted as % of original weight over time post transfer. Initial body weights averaged 18.8 ± 0.8 g. One recipient died naturally from severe colitis at 6 weeks post transfer when the experiment was terminated.
Results

Figure 3.1 B - FACS plot depicting intracellular stainings of IFN-γ and IL-17 in one H\(^+\) RAG\(^{-/-}\) mouse with colitis, 4 weeks post adoptive transfer, compared to naïve T cells derived from wt donors. Splenocytes and colonic lymphocytes were stimulated \textit{in vitro} with PMA, ionomycin and brefeldin before staining.

Figure 3.1 C - Colon transverse histological sections indicate individual colitis scores (H&E staining; original magnification - x10). Recipients of the same transfer depicted in Fig. 3.1A.

Figure 3.1

Effect of transfer naïve CD4\(^+\)CD45RB\(^{\text{high}}\) T cells into H\(^+\) RAG1\(^{-/-}\) (B10.PL)

H\(^+\) RAG\(^{-/-}\) mice (n=11, 6-8 weeks old) received 3x10\(^5\) CD4\(^+\)CD45RB\(^{\text{high}}\) T lymphocytes (purity 100%) isolated from syngeneic H\(^+\) Thy1.1\(^+\) donors.
In a separate experiment, to assess if the *Helicobacter* status of the recipient determines the rapidity of onset and the severity of T cell-induced colitis, H+ wt donor naïve T cells were transferred to H+ and H− RAG−/− recipients (the latter re-derived by embryo transfer into a *Helicobacter*-free environment). In this transfer, CD25 exclusion by sorting of the naïve T cell population and subsequent use of CD4+CD45RB<sup>high</sup>CD25<sup>−</sup> T cells further restricted the number of Foxp3+ T lymphocytes in the transfer. Analysis of Foxp3 expression in a wt Thy1.1 donor before transfer reveals that it is reduced from 1.7 % in the CD4+CD45RB<sup>high</sup> to 0.35 % in the CD4+CD45RB<sup>high</sup>CD25<sup>−</sup> T cell population (Fig. 3.2).

![Frequency of Foxp3 among CD4<sup>+</sup> cells in CD45RB<sup>high</sup> and CD45RB<sup>high</sup>CD25<sup>−</sup> T cell pools](image)

**Figure 3.2**

*Frequency of Foxp3 among CD4<sup>+</sup> cells in CD45RB<sup>high</sup> and CD45RB<sup>high</sup>CD25<sup>−</sup> T cell pools*

Representative FACS analysis of pooled mLn and nmLn obtained from a Thy1.1<sup>+</sup> wt donor. Number inside dot plots represents the frequency of Foxp3 among CD4<sup>+</sup> cells in the CD45RB<sup>high</sup> and CD45RB<sup>high</sup>CD25<sup>−</sup> T cell pools.

As shown in Fig. 3.3 A, colitis in the H+ had an earlier onset and caused more severe weight loss and spontaneous mortality than in H− recipients. This was reflected in the differences in colitis scores which were higher in the H+ mice (Fig. 3.3 B). In this transfer, the induced colitis was more severe than in the transfers shown in Fig. 3.1, possibly due to a heavier colonization by *Helicobacter* recipient mice (Fig. 3C). Mice were sacrificed at five weeks post transfer and FACS analysis revealed the presence of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells. These were reduced, both in percentage and number, in the H+ compared to the H− recipients both in the mLn and nmLn (Fig. 3.3 D).
**Figure 3.3 A** - Number of mice surviving natural death

<table>
<thead>
<tr>
<th>Condition</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAG(^{-/}) No transfer</td>
<td>0/9</td>
</tr>
<tr>
<td>H(^{-}) recipient</td>
<td>6/9</td>
</tr>
<tr>
<td>H(^{+}) recipient</td>
<td>8/9</td>
</tr>
</tbody>
</table>

**Figure 3.3 B** - Representative colon transverse histological section from recipient mice analysed at 5 weeks post transfer. Individual colitis scores are indicated (H&E staining; original magnification - x10)
Figure 3.3 continued

**Figure 3.3 C** - Shown are bands corresponding to amplification of *Helicobacter spp* positive DNA from faecal material in RAG−/− recipient mice depicted in Fig. 3.1A and 3.3. NTC: No Template Control, C: Control

**Figure 3.3 D** - Plots show number of CD4⁺ T lymphocytes and frequency and number of CD4⁺Foxp3⁺ T cells in mLn and nmLn at 5 weeks post transfer

**Figure 3.3**

**Effect of the Helicobacter status of the recipient on the severity of the T cell-induced colitis**

H⁺ and H⁻ RAG⁺⁺ recipients (6-8 weeks old) of 3x10⁵ CD4⁺CD45RB⁺⁺CD25⁻ T lymphocytes (99.5 % purity) from H⁺ syngeneic donors. Weight over time expressed as a percentage of the weight at the beginning of the experiment. Initial body weights averaged 20.7 ± 1.8 g.
3.3.2. The specificity of the immune response to *Helicobacter*

To determine if T cell-induced colitis in H+ recipients occurs in the presence of a predominant immune response to *Helicobacter* in the gut, T cell-induced colitis was induced by transfer of 2x10^5 CD4^+CD45RB^high^CD25^-^ T lymphocytes (purity 99%) pooled from H- wt donors into H+ RAG^-/-^ recipients. At 6 weeks post transfer, when weight loss corresponded to 87% of the original value, 3x10^5 CD4^+CD25^-^ T lymphocytes were purified from 1 mouse (% original weight shown in Fig. 3.4 A) and re-transferred to H- RAG^-/-^ recipients (n=4). Mild weight loss or diarrhoea were only present from 14 weeks post transfer in two out of four mice (Fig. 3.4 B), signalling the presence of late onset mild colitis, with intestinal mucosal hypertrophy and a mild cellular infiltrate documented at 15 weeks post transfer (Fig. 3.4 C). The other two mice remained healthy.

**Fig. 3.4 (A-D)**

![Figure 3.4 A](image)  **H+ RAG^-/-^**  7-week old recipient of naïve 2x10^5 CD4^+CD45RB^high^CD25^-^ T lymphocytes (99 % purity) from syngeneic wt donors. Weight over time is expressed as a percentage of the weight at the beginning of the experiment. Initial body weight = 17.4 g. At 6 weeks post transfer, when weight loss corresponded to 87% of the initial value, 3x10^5 CD4^+CD25^-^ T lymphocytes were purified and transferred to recipients in B. Change in weight over time is expressed as a percentage of the weight at the beginning of the experiment.

![Figure 3.4 B](image)  **H+ RAG^-/-^**  6-week old recipients (n=4) of 3x10^5 CD4^+CD25^-^ T lymphocytes (99 % purity) from mouse in A (Initial body weight 18.3 g ± 1.6 g)
Fig. 3.4 continued

No diarrhoea
Score 0/9 (n=2)

No diarrhoea
Score 3/9 (n=2)

Figure 3.4 C - Representative colon transverse histological section from mice in Fig. 3.3B analysed at 15 weeks post transfer. Colitis scores are indicated (H&E staining; original magnification x10)

Figure 3.4 D - H⁻ (n=3) and H⁺ (n=1) RAG⁻/⁻ 7-week old recipients of 3x10⁵ CD4⁺CD25⁻ T lymphocytes derived from 2 mice with T cell-induced colitis at 5 weeks post transfer. Initial body weight of 20.8 g ± 0.7 g and 19.5, for H⁻ and H⁺ respectively). The H⁺ recipient died at 8 weeks post transfer with severe colitis

Figure 3.4 (A – D)

Immune response to Helicobacter in colonized recipients with T cell-induced colitis.

A repeat experiment compared the transfer of 3x10⁵ CD4⁺CD25⁻ T lymphocytes from mice with ongoing T cell-induced colitis between H⁺ and H⁻ recipients in the recipients. Diarrhoea onset was much earlier in H⁺ than in H⁻ recipients (at 1.3 versus 5.3 weeks respectively). Weight loss was more severe in the H⁺ recipient that died at eight weeks post transfer while the H⁻ recipients looked healthy at the time of sacrifice, 15 weeks post transfer (Fig. 3.4 D). In both sets of
results 132 experiments (described in Fig. 3.4 A and D), a chronic halo of hair loss was noted in a periorbital distribution, from seven weeks post transfer. The presence of hair loss in a periorbital distribution was sporadically seen in other cell transfers of naïve T cells, where mice survived more than six weeks after transfer, and its significance is uncertain. It may reflect the presence of physiological self-reactivities in the donor.

3.3.3. Effect of ongoing colitis on regulatory T cells
To evaluate the frequency of donor CD4+ T cells expressing Foxp3 in the course of T cell-induced colitis (histologies in Fig. 3.1 C), one animal was sacrificed for weekly FACS analysis. The frequency of CD4+Foxp3+ T cells of donor origin was found to increase progressively reaching a peak at four weeks post transfer with most of the Foxp3+ T cells expressing CD25+ (Fig. 3.5 A). From week four post transfer, the fold increase of Thy1.1+CD4+Foxp3+ T cells was higher than that of the CD4+Foxp3− counterpart indicating preferential accumulation of Treg (Fig. 3.5 B).

Figure 3.5 A - Weekly FACS analysis during the course of colitis induced by transfer of 3x10^5 CD4+CD45RB<sup>high</sup> T cells (experiment in Fig. 3.1)
Fig. 3.5 continued

![Graph showing the response of regulatory T cells to inflammation](image)

**Figure 3.5 B** - Fold increase of Thy1.1^CD4^Foxp3^+ T cells is calculated according to the number of lymphocytes recovered from mLn, nmLn and Sp, divided by the number of cells injected per mouse. The number of CD25^+ T cells inoculated was calculated from the average frequency of CD4^Foxp3^+T cells found in the CD45RB<sup>high</sup> population, obtained from 4 separate wild type mice and yielding a percent value of 1.2 ± 0.4. Every transfer of 3x10^5 sorted CD4^CD45RB<sup>high</sup>T lymphocytes included 3780 CD4^Foxp3^+ T cells.

3.3.4. Recipient analysis after Foxp3^- T cell transfer

In this study the question of *de novo* conversion of naïve to Foxp3^+ Treg was examined by purifying donor cells from Foxp3<sup>gfp</sup> reporter knockin mice, whereby sorting of green fluorescent protein (GFP) negative T lymphocytes allowed for exclusion of contaminating Foxp3^+ T cells. A highly pure CD4^+CD45RB<sup>high</sup>GFP^- T cell population was transferred to RAG^-/-H^- recipients and GFP^-CD4^+ T cells were detected in the surviving recipient, a result indicating of conversion of naïve to Treg T cells (Fig. 3.6 A). Even though there was no particular difference in the weight curves (Fig. 3.6 B), reflecting a similar severity of T cell-induced colitis with maximal scores at nine weeks post transfer (Fig 3.6 C), there was no mortality in the recipients of GFP unsorted CD4^+ T cells contrasting with two thirds of the recipients that received GFP^- T cells and died.
Figure 3.6 A - FACS analysis displays the frequency of GFP expressing cells in sorted CD4+CD45RB<sup>high</sup> and CD4+CD45RB<sup>high</sup>GFP<sup>-</sup> donor populations from Foxp3<sup>gfp</sup> knockin mice and the corresponding frequency in recipients sacrificed for analysis at nine weeks post transfer.

Figure 3.6 B - Survival and weight curves of H<sup>-</sup> syngeneic RAG<sup>−/−</sup> mice recipients of 3x10<sup>6</sup> CD4+CD45RB<sup>high</sup> or 3x10<sup>6</sup> CD4+CD45RB<sup>high</sup>GFP<sup>-</sup> T cells.
3.4. Discussion

3.4.1. The *Helicobacter* status of the recipient determines the rapidity of onset and the severity of T cell-induced colitis

This is the first report of T cell-induced colitis in B10.PL mice. Taken together, the weight curve, cytokine expression of effector T cells and the histological scores allow for the conclusion that T cell-induced colitis in B10.PL H2u RAG-1−/− recipients follows a course similar to that which occurs in Balb SCID and C57BL/10 RAG-2−/− mice (Powrie et al., 1993; Powrie et al., 1997; Kullberg et al., 2002).

Typically, an initial weight gain, up to approximately 110% of the original weight in the first week is followed by a weight loss from two to five weeks onwards. It should be noted that in our SPF *Helicobacter* free animal facility, T cell-induced colitis in B10.PL mice is well established at four weeks post transfer compared to 12 weeks in C57BL/6 mice. This may be particular to the breeding and housing conditions of donor and recipient B10.PL mice, which are located in different facilities from those of C57BL/6 mice or indeed reflect a greater susceptibility of B10.PL mice to T cell mediated colitis induction. In the present experiments, we are unable to distinguish
Discussion

genetic background from the influence of environmental factors such as gut bacteria that drive the inflammatory response.

At one end of the spectrum, we describe that B10.PL RAG-1 mice heavily *Helicobacter* colonized recipients of H+ donors develop severe wasting. This high mortality is comparable to that of SCID mice directly infected with *H. hepaticus* (Cahill et al., 1997). At the other extreme, H- B10.PL RAG-1-recipients, less colonized by *Helicobacter* developed a milder form of colitis after receiving H+ donor cells.

3.4.2. The immune response appears to be specific to *Helicobacter* in colonized recipients with T cell-induced colitis

These experiments suggest that in T cell-induced colitis in the presence of *Helicobacter*, the specificity of effector T cells to *Helicobacter* predominates over specificities for other gut bacteria. *H. hepaticus* Ag specific CD4+ Th1 clones have actually been shown to transfer disease to *H. hepaticus*-infected T cell-deficient RAG-1- hosts (Kullberg et al., 2003) and we have reproduced this finding with the transfer of colitogenic T cells. What is novel in this work is the demonstration that upon re-transfer to H- recipients, T cells from H+ mice with colitis are unable to reproduce the colitis they caused in the original donor as compared to the full blown colitis in the H+ recipient. This is in direct contrast with the fact that albeit with a delayed onset, severe colitis occurs in H- RAG-1-recipients after a naïve T cell transfer and is highly suggestive that the origin of disease is not autoimmune. If T cell-induced colitis was an AID then it would be expected that the CD25- transfer would yield severe fast disease in both *Helicobacter* positive and negative recipients, the opposite of what is herein described.

The possibility remains that DC require *Helicobacter* or other pathogens for activation and self Ag presentation, in order to promote T helper function. Indeed, this DC requirement may explain the absence of T cell-induced colitis in GF alymphoid recipients and this absence of T cell colitis induction after a transfer to H- recipients with colitogenic T cells that have already been primed. In any case, mice colonized with bacteria other than *Helicobacter* developed T cell colitis after a naïve T cell transfer, albeit milder, and the H- recipients of the colitogenic transfer must have been colonized by other bacteria. In the present work, results suggest that the model of T cell-induced colitis represents the immunopathology caused by an infection with specific gut bacteria, featuring *Helicobacter* at the top of the list.

In addition, these findings may provide an indirect explanation why CD4+CD45RBlow T cells from GF mice are capable of inhibiting colitis induced by CD45RBhigh T cells also derived from GF mice (Annacker et al., 2000), while in apparent contradiction, colitis in *H. hepaticus*...
infected RAG⁻/⁻ animals induced by the transfer of CD4⁺ T cells from infected colitic IL-10⁻/⁻ mice is only prevented by the co-transfer of CD45RB<sup>low</sup> cells from *H. hepaticus*-infected wt mice and not from uninfected wt animals (Kullberg et al., 2002). In wt mice, colonization with *Helicobacter* may lead to the development of Ag-specific Treg, which in turn protect the host from colitis and the phenomenon of Treg conversion, such as we describe, may well be the mechanism involved. If *Helicobacter* responses become immunodominant during the development of colitis then this fact may well explain the paradox that, in the presence of *Helicobacter*, Treg Ag specificity is required for protection. During colitis developing in the absence of *Helicobacter*, there are reactivities against more than one Ag and a polyclonal Treg population has a greater potential than a monoclonal population for expanding at least some of the correct specificities, even if it comes from GF mice. Therefore it is argued that control of disease by a polyclonal Treg population does not equal lack of specificity. It may well be that in humans, effector and Treg specificities vary on an individual basis depending on environmental factors and, in that context, the T cell colitis model may provide a useful research guide in humanized animal models.

3.4.3. Treg Cells accumulate selectively in T cell–induced colitis

An expanded *Helicobacter* specific effector T cell population of H⁺ donor origin may have been the most important contributing factor to higher disease severity in H⁺ compared to H⁻ recipients. Upon CD4⁺ T cell transfer to alymphoid recipients, the presence of Treg has actually been demonstrated in the lamina propria (Leithauser et al., 2006). The contribution of Treg must not be ignored as in Ln, Treg were found in smaller numbers in H⁺ than H⁻ recipients and differences in Treg distribution may be a cause or a consequence of colitis severity. However, we did not succeed in measuring Foxp3 in the colon and therefore whether this phenomenon is a direct consequence of migration pressure to a more inflamed colon remains an open question. It is purely speculative but worth considering that *Helicobacter* could have an inhibitory effect on Treg, resulting in more severe pathology.

This preliminary work demonstrates that even in the presence of an overwhelming inflammatory disease such as T cell-induced colitis and in the presence of activated T lymphocytes, Treg accumulate either due to expansion of the small amount of CD45RB<sup>high</sup>Foxp3<sup>+</sup> present in the inoculum and/or from conversion of naïve T cells in the transfer.

3.4.4. Conversion from Foxp3⁻ to Foxp3⁺ T cells in ongoing T cell-induced colitis

Previous *in vivo* studies report conversion of naïve T cells to Treg, with a frequency of converted Foxp3<sup>+</sup> T cells up to 25%, but these have been performed within days of transfer, in
the absence of inflammation and particularly in the absence of T cell-induced colitis (Curotto de Lafaille et al., 2004; Apostolou and von Boehmer, 2004; Liang et al., 2005; Kretschmer et al., 2005). In the present study, the presence of GFP+ T cells in the recipients of total CD4+CD45RBhighGFP− T cells is highly indicative of peripheral conversion from naïve to Foxp3+ Treg and occurring in the presence of T cell-induced colitis. This is in contrast with a previously reported insignificant conversion frequency to Foxp3+ T cells up to 6 weeks post naïve T cell transfer, also in the present of T cell-induced colitis and using a similar stringent system of exclusion of Foxp3+ donor cells from FOXP3-IRES-MRFP knockin mice [Wan and Flavell, 2005]. The protective role of the Treg inoculum is indicated by the lack of mortality in the recipients of the total CD4+CD45RBhighGFP containing transfer. It remains to be tested whether co-transfer of this converted Treg population with naïve T cells protects from colitis and whether converted Treg may be more efficient suppressors than natural Treg. This result suggests that inflammation promotes Treg conversion.

3.4.5. Concluding remarks and implications for clinical practice

Several additional factors, besides the Helicobacter load of the recipients, may have contributed to the disparity in the course of colitis between the H+ recipients. The CD45RBhighCD25− population contains approximately one third of the Foxp3+ T cells in the CD45RBhigh transfer unfractioned for CD25 and therefore may have contained less Treg. Furthermore, the donor T cells were from an H+ colony in the milder colitis group, and therefore, the small Treg inoculum which was transferred may have contained more Helicobacter-specific protective Treg (and arguably more Helicobacter-specific effector T cells). Our results suggest that, in ongoing T cell-induced colitis, the population of Treg accumulates and recipients of a larger and more likely to be Ag-specific Treg inoculum have improved disease control. Formal testing through transfer of differential sorted populations from the same pool of donors still needs to be undertaken. Overall, the combination of an H+ donor population, a small and non-specific Treg inoculum and a heavy Helicobacter load, likely places the recipient in a disadvantageous position for colitis development. Due to natural infection with Helicobacter and a probable increase in the Helicobacter load with the passage of time, the findings may not be reproduced unless the degree of Helicobacter colonization is accurately quantified and controlled.

In conclusion, we confirm the influence of luminal content on colitis outcome. We determine that, when colitis occurs in the presence of Helicobacter, the immune response is dominant ie it is specific for this microorganism, and we theorize this is the reason why it requires an Ag-specific Treg response for disease control, in contrast to the apparent lack of specificity.
requirement in non-Helicobacter induced colitis. Even in the presence of severe inflammation due to ongoing T cell colitis, Foxp3+CD4+ T cells are able to accumulate selectively and conversion from naive to Foxp3+ T cells also occurs. These Treg play an important role in regulation by decreasing mortality in the T cell-induced colitis model and therefore this work is highly suggestive that inflammation promotes Treg expansion and function but this remains to be formally tested.

No completely effective therapeutic strategy has been established because the etiology of IBD remains largely unknown. Current medical treatments for IBD rely on the use of nonspecific anti-inflammatory agents and immunosuppressive drugs that cause severe side effects, and in significant percentage of the patients, do not induce long-term benefit (Sandborn and Targan, 2002; Baert et al., 2004). Induction of remission has been improved by the use of Tumour Necrosis Factor-α neutralising antibodies (Akobeng and Zachos, 2004; Lawson et al., 2006) but recurrence of disease activity remains frequent (Sandborn et al., 2007). However, anti-TNF therapy may be associated with considerable morbidity (Kwon and Farrell, 2005) and there remains a need for new and well tolerated therapies that effectively induce remission and alter the natural course of the disease.

Even though the presence of Treg has been demonstrated in the inflamed mucosa and lymph nodes of patients with IBD (Pallone et al., 1987; Leach et al., 1996; Kelsen et al., 2005; Uhlig et al., 2006; Yu et al., 2006; Yu et al., 2007), Treg frequencies are reportedly lower in IBD than in patients with other inflammatory gut pathologies such as diverticulitis (Maul et al., 2005) but are expanded in mucosal lymphoid tissues (Saruta et al., 2007). A translation of these together with our findings into clinical practice effectively means that even a very small number of Treg is probably beneficial in ongoing disease. The realization that inflammation is an important stimulus for Treg stresses the potential therapeutic role of Treg supplementation during anti-inflammatory/ immunosuppressive therapy. It is likely to be long until we know whether in individual humans, IBD reflects an autoimmune disease, a defective immune system or specific gut colonization by specific microbes. In parallel to the role of Helicobacter colonization in animal models, the present work indicates that a search for microbes which colonize human IBD patients and elicit an immunodominant response is worthwhile, as for these cases, there may be a requirement for Ag specificity in Treg based therapies.
Current medical treatments for EAE rely on the use of immunosuppressive drugs that cause severe side effects, and in a significant percentage of the patients, do not lead to a cure. Cellular immunoregulatory therapy may provide a well tolerated therapeutic alternative, which could effectively induce remission and alter the natural course of the disease. Based on the immunopathological nature of EAE, several strategies have been proposed, such as attempts to deviate the CD4 pathogenic T cells from Th1 to Th2 phenotype or alter cytokine patterns but these have met with limited success. In accordance with the hygiene hypothesis, the notion that organ-specific autoimmune disease is poorly induced in animal colonies of relaxed biocontainment rules inspired the present work. Based on the evidence that T-cell induced colitis results in an inflammatory immune response, we directly tested whether this entity could affect the course of a bona fide AID. Colitis was induced by transfer of naïve T cells in anti-MBP TCR transgenic mice that develop spontaneous EAE. We first evidenced that T cell-induced colitis completely prevented EAE development but was unable to suppress established EAE. Prevention of EAE only occurred with rapid colitis induction, in the presence of Helicobacter colonization of recipient mice, and within a time window which corresponded to that expected from protection with a regulatory T cell transfer. Protection was found to be dependent on the presence of the transferred T lymphocytes, as chemical colitis was unable to protect from EAE. Next we investigated whether this protection was regulatory T cell mediated and documented donor Foxp3+ regulatory T cell
accumulation. The results indicated that the protective immune responses that followed the acute phase of T cell-induced colitis were mediated by donor CD4+CD25− T cells and revealed the protective nature of the Treg population, through a milder form of colitis elicited upon re-transfer. Foxp3 expression was also induced in the transgenic T cell population upon colitis development. These anti-MBP Foxp3+ T cells alone did not prevent EAE but may have contributed to: (i) the reduced proliferation of transgenic cells from “T-cell colitis protected mice” in response to MBP; (ii) the milder form of EAE that resulted from cure of colitis by donor T cell depletion and from the transfer of cells derived from the “T-cell colitis protected mice” to RAG−/− recipients. Differential migration of the tg population to the bowel may also have contributed to EAE protection. This work is suggestive that a high Foxp3 expression may be a consequence of AID and chronic inflammation and while the presence of Foxp3 is not synonymous with full tolerance, it may still modulate immunopathology and partially control autoimmunity. Further work, such as the identification of chemokine, adhesion molecule and cytokine patterns involved will elucidate specific colitogenic T cell requirements for EAE protection.

4.1. Introduction

4.1.1. Immune mechanisms in immuno inflammatory diseases

Experimental models of experimental autoimmune encephalomyelitis (EAE) have provided substantial information on disease pathogenesis. Chemical or T-cell induced colitis have an effect on different arms of the immune system, namely the innate and adaptive immune responses respectively, allowing for the study of differential interactions between these two forms of colitis and EAE. The description that follows aims to identify potential levels of interaction between these models of colitis and the mechanisms involved in EAE pathogenesis, with a special focus on the inhibition of autoimmunity.
4.1.1.1. Experimental auto-immune encephalomyelitis

4.1.1.1.1. Experimental models

EAE reflects a collection of animal models first described in monkeys (Rivers et al., 1933) and recently reviewed by Steinman and Zamvil (2006), developed to reproduce different features of multiple sclerosis, a demyelinating human auto-immune disease (AID) of the central nervous system (CNS). As a model, it has been subjected to many refinements and variations over the past 75 years. Originally designated “experimental allergic encephalomyelitis”, the name EAE evolved to experimental autoimmune encephalomyelitis, once the pathogenic potential of myelin specific T lymphocytes was identified, nearly thirty years ago, by myelin basic protein (MBP) immunization of Lewis rats (Ortiz-Ortiz and Weigle, 1976), by EAE induction following lymphocyte re-transfer to irradiated recipients (Paterson, 1966; Paterson and Harvey, 1978) and more recently by the identification, in wt mice (Fritz et al., 1983) and healthy humans (Ota et al., 1990), of T cells responsive to MBP.

EAE can be induced by a broad spectrum of T cells that react against a diversity of CNS antigens. Experimental models include (i) active immunization with CNS tissue or myelin components; (ii) adoptive transfer of sensitized T lymphocytes from animals with EAE or established cell lines or (iii) genetically manipulated mice with a TCR specific for one of the myelin antigens, allowing for the study of EAE which occurs spontaneously or with minimal induction. While in isolation none of these models correspond exactly to human MS, together they complement each other. In actively induced EAE, immunization with several encephalitogenic proteins from myelin, such as MBP, proteolipid protein (PLP) or myelin oligodendrocyte glycoprotein (MOG) leads to Ag-specific T cell activation, proliferation and migration with invasion of the CNS, causing autoimmune inflammation and subsequent paralysis.

Clinical variations in human MS are reproduced in animal models depending on the genetics of the experimental animals and the specific antigen used for induction. In Lewis rats, MBP immunization leads to a monophasic illness with no relapses which may be lethal, and is characterized histologically by severe inflammation, axonal damage but no demyelination (Levine and Wenk, 1965). In contrast, MOG immunization in Lewis rats results in chronic demyelinating disease (Storch et al., 1998). In H2u mice, MBP induced demyelination is mild and self-contained (Zamvil et al., 1986) and in the SJL/J mouse is characterized by a relapsing and remitting pattern (Brown and McFarlin, 1981; Lublin et al., 1981). In B6 mice, MOG immunization varies according to the strength of the adjuvant used for induction, resulting in remitting disease with mild induction (McGeachy et al., 2005) or in a progressive non-remitting and often lethal form of disease with strong stimulation (Mendel et al., 1995; Chora et al., 2007). The mechanisms determining these
different disease manifestations are largely unknown. Adoptive transfer models display the same type of variability according to the specificity of the T cells and recipient strain and have provided proof of the role of pathogenic T cells in EAE induction, allowing for recognition of major antigenic epitopes and characterization of the secretion pattern of the proinflammatory mediators, cytokines and chemokines that lead to demyelination.

4.1.1.1.2. The blood brain barrier
The brain has been classically considered to be a site of limited reactivity because allografts usually fare better in the brain than in other organs. This concept, termed immune privilege, has been attributed to several factors such as the presence of a blood brain barrier (BBB), an absent conventional lymphatic system, a paucity of antigen-presenting cells (APC), an inhibition of major histocompatibility complex (MHC) class II molecule expression and the presence of cytokines that inhibit the immune response in the brain. This limited reactivity was attributed to regulation rather than lack of immune response once it was realized that there seems to be a continuous and highly regulated communication between the brain and the immune system via cervical lymphatics and the BBB (Cserr and Knopf, 1992).

Differences in permeability of the cerebral capillaries to dyes, bacterial toxins, ions, metabolites and drugs when compared to capillaries from other tissues have led to the coining of the term “blood brain barrier”. This barrier has been attributed to morphological characteristics of the cerebral capillaries, where, unlike non-neural capillaries, the presence of an elaborate network of complex of tight junctions, narrow intercellular gaps, paucity of pinocytosis and an intact basement membrane inhibit transcellular passage of hydrophilic molecules. At the same time, the barrier has to meet the high metabolic needs of the CNS tissue and, as such, specific transport systems selectively expressed in the capillary brain endothelial cell membranes mediate the directed transport of nutrients and toxic metabolites in and out of the CNS, respectively (Sage, 1982). Cerebral capillaries have a closely investing glial sheath, composed of the “end-feet” of astrocytes which have been shown to be responsible for the BBB properties (Janzer and Raff, 1987). Using ovalbumin- and MBP- specific T blasts labelled with 14C-thymidine, Wekerle, Linnington at al (1986) demonstrated that the endothelial BBB regulates lymphocyte entry into the CNS by limiting it to freshly activated (but not resting) T lymphocytes of any specificity. Under physiological conditions lymphocyte entry into the healthy CNS across the BBB is therefore kept at a low level.
4.1.1.1.3. Effector lymphocyte activation

Only upon activation by specific antigens do encephalitogenic T cells initiate disease (Wekerle et al., 1987; Hickey, 1991). Adoptive transfer models have revealed that low numbers of activated encephalitogenic T cells cross the BBB and infiltrate the CNS within hours of peripheral transfer, encountering their antigen presented by local APC. It is proposed that the brain-Ag specific T cells are reactivated within the CNS and start the molecular events leading to inflammation, resulting in loss of BBB permeability, a second massive wave of specific and non-specific T cell infiltration and finally demyelination. The tracking of myelin specific encephalitogenic GFP+ T cell lines has confirmed these findings and further showed a pre-CNS down-regulation of activation markers concomitantly with an upregulation of chemokine receptors specific for a series of chemokines known to be produced by CNS cells (Flugel et al., 2001). The severity of disease seems to be correlated with the level of T cell activation and resultant macrophage recruitment and effector capacity within the CNS (Kawakami et al., 2004).

The activation, differentiation and termination of T cell responses require both antigen/MHC recognition and co-stimulatory signals through B7-1 (CD80), B7-2 (CD86) and programmed death-1 (PD1) on the APC and their respective receptors CD28, cytotoxic T lymphocyte antigen (CTLA-4) and PD1 ligand on the T lymphocyte, of which CTLA-4 and PD-1 are inhibitory to the immunological synapse. Induction of EAE can be modulated in mice that are knock-out for these signals or that are subjected to their administration or antagonism. EAE can be induced in CD28−/− mice suggesting an alternative pathway (Chitnis et al., 2001), CTLA-4 blockade has been shown to exacerbate EAE or accelerate disease relapse in susceptible strains of mice (Karandikar et al., 1996; Perrin et al., 1996; Hurwitz et al., 1997) and CTLA-4 administration diminishes disease (Perrin et al., 1995). Contrary to E- and P-selectin, L-selectin expression on T lymphocytes is an absolute requirement for active EAE induction (Grewal et al., 2001; Li et al., 2006; Doring et al., 2007).

The multi-step model of lymphocyte endothelial interaction (Butcher et al., 1999) involving adhesion and signalling molecule activation in both lymphocyte and endothelium applies to the BBB. Enhanced leucocyte traffic into the CNS, during EAE, implies that the BBB endothelium expresses a particular pattern of adhesion molecules and chemokines which is matched by the phenotype of the ligands of activated but not resting T cells. In brief, and according to a recent review by Engelhardt (2006), BBB constitutively expresses VCAM-1 which mediates the G-protein independent prompt arrest (capture) and adhesion of circulating encephalitogenic T cell blasts via α4-integrin. Expression of CCR7 is detected on encephalitogenic T cells, which chemotax specifically towards both CCL19 and CCL21 in a
Introduction

concentration dependent and pertussis toxin sensitive manner. Engagement of T cells via LFA-1 on endothelial ICAM is required for transmigration.

Just like for most AID, it is not known how MS develops. As opposed to active EAE and passive transfer models, where T lymphocytes are forcibly activated, lymphocyte activation occurs spontaneously in tg mouse models. The BBB is normally a barrier to resting T cells but the chance that an MBP-specific CD4 T lymphocyte reaches the parenchyma of the brain is much higher in tg than in wt mice. Even if the latter was true, activation and clonal proliferation within the CNS are unlikely, as immune reactions within the CNS favour immunosuppression (Cserr and Knopf, 1992). Therefore, even if the initiation of the autoimmune attack were to occur in the target organ, it is most probably followed by myelin reactive lymphocyte activation in the Ln.

4.1.1.1.4. Role of cytokines

Over the past 20 years, the direct administration of cytokines, their receptors or antibodies and mouse knock-out technology applied to EAE models have identified cytokines that play a role in EAE potentiation or inhibition, acting directly on the CNS or via immune response control in the periphery. Functional pleiotropism, overlapping and, in part, redundant functions at the induction and effector phases of EAE prevent a simple statement about the role of individual cytokines. Furthermore cytokines act via multi-component receptor molecules that are themselves common to several cytokines, examples of which are IL-2 and IL-4, that use receptors that share the common $\gamma$-chain ($\gamma_c^3$) as one of their receptor subunits. As new cytokines and new polarized immune responses are identified, the system allows for the unravelling of seemingly puzzling contradictory results.

Within the inflamed CNS, lymphocytes, astrocytes, macrophages, and microglia have been identified as sources of cytokine production (Link, 1998). As recently summarized (Harrington et al., 2005), sub-populations of CD4 T lymphocytes have been assigned to the T helper type 1 (Th1) or Th2 lineage based on distinct cytokine profiles: Th1 cells are defined on the basis of their production of IFN-$\gamma$ and Th2 cells produce IL-4, IL-5 and IL-13. Th1 and Th2 development diverges rapidly after antigen priming to produce mature effector cells with mutually exclusive expression of IFN-$\gamma$ and IL-4, respectively, IFN-$\gamma$ and IL-27 induce STAT1 signalling and T-bet expression in antigen-activated, naïve CD4$^+$ T cells, leading to up-regulation of the IL-12 receptor (IL-12R) on developing Th1 cells and suppression of GATA-3. Similarly, IL-4 produced by mature Th cells initiates Th2 cell development through its up-regulation of GATA-3 via STAT6 and suppresses Th1 cell development by blocking IL-12R expression. These two Th
cell populations cross-regulate one another because their respective cytokines act antagonistically.

Earlier work in an active EAE model identified IFN-γ and TNF-α peaking at the height of the acute phase and relapses and conversely, IL-4, IL-10 and TGF-β in the CNS were associated with recovery from EAE in mice and rats (Kennedy et al., 1992; Khoury et al., 1992; Begolka and Miller, 1998; Begolka et al., 1998). The naïve belief that those cytokines that were found circulating or in CNS lesions were actually contributing to disease pathogenesis led to initial efforts to inhibit EAE through cytokine manipulation. These attempts, using supplementation of those cytokines that were present in the recovery period or antagonism of those that were present at the peak of disease, proved meaningless. Major studies on the effect of cytokine manipulation in EAE models are summarized in Table 4.I.

For example IFN-γ, known to be important for the generation of Th1 effector T cells, was found to be peaking at the height of EAE. Nevertheless, studies indicate a suppressive rather than pathogenic role for IFN-γ in EAE. Administration of anti–IFN-γ Ab or inactivation of either the IFN-γ or IFN-γ R gene leads to exacerbation of disease in susceptible strains of mice and to loss of strain resistance to EAE induction. Administration of IFN-γ to mice with EAE inhibited the disease and prevented further relapses but, disappointingly, in humans with established disease this therapy was associated with exacerbations (Panitch et al., 1987). As interest in IFN-γ waned, chronic IFN-β therapy was marginally successful in clinical trials and is now routine treatment for MS despite a modest impact on disease progression, reducing disease exacerbations by only about 30%. Its mode of action has been attributed to IL-4 and IL-10 induction, inhibition of NFκB activity and IL-17, and reduction of cell adhesion to the BBB (Sospedia and Martin, 2005; Martin-Saavedra et al., 2007). IFN-β−/− mice are more susceptible to EAE than their wt littermates (Teige et al., 2003). IL-4, IL-10 and TGF-β seem to have a role in illness limitation. IL-10−/− but not IL-4−/− mice are more susceptible to EAE induction with both developing more severe disease than wt mice (Liblau et al., 1997; Bettelli et al., 1998).

The understanding of the contribution of cytokines for the pathogenesis of EAE is evolving, an example of which is IL-12, recently reviewed (Cheng et al., 2007). IL-12 is composed of a 40 KDa heavy chain (p40) and a 35 KDa light chain (p35). IL-12p40-deficient mice are resistant while IL-12p35-deficient mice are susceptible to EAE induction. IL-12 receptor β1-deficient mice are completely resistant while IL-12 receptor β2-deficient mice develop very severe EAE. Recent data have shown that the perceived role of IL-12 is actually due to IL-23, a closely
related cytokine sharing the p40 subunit and the beta1 receptor chain with IL-12 (Gran et al., 2004).

**Table 4.1 - T lymphocyte subset, receptor and cytokine requirements for EAE induction**

<table>
<thead>
<tr>
<th>Knock-out</th>
<th>Antibody</th>
<th>Cytokine</th>
<th>Strain - Peptide</th>
<th>Adoptive</th>
<th>EAE</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>T-bet</td>
<td>-</td>
<td></td>
<td>B6 - MOG</td>
<td>Inhibition</td>
<td></td>
<td>Bettelli, et al., 2004</td>
</tr>
<tr>
<td>IFN-γ R</td>
<td>-</td>
<td></td>
<td>SJL - MOG</td>
<td>Potentiation</td>
<td></td>
<td>Willenborg, et al., 1996</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>+</td>
<td></td>
<td>Balb - MBP</td>
<td>Potentiation</td>
<td></td>
<td>Krakowski and Owens, 1996</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>+</td>
<td></td>
<td>B6 – spinal cord</td>
<td>Potentiation</td>
<td></td>
<td>Billiau, et al., 1988</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>+</td>
<td></td>
<td>SJL – MBP</td>
<td>Potentiation</td>
<td></td>
<td>Duong, et al., 1992</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>+</td>
<td></td>
<td>SJL – MBP</td>
<td>Potentiation</td>
<td></td>
<td>Lublin, et al., 1993</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>+</td>
<td></td>
<td>Balb – MBP</td>
<td>Potentiation</td>
<td></td>
<td>Duong, et al., 1994</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>+</td>
<td></td>
<td>B6 – spinal cord</td>
<td>Potentiation</td>
<td></td>
<td>Willenborg, et al., 1996</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>+</td>
<td></td>
<td>SJL – MBP</td>
<td>Inhibition</td>
<td></td>
<td>Billiau, et al., 1988</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>+</td>
<td></td>
<td>Lewis – MBP</td>
<td>Inhibition (i.v. no effect)</td>
<td></td>
<td>Voothuis, et al., 1990</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>+</td>
<td></td>
<td>Human Therapy</td>
<td>Potentiation</td>
<td></td>
<td>Panitch, et al., 1987</td>
</tr>
<tr>
<td>IFN-αβγ R</td>
<td>-</td>
<td></td>
<td>129/SvEv - MOG</td>
<td>Potentiation</td>
<td></td>
<td>Fabis, et al., 2007</td>
</tr>
<tr>
<td>IFN-β</td>
<td>-</td>
<td></td>
<td>B10.PL - MBP</td>
<td>Potentiation</td>
<td></td>
<td>Teige, et al., 2003</td>
</tr>
<tr>
<td>IFN-β</td>
<td>+</td>
<td></td>
<td>SJL - MBP</td>
<td>Inhibition</td>
<td></td>
<td>Martin-Saavedra, et al., 2007</td>
</tr>
<tr>
<td>IFN-β</td>
<td>+</td>
<td></td>
<td>Human Therapy</td>
<td>Inhibition</td>
<td></td>
<td>Jacobs, et al., 1996</td>
</tr>
<tr>
<td>IFN-β</td>
<td>+</td>
<td></td>
<td>Human Therapy</td>
<td>25% non-response</td>
<td></td>
<td>Jacobs, et al., 2000</td>
</tr>
<tr>
<td>IFN-β</td>
<td>+</td>
<td></td>
<td>Human Therapy</td>
<td>30% non-response</td>
<td></td>
<td>Rudick et al., 2004</td>
</tr>
<tr>
<td>IFN-β</td>
<td>+</td>
<td></td>
<td>Human Therapy</td>
<td></td>
<td></td>
<td>Waubant, et al., 2003</td>
</tr>
<tr>
<td>TNF-α</td>
<td>-/-</td>
<td></td>
<td>B6 – MOG</td>
<td>Inhibition</td>
<td></td>
<td>Kassiotis and Kollias, 2001</td>
</tr>
<tr>
<td>TNF-α</td>
<td>+</td>
<td></td>
<td>B6 – MOG</td>
<td>Inhibition</td>
<td></td>
<td>Fabis, et al., 2007</td>
</tr>
<tr>
<td>TNF-α</td>
<td>+</td>
<td></td>
<td>Biozzi AB/H – Sp cord</td>
<td>Inhibition</td>
<td></td>
<td>Baker, et al., 1994</td>
</tr>
<tr>
<td>TNF-α</td>
<td>+</td>
<td></td>
<td>SJLUJ – MBP</td>
<td>Inhibition</td>
<td></td>
<td>Ruddle, et al., 1990</td>
</tr>
<tr>
<td>TNF-α</td>
<td>+</td>
<td></td>
<td>SJLUJ – MBP</td>
<td>Inhibition</td>
<td></td>
<td>[Selma, 1995 #9577]</td>
</tr>
<tr>
<td>TNF-α</td>
<td>+</td>
<td></td>
<td>Human Therapy</td>
<td>Potentiation</td>
<td></td>
<td>van Oosten, et al., 1996</td>
</tr>
<tr>
<td>IL-2 R</td>
<td>+</td>
<td></td>
<td>Lewis – MBP</td>
<td>No effect</td>
<td></td>
<td>Robinson, et al., 2001</td>
</tr>
<tr>
<td>IL-2</td>
<td>+</td>
<td></td>
<td>SJL – MBP</td>
<td>No effect</td>
<td></td>
<td>Mohan, et al., 2001</td>
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<tr>
<td>IL-4</td>
<td>-/-</td>
<td></td>
<td>B6 - MOG</td>
<td>Potentiation</td>
<td></td>
<td>Lilliu, et al., 1997</td>
</tr>
<tr>
<td>IL-6</td>
<td>-/-</td>
<td></td>
<td>B6 – MOG</td>
<td>Inhibition</td>
<td></td>
<td>Bettelli, et al., 1998</td>
</tr>
<tr>
<td>IL-6</td>
<td>-/-</td>
<td></td>
<td>B6 – MOG</td>
<td>Inhibition</td>
<td></td>
<td>Okuda, et al., 1998</td>
</tr>
<tr>
<td>IL-6</td>
<td>-/-</td>
<td></td>
<td>B6 – MOG</td>
<td>Inhibition</td>
<td></td>
<td>Eugster, et al., 1998</td>
</tr>
<tr>
<td>IL-6</td>
<td>-/-</td>
<td></td>
<td>DA rat – spinal cord</td>
<td>Inhibition</td>
<td></td>
<td>Mendel, et al., 1998</td>
</tr>
<tr>
<td>IL-6</td>
<td>+</td>
<td></td>
<td>Human therapy</td>
<td>Not done</td>
<td></td>
<td>Di Marco, et al., 2001</td>
</tr>
</tbody>
</table>

continues
Table 4.1 - continued

<table>
<thead>
<tr>
<th>Knock-out</th>
<th>Antibody</th>
<th>Cytokine</th>
<th>Strain - Peptide</th>
<th>Adoptive</th>
<th>EAE</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-10 -/-</td>
<td>TGF-β Human therapy - Phase 1 (n=11)</td>
<td>TGF-β</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Transfer of IL-10 -/- Treg</td>
</tr>
<tr>
<td>IL-12 + +</td>
<td>B6 - MOG</td>
<td>B6 - MOG</td>
<td>Inhibition (IFN-γ Dependent)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-22 -/-</td>
<td>+</td>
<td>+</td>
<td>B6 - MOG</td>
<td></td>
<td></td>
<td>Not affected</td>
</tr>
<tr>
<td>IL-23 (induces Th17) -/-</td>
<td>+</td>
<td>+</td>
<td>B6/129 - MOG</td>
<td></td>
<td></td>
<td>Inhibition</td>
</tr>
<tr>
<td>IL-25 (inhibits IL-23) -/-</td>
<td>+</td>
<td>+</td>
<td>B6 - MOG</td>
<td></td>
<td></td>
<td>Potentiation</td>
</tr>
<tr>
<td>IL-25 (inhibits IL-23) -/-</td>
<td>+</td>
<td>+</td>
<td>B6 - MOG</td>
<td></td>
<td></td>
<td>Inhibition (IL-13 dependent)</td>
</tr>
<tr>
<td>IL-17 -/-</td>
<td>+</td>
<td>+</td>
<td>B6 - MOG</td>
<td></td>
<td></td>
<td>Inhibition</td>
</tr>
<tr>
<td>IL-17 +</td>
<td>+</td>
<td>+</td>
<td>B6 - MOG</td>
<td></td>
<td></td>
<td>Inhibition</td>
</tr>
<tr>
<td>IL-27 R -/-</td>
<td>+</td>
<td>+</td>
<td>B6 - MOG</td>
<td></td>
<td></td>
<td>Potentiation</td>
</tr>
<tr>
<td>IL-27 (inhibits IL-17) +</td>
<td>+</td>
<td>+</td>
<td>B6 - MOG</td>
<td></td>
<td></td>
<td>Inhibition</td>
</tr>
</tbody>
</table>

IL-23 induces naïve precursor cells to differentiate into Th-17 cells independently of the transcription factors STAT1, T-bet, STAT4 and STAT6 (Harrington et al., 2005). In particular, IL-22-secreting Th17 cells were shown to specifically mark the highly pathogenic population of self-reactive T cells in EAE but IL-22 -/- mice turn out to be fully susceptible to EAE induction (Kreymborg et al., 2007), in contrast to IL-23 deficient mice that are resistant (Cua et al., 2003). Of note, Th1 cell development is dominant in the setting of IFN-γ and IL-17 availability but the importance of the Th17 pathway has been revealed by the demonstration that IL-17 -/- mice
develop milder and delayed EAE upon active induction (Komiyama et al., 2006). Moreover, the Th17 pathway explains previous unexpected results showing that anti-IFN-γ-treated mice, IFN-γ- or IFNR-deficient mice develop EAE.

4.1.1.1.5. Role of regulatory T cells

In parallel with the knowledge that a selected set of cytokines is detected in the recovery phase of EAE, the realization that Treg also contribute to spontaneous improvement has been put forward from as early as 1993, at a time when Treg markers had not yet been identified. CD4+ T lymphocytes were subsequently identified as inhibitors of the proliferative response to Ac1-9, able to protect H2u mice from disease induction and mediators of spontaneous recovery from MBP induced EAE, imparting resistance to disease re-induction (Kumar and Sercarz, 1993). Spontaneous EAE in TR- mice (the anti-MBP TCR tg model developed by Lafaille is reviewed in section 2) was subsequently shown to be prevented by transfer of CD4+ splenocytes or thymocytes from wild-type syngeneic mice (Olivares-Villagomez et al., 1998; Van de Keere and Tonegawa, 1998) considered to contain a polyclonal Treg population. Protective Treg need not express the CD25 marker, as CD4 T cell populations depleted of CD25+ T cells were just as effective (Furtado et al., 2001). CD4+ T cells were actually determined to be protective in TR+ mice (reviewed in section 2), that remain healthy despite a quasi-monoclonal tg repertoire, as long as the non-MBP-specific CD4+ T cell population contains Treg (Olivares-Villagomez et al., 2000). Of note, in this model, Treg contained in the double expressor subpopulation are more potent than those exclusively encoding non-Tg TCR chains indicated that it is the tg TCR which confers the specificity through which Treg exert their protective function (Hori et al., 2002). Natural polyclonal regulatory CD4+ activity also reduce the clinical signs of EAE in active immunization models (Kohm et al., 2002; Zhang et al., 2004; McGeachy et al., 2005; Gartner et al., 2006).

In the Lafaille experimental system, few cytokine manipulations have been described in the study of EAE induction and effector requirements (Table 4.II). Against current dogma, that EAE induction occurs with Th1 effector cells alone, CD4+ T cells from TR+ mice, when stimulated with MBP peptide in the presence of IL-4 generate Th2 cells, causing a delayed form of EAE in RAG-1 mice (Lafaille et al., 1997). More recently, IFN-γ disruption in an MBP specific TCR transgenic mouse system crossed into the RAG-1 deficient background was shown to result in a severe nonclassical form of spontaneous EAE characterized, most strikingly, by intense eosinophilia localized predominantly in the brainstem and cerebellum (Wensky et al., 2005).
Table 4.II - EAE mouse models and regulatory T cells

<table>
<thead>
<tr>
<th>Induction (strain-peptide)</th>
<th>Spontaneous Disease</th>
<th>Transfer</th>
<th>CD depletion</th>
<th>EAE outcome</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-MBP TCR tg (TR-)</td>
<td></td>
<td>wt CD4&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Prevention</td>
<td>Olivares-Villagomez, et al., 2000.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>wt CD4&lt;sup&gt;+&lt;/sup&gt;CD25&lt;sup&gt;-&lt;/sup&gt;</td>
<td></td>
<td></td>
<td>Furtado, et al., 2001</td>
</tr>
<tr>
<td></td>
<td>IL-2&lt;sub&gt;−/−&lt;/sub&gt; CD4&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>Prevention</td>
<td></td>
<td>Furtado, et al., 2002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD25&lt;sup&gt;-&lt;/sup&gt;/CD4&lt;sup&gt;+&lt;/sup&gt; T cells</td>
<td>No Prevention</td>
<td></td>
<td>Furtado, et al., 2002</td>
<td></td>
</tr>
<tr>
<td>B6 - MOG</td>
<td>wt CD25&lt;sup&gt;+&lt;/sup&gt; T cells before induction</td>
<td>Prevention</td>
<td></td>
<td>Kohm, et al., 2002</td>
<td></td>
</tr>
<tr>
<td>B6 - MOG blasts</td>
<td>wt CD25&lt;sup&gt;+&lt;/sup&gt; T cells Co-transfer</td>
<td>Inhibits severity</td>
<td>Kohm, et al., 2002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SJL - PLP</td>
<td>wt CD25&lt;sup&gt;+&lt;/sup&gt; T cells before induction</td>
<td>Inhibits severity</td>
<td>Zhang, et al., 2004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B10.S - PLP</td>
<td>PC61</td>
<td>Increases severity</td>
<td>Reddy, et al., 2004</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B6 - MOG</td>
<td>PC61</td>
<td>Increases severity</td>
<td>McGeachy, et al., 2005</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SJL/J - PLP</td>
<td>PC61</td>
<td>Increases relapse</td>
<td>Gartner, et al., 2006; Zhang, et al., 2004</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Treg express IL-2Rα (CD25), IL-2Rβ (CD122) and the γ<sub>R</sub>, all of which are subunits required to express a functional IL-2R. Treg cells are reduced in IL-2<sup>−/−</sup>, IL-2R α<sup>−/−</sup> and II-2R β<sup>−/−</sup> mice (described in table 1.I), correlating with the development of AID. The anti-MBP TCR tg model developed by Lafaille was further used to show that the adoptive transfer of CD4<sup>+</sup> T cells from II-2<sup>−/−</sup> mice prevented the development of spontaneous EAE in IL-2<sup>+</sup> TR<sup>−</sup> hosts but CD4 T cells from CD25<sup>−/−</sup> donor cannot prevent EAE (Furtado et al., 2002). Restoring IL-2 production led to the reconstitution of the Treg population because IL-2 is essential for Treg function also promoting suppressor function in vitro (Tang et al., 2004; Thornton et al., 2004). All of these studies highlight the protective effectiveness of a polyclonal Treg population in EAE protection. Antigen-specific regulation has also been described through induction of Treg in PLP1-specific 5B6 TCR transgenic mice with suppression of PLP1 peptide-induced EAE in SJL/J mice upon transfer (Yu et al., 2005).

Two other subsets of Treg have been described in EAE models. A subset of so-called Tr1 adaptive regulatory IL-10 producing Foxp3-CD4<sup>+</sup> T lymphocytes has been described as being
induced, *in vitro*, by culture of CD4+ T cells with DEX and vitamin D3 and to inhibit CNS inflammation (Barrat *et al.*, 2002). Another subset, Th3 cells, originally identified in mice orally tolerized to MBP, inhibit EAE in a TGF-β dependent manner (Chen *et al.*, 1994). There is evidence that IL-10 secreting Tr1 cells dampen immune responses in infections, as previously reviewed (McGuirk and Mills, 2002). Just as importantly, from a historical perspective, neurons have been neglected as cells with a major immune-regulatory function because they do not express MHC class II. The importance of neuronal interaction with CD4+ T lymphocytes is highlighted by the finding that the *in vitro* interaction between neurons and T cells results in the conversion of encephalitogenic T cells to CD25+ TGF-β1+ CTLA-4+ FoxP3+ Treg that inhibit EAE (Liu *et al.*, 2006).

Finally, in humans, studies in patients with MS are contradictory in terms of how frequencies of Treg in peripheral blood (PB) and in cerebrospinal fluid (CSF) compare to healthy controls, but are unanimous in reporting that Treg show reduced suppressive capacity (described in table 1.V).

### 4.1.1.2. T cell-induced colitis

As already mentioned in section 3, the T cell-induced colitis model involves the adoptive transfer of wt naïve CD4+ T cells into SCID or RAG-2−/− mice resulting in a disease with the morphological features of inflammatory bowel disease in humans (Morrissey *et al.*, 1993; Powrie *et al.*, 1993). Colitis is Th1 (Powrie *et al.*, 1994; Leach *et al.*, 1996; Mackay *et al.*, 1998) and Th17 (Kullberg *et al.*, 2006) effector cell mediated involving more than one Ag (Matsuda *et al.*, 2000), the net result of which is bowel inflammation and epithelial damage due to an immune response and resulting immunopathology. The colon becomes markedly thickened due to both hyperplasia of the epithelium and infiltration of the lamina propria and submucosa by lymphocytes and macrophages (Leach *et al.*, 1996). Direct infection with *H. hepaticus* severely aggravates colitis in SCID or RAG-2−/− mice reconstituted with CD45RB<sup>high</sup> T cells (Cahill *et al.*, 1997) and in Interleukin (IL-10)<sup>−/−</sup> mice that spontaneously develop colitis in SPF conditions (Kullberg *et al.*, 1998).

The administration of CD45RB<sup>low</sup>CD4+ T cells to T cell deficient mice along with the pathogenic CD45RB<sup>high</sup> population leads to complete inhibition of colitis development (Powrie *et al.*, 1993; Powrie *et al.*, 1994; Aranda *et al.*, 1997). These Treg cells block colitis through a mechanism involving TGF-β (Powrie *et al.*, 1996) and IL-10 (Asseman *et al.*, 1999) and recent reports have further established that a CD4+CD25+ T cell transfer cures already established colitis (Mottet *et al.*, 2003). The TCR specificity of these protective cells remains unknown. Neither
T cell-induced colitis on the repression of encephalomyelitis in a transgenic mouse model

pathogenic nor protective T cells have to be primed by gut flora before cell transfer, as germ free derived cells, are effective in disease induction or protection upon transfer to flora-containing SCID mice (Annacker et al., 2000). This was interpreted to reveal the autoimmune nature of T cell-induced colitis as it was presumed that Treg were self-reactive. Alternative explanation would be Treg TCR cross-reactivity to foreign antigens or that Treg are induced from the naïve T cells, as reviewed in table 3.I). Results from the present work, presented in section 3 suggest that the immune response in T cell-induced colitis is specific for a bacterial antigen, opposing an autoimmune nature of T cell-induced colitis.

In contrast with the finding that Treg from germ free mice are protective, IL-10 deficient, but not wt mice, have been described to develop colitis after infection with Helicobacter hepaticus, suggesting that infected wt mice mount a disease protective response to the bacterium, possibly a Treg type of response. This is confirmed by the observation that only CD4+ T cells from H+ (colonized with Helicobacter spp.) wt recipients but not H+ donors protected from colitis induced by an IL-10 deficient T cell transfer to H+ alymphoid recipients. These protective cells were enriched in the CD45RBlowCD25− T cell compartment (Kullberg et al., 2002) and were not tested for Foxp3 expression but their presence highlights the foreign antigen specificity of Treg.

The fact that polyclonal Treg are capable of controlling T cell-induced colitis and EAE upon transfer does not rule out a requirement for Ag specificity which could be present in a particular Treg clone, naturally selected in the thymus or peripherally induced.

4.1.1.3. Chemical colitis

Experimental colitis may be induced in mice by dextran sodium sulphate (DSS) administration in their drinking water (Okayasu et al., 1990; Cooper et al., 1993; Dieleman et al., 1994; Dieleman et al., 1998; Mahler et al., 1998). DSS is a sulphated polymer that is thought to induce mucosal injury and inflammation through a direct toxic effect on epithelial cells and exposure of the lamina propria to luminal bacteria. The clinical features of this model include weight loss, diarrhoea, and rectal bleeding. Histopathological analysis typically reveals extensive crypt and epithelial cell damage, significant infiltration of granulocytes and mononuclear cells and tissue oedema, often accompanied with severe ulceration (Cooper et al., 1993).

The severity of colitis varies with the concentration, number of cycles and duration of DSS therapy as well as with a genetically determined variation in susceptibility to DSS-induced colitis among inbred strains of mice. Histological changes in B6 mice subjected to a seven day course of 3% DSS are still present 1 month after cessation in comparison to Balb mice subjected
to 5% DSS that have regained normal histology (Melgar et al., 2005). In addition, in B6 mice, a 7 day course of 2.5% DSS (one cycle) in drinking water is reported to cause progressive weight loss reaching more than 70% of the original weight within one week and leading to 100% mortality 10 days after the first DSS administration. Three 5-day cycles of 1.5% DSS with 1 week interval (each cycle followed by regular water) after the first DSS exposure and 5-day intervals after the second exposure resulted in 20% body weight loss in C57BL/6, whereas a more vigorous regime, 5% DSS (for 5 days with 5-day intervals), is required to obtain 15% weight loss in BALB/c mice (Aharoni et al., 2006).

DSS-induced colitis is a T cell–independent model because it can be induced in severe combined immunodeficient (SCID) mice lacking both T and B lymphocytes (Dieleman et al., 1994) and is not affected by CD4 or natural killer (NK) lymphocyte depletion (Axelsson et al., 1996). Adoptive transfer of dendritic cells (DC) exposed ex vivo to DSS worsens the severity of the disease and DC ablation attenuated disease, leading to the idea that DSS first injures colonic epithelium and subsequently activates DC, responsible for the production of chemokines and pro-inflammatory cytokines (Berndt et al., 2007). In fact bone marrow-derived-DC are able to produce IL-12 and TNF-α, but not IL-4, IL-10, or IFN-γ when stimulated by DSS (Berndt et al., 2007). NK lymphocytes responsible for the production of a variety of cytokines, including IFN-γ, IL-1 beta, IL-2, IL-3, IL-4, IL-5, and IL-6 (Saito et al., 1993), monocytes, epithelial cells, endothelial cells, fibroblasts and intestinal macrophages are all major sources of cytokines and are present in T and B cell deficient mice. In RAG−/− mice, except for IL-17 which will be absent in DSS colitis, there may be a common cytokine pattern in DSS and T cell-induced colitis, notwithstanding a preponderance of Th1 and Th2 cytokines such as IL-2, IFN-γ and IL-4 in T cell-induced colitis.

4.1.2. The hygiene hypothesis

The “hygiene hypothesis” has been proposed to explain a fast and recent rise in immune mediated disorders such as allergy and AID in wealthier societies, inversely correlated to childhood infections and poverty. It was originally put forward, supported by several epidemiological studies, reporting that: (i) hay fever and eczema were inversely related to the number of children per household (Strachan, 1989; Wold, 1998), facts that were interpreted as evidence that allergy was due to lack of infections caused by unhygienic contact with older siblings; (ii) antibiotic use early in life increased the risk of asthma (Cohet et al., 2004) and; (iii) childhood diabetes was inversely correlated to birth order (Bingley et al., 2000) and day care attendance in early infancy (McKinney et al., 2000). This was extended to the concept that in ‘Westernized’ countries, a small family size, affluent urban homes, a stable Intestinal microflora,
high antibiotic use, low or absent helminth burden, good sanitation and a low orofaecal burden are associated with a high frequency of asthma, eczema and rhinitis, in contrast with rural homes, where livestock, a large family size, a variable intestinal microflora, low antibiotic use, high helminth burden, poor sanitation and high orofaecal burden maintain developing countries free from allergic diseases (Wills-Karp et al., 2001).

The “hygiene hypothesis” postulates that the immune system naturally evolved to deal with a “dirty world” and as the environment became increasingly sanitized, the immune system stopped being used for its original purpose starting to react against self, due to a deregulation from lack of exposure. It takes into account how the entire infectious history of an individual might determine the overall immune status and consequent susceptibility to AID. As recently brought to light, the contemporary western lifestyle is also associated with an increase in the prevalence of organ-specific autoimmune diseases (Bach, 2002) and an increase in the frequency of AID and allergy in the same patient (Airaghi and Tedeschi, 2004). Originally explained on the basis of the classical Th1 and Th2 paradigm, whereby a high parasitic burden, typically associated with a Th2 polarizing response, was thought to paradoxically inhibit Th2 responses involved in asthma and allergy, the concept has extended to explain a common factor underlying the development of Th1 and Th2 mediated diseases,

Counter-regulatory mechanism involving Treg stimulation by the infectious environment and subsequent IL-10 inhibition of allergy and AID have been proposed (Wills-Karp et al., 2001). The fact that polyclonal Treg in germ free mice are not as potent suppressors in vitro as those from conventional animals suggests that the presence of a microbial flora favours the development of a fully functional Treg population (Ostman et al., 2006) and a lack of infections in the western world may contribute to a decrease in both the number and function of Treg. Other protective mechanisms that have been recently proposed include: (i) antigenic competition where immune responses against pathogens compete with autoimmune and allergic responses for self MHC peptide recognition and growth factors; (ii) bystander suppression by immunoregulatory cells other than Th2 and CD25+, such as Tr1 and NK T cells or cells recognizing specificities other than those which triggered their differentiation; (iii) binding to specific receptors such as Toll-like receptors (TLR) on macrophages or the presence of Treg and immunosuppressive proteins from the infection (Bach, 2005). There are examples of AID inhibition by parasites - such as in experimental models of lupus (Greenwood et al., 1970), diabetes (Saunders et al., 2007) inflammatory bowel disease (Summers et al., 2005) and multiple sclerosis (Correale and Farez, 2007) - providing proof of principle for this hypothesis, which predicts an inverse correlation between the incidence of most autoimmune disorders and the incidence of common infections.
Introduction

(Leon et al., 2004) but does not rule out that mechanisms such as antigenic mimicry may lead to specific autoimmune disorders after particular infections, as recently reviewed (Christen and von Herrath, 2005).

4.1.3. Regulatory T cell homeostasis
The driving force behind the proliferation of a polyclonal naïve T cell transfer into hosts lacking lymphocytes and a functional thymus has for long been a matter of debate where a distinction is often made between lymphopenia induced proliferation (LIP) to self antigens and proliferation in response to cognate antigen. Self or foreign antigens, space and resources availabilities have all been tested in several experimental systems and each has been found to influence homeostasis (Jameson, 2002).

MHC / peptide specificity (Ernst et al., 1999) and TCR signalling (Wang et al., 2001) have all been shown to be essential for naïve CD4+ T cell proliferation. As an example, minimal CD4+ T cell proliferation in SCID mice maintained in germ free conditions is very suggestive that naïve T cell proliferation upon transfer is actually driven by foreign antigens (Kieper et al., 2005). RAG−/− recipient mice are actually considered to be highly susceptible to chronic infection and thus may present a spectrum of microbial Ag to the adoptively transferred T cells (Kieper et al., 2005), together with an increase in space availability for a proliferating lymphocyte population. Furthermore, T cell-induced colitis in immunodeficient hosts is only observed in the presence of Ag from specific strains of bacteria, required to drive rapid T cell proliferation and differentiation into pathogenic cells and cannot be induced in germ-free mice (Powrie et al., 1997). In contrast, TCR transgenic cells in RAG-deficient backgrounds undergo proliferation in lymphopenic hosts, implying that they are driven by MHC molecules loaded with self rather than foreign peptides (Ernst et al., 1999).

4.1.4. Objectives
Current medical treatments for EAE rely on the use of immunosuppressive drugs that cause severe side effects, and in a significant percentage of the patients do not induce long-term benefit. Cellular immunoregulatory therapy may provide a well tolerated therapeutic alternative which effectively induces remission and alters the natural course of the disease. Based on the immunopathological nature of EAE several strategies have been proposed, such as attempts to deviate the CD4 pathogenic T cells from Th1 to Th2 phenotype or to alter cytokine patterns but these have met with limited success. In accordance with the hygiene hypothesis, the notion that it is difficult to generate organ-specific autoimmune disease in animal colonies of relaxed
biocontainment rules inspired the present work, aiming to understand the mechanism through which T-cell induced colitis is capable of protecting TR- mice from EAE and whether this protection is regulatory T cell mediated.

4.2. Material and methods

4.2.1. Mice
Mice strains described in 2.2.1 and 3.2.1 of the present study. Re-derivation of the TR- colony was performed by embryo transfer (Hogan, 1994).

4.2.2. Antibodies and reagents
DSS, M.W. 36000–50000 (MP Biomedicals, LLC). DSS 1.5% in drinking water on alternate weeks was not enough to induce colitis which only occurred when a concentration of 3% was used. Remaining antibodies as described in 2.2.4 and 3.2.3.

4.2.3. Cell purification and transfer
As described in 3.2.3

4.2.4. Cell recovery and flow cytometric analysis
Cell suspensions from Sp or mLn were prepared, stained, counted and analysed as described in 3.2.4. Leucocytes were isolated from the CNS as described by Vigario et al. (2007). Briefly, mice were killed and perfused intracardially with PBS to remove circulating RBC and leucocytes from the brain. The brain, spinal cord and intestine were removed and lymphocytes were isolated after tissue homogenization in PBS supplemented with 2% FCS and 2 U/ll DNAse (Sigma). Tissue extracts were then centrifuged and the pellet further puried by centrifugation at 1,100g for 20 min in 30% (v/v) Percoll (Pharmacia) at room temperature. Cell suspensions from CNS, colon, sp, or Ln (inguinal, axilary, mesenteric and brachial) were first incubated with saturating amount of Fc-block (in-house produced) before Ab staining. For intracellular cytokine staining, cells were first stimulated for 4h with ionomycin (500 ng/ml, Calbiochem) and brefeldin A (10 μg/ml, Sigma) was added for the final 2 h of stimulation.

4.2.5. Cell culture and proliferation assay
All cultures were set in RPMI-1640 supplemented with 10% FCS, 100 U/ml penicillin, 100 g/ml streptomycin, 50 g/ml gentamycin, 50 μm 2β -ME, 10 mM Hepes, and 1 mM sodium pyruvate (all from Life Technologies). Tg T cells were plated at 2.5x10⁴/well in U-shape 96-well plates together
Material and methods

with $10^5$ APC and anti-CD3 mAb (1 μg/ml). Erythrocyte-depleted splenocytes were irradiated at 30 Gy and used as a source of APC. Each dilution was set in triplicate and culture was maintained for 3 days. All proliferations were monitored by addition of [$^3$H] thymidine (1 Ci/well; Amersham Biosciences) for the last 6 h of culture.

4.2.6. Histological evaluation

Mice were perfused with PBS followed by 10% formalin after which the brain and spinal cord were removed, embedded in paraffin and stained with hematoxylin and eosin (H&E) or with Luxol fast blue (LFB) stains.

The colon was removed after T cell reconstitution and fixed in 10% formalin. Paraffin-embedded sections were cut and stained with hematoxylin and eosin for assessment of morphology. Inflammation was scored in a blinded fashion, on a scale of 0–9 as previously described (Liu et al., 2000).

4.2.7. Data analysis

Statistical analysis was performed with GraphPad prism 4.0 (GraphPad Software Inc, San Diego, California). Statistical significance was determined using the two-tailed Student’s t test. $P \leq 0.05$ was considered significant (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$). Results were expressed as mean ± standard deviation.

4.3. Results

4.3.1. Effect of T cell-induced colitis upon spontaneous EAE

The transfer of $3 \times 10^5$ CD4$^+$CD25$^-$CD45RB$^{high}$T cells caused colitis and simultaneously protected TR- mice from the development of spontaneous EAE. At nine weeks post transfer, the weight decrease in the control group of TR mice that did not receive a cell transfer corresponded to EAE onset and in the experimental group, that received the cellular transfer, to colitis development (Fig 4.1 A). Histologically, in the experimental group, there was no evidence of CNS cellular infiltration or demyelination which were present in the control group (Fig. 4.1 B). This finding was reproduced by the simultaneous or sequential transfer of anti-MBP TCR tg and naïve T cells into RAG$^{-/-}$ mice, the net result of which was colitis and full EAE protection, in contrast with fulminant lethal EAE produced by the transfer of anti-MBP TCR tg T cells alone, as shown in Fig. 4.1 C. Of note, colitis abrogated the expected increase in weight due to growth.
**Figure 4.1 (A-C)**

**Figure 4.1 A** - Serial weights, cumulative EAE incidence and mean EAE score. Initial body weights averaged 9.2 ± 1.6 g (control) and 9.4 ± 2.2 g (transfer). In the transfer group, one recipient died naturally from severe colitis and seven were sacrificed for analyses at 8 and 10 weeks of age. Results pooled from 3 separate experiments.

**Figure 4.1 B** - Representative H & E (top) and luxol fast blue (bottom) transverse spinal cord staining of the untreated TR- mice before disease onset (score 0 - healthy) or after disease onset (score ≥ 2 - sick) and of TR- mice with colitis induced by T lymphocyte transfer before 4 weeks of age (Protected). Meningeal and parenchymal cellular infiltrates with areas of demyelination are shown in the Sick TR- and absent in the other stainings. TR- mice with colitis were age matched to the sick TR- and analyzed at 11 weeks when 100% of untreated TR- have EAE. (original magnification x40).
Results

Figure 4.1 continued

*Figure 4.1 C* - Serial weights and cumulative EAE incidence in RAG−/− recipients of 3x10^5 CD4+ anti-MBP TCR tg T cells obtained from TR− mice 3.5 weeks old transferred either alone (n=2, grey squares), simultaneously (n=4, white square) or sequentially 2 weeks after sorted 3x10^5 CD4+CD45RBhighCD25− T cells isolated from syngeneic mice (n=4, black squares). Weights not measured in the single transfer.

**Figure 4.1**

**Effect of T cell-induced colitis upon the incidence of spontaneous EAE**

The transfer was partially protective in H− recipients that developed mild colitis as EAE occurred in seven out of eight H− recipients (Fig. 4.2 A and B). EAE was mild and there was with no mortality. Full protection only occurred when TR− mice were colonized with *Helicobacter* spp. allowing for more rapid colitis development, with diarrhoea starting at one week (H+) vs three weeks (H−).
**Figure 4.2 A** - Cumulative EAE incidence, mean EAE score and serial % of initial weights in H⁺ and H⁻ recipients of a T cell transfer from the same donor pool before 4 weeks of age. Transfers performed with sorted 3x10⁵ CD4⁺CD45RB⁺⁺CD25⁻ T lymphocytes from wt donors.

**Figure 4.2 B** - Representative H&E transverse colon stain of H⁺TR⁻ mouse that developed colitis and were protected from EAE (right) compared to the mild colitis (left) with little diarrhoea in the H⁻TR⁻ mice (original magnification x10). When experiments were terminated all the surviving mice had an EAE score of 2.

**Figure 4.2**

Effect of *Helicobacter* recipient status upon EAE incidence after a T cell transfer
Results

These results indicate that T cell mediated colitis protects anti-MBP TCR Tg mice on a RAG−/− background from spontaneous EAE, with absence of CNS inflammation and block of demyelination and that protection hinges on the rapid induction of severe colitis, which in turn depends on the presence of Helicobacter in recipient mice.

A transfer of naïve T cells after 5 weeks of age failed to protect H+ TR− from EAE, with an incidence and mean EAE score similar to control TR− mice, associated with a high mortality (Fig. 4.3). The same donor pool was transferred to H+ RAG−/− mice eliciting full blown colitis, indicating the naïve T cells were competent effectors.

Figure 4.3
Effect of a T cell transfer upon EAE incidence in TR− mice older than 4 weeks of age
Cumulative EAE incidence, mean EAE score and mortality of H+ TR− recipients of sorted 3x10^5 CD4+CD45RB^{high}CD25− T lymphocytes from wt donor pool, transferred before (n=16, black squares) or after (n=12, grey squares) 4 weeks of age. Results in each panel pooled from 2 experiments.
4.3.2. Effect of chemical induced colitis upon spontaneous EAE

In order to dissociate potential EAE inhibitory effects of gut inflammation per se from those mediated by T lymphocytes, we used a model of innate mediated DSS induced colitis. DSS 3% administered to TR− mice from 3.5 weeks of age, in four alternate weekly cycles induced severe colitis and failed to prevent EAE in these mice (Fig 4.4 A and B).

Figure 4.4 A - Serial weights and cumulative EAE incidence. Initial body weights averaged 8.4 ± 1.1 and 9.2 ± 0.6 g respectively. Two recipients died from severe colitis before 7 weeks of age

Figure 4.4 B - Representative haematoxylin and eosin transverse colon staining of control TR− (no colitis), T cell and DSS colitis induced in H+TR− mice before 4 weeks of age. Samples were taken at 8 wks post cell transfer and after 4 alternate weekly cycles. There is loss of crypt architecture and goblet cells and an inflammatory infiltrate spanning the entire lamina propria, submucosa and muscularis. The large ulcer is typically caused by DSS, (original magnification x10).

The effect of chemical colitis upon EAE incidence in TR− mice
There was colitis related mortality in that 2 out 14 mice died before 7 weeks of age, most probably from severe colitis, without any signs of severe EAE. EAE scores were similar to those of un-reconstituted TR− mice. This finding suggests that severe inflammation alone is not sufficient to prevent EAE and that EAE prevention by colitis is T cell mediated.

4.3.3. Effect of donor T cell depletion in TR− mice with T cell-induced colitis

After colitis induction by naïve T cell transfer, serial weekly depletions (3 in total) of donor Thy1.1+ T cells by the use of a Thy1.1 depleting monoclonal antibody cures T-cell induced colitis and is followed, 4 weeks later, by the appearance of EAE in a mild non-progressive form (Fig. 4.5). After donor depletion, EAE may have been caused by newly thymic differentiated tg T lymphocytes and/or by tg T lymphocytes released from inhibition. This observation does not allow for a differentiation between the possible effects of inflammation or T-cells, as depletion leads to both removal of colitogenic T cells and cure of colitis. It also does not exclude that EAE protection is related to the establishment of a tolerant state mediated by donor Treg as depletion would have removed this group of potentially protective cells.

**Figure 4.5**

The effect of donor T cell depletion in TR− with T cell-induced colitis

Serial weights, cumulative EAE incidence and mean EAE score of H+ 3.5 week-old TR− recipients of sorted 3×10^5 CD4+CD45RB<sup><small>hi</small></sup>CD25−Thy1.1+ T lymphocytes from wt donors, subjected to 3 weekly administrations (arrows) of thy1.1 depleting mAb (0.5 mg/animal) starting at 4 weeks post transfer (n=3, black squares), compared to non-depleted recipient controls (n=2, white squares). Diarrhoea started 9 days post transfer and stopped after the first depletion accompanying the gain in weight. EAE developed in all recipients thirty days after the last depletion. At 9 weeks post transfer, the non-depleted TR− naturally died from colitis.
4.3.4. Phenotypic characterization of T cells in recipient mice

Accumulation of Foxp3+ T cells of donor origin reaches a peak at 6 weeks post transfer when the great majority of CD25+ donor T cells were Foxp3+ (Fig. 4.6 A). Donor Foxp3+ T cells accumulated in the TR− recipients with T cell-induced colitis (Fig 4.6 B) with a fold accumulation which reached up to 20 times that of the transferred non-Foxp3+ population in H− recipients (Fig. 4.6 D). There were no significant differences between H+ and H− recipients in terms of the frequency of Foxp3 expression but more lymphocytes were recovered in H− recipients, possibly because of lymphocyte migration to the colon, with more severe colitis (Fig. 4.6 C and D). The transfer of sorted CD4+CD45RBhigh naïve T cells always contained a small frequency of Foxp3+ T cells estimated at approximately 1% of the cell number (described in Fig. 3.5 B of section 3). Anti-MBP TCR tg T cells acquired Foxp3 expression in DSS colitis and after CD4+ and naïve T cell transfer (Fig. 4.7).

**Figure 4.6 A** - Representative FACS plots showing average percentage (inside top) and number (inside bottom) of CD25+ donor T cells and Foxp3 in CD25+ donor CD4+ T cells in mLn and nmLn of TR− with T cell-induced colitis at 4 (n=3), 6 (n=3) and 8 (n=3) weeks post transfer of sorted 3x10^5 CD4+CD45RBhighCD25−Thy1.1+ T lymphocytes from wt donors.

continues
Figure 4.6 B - FACS plots of frequency and number of donor naïve T cells (Thy1.1+) and donor derived Treg (Thy1.1+Foxp3+) in TR+ recipients (H- n=3, H+ n=2) at 8 weeks post transfer. These results pertain to the transfer in Fig. 4.2 A and B.

Figure 4.6 C - Fold accumulation is calculated according to the number of Thy1.1+ lymphocytes recovered from mLn, nmLn and Sp from recipients in (C), divided by the number of cells injected per mouse. The number of CD25+ T cells injected was not analyzed in this particular transfer and was calculated from the average frequency of Foxp3+ T cells found in the CD4+CD45RBhighCD25− population, obtained from wt mice and yielding a percent value of 0.4. It was calculated that for 3x10⁵ sorted CD4+CD45RBhigh T lymphocytes, 1200 Foxp3+ T cells were transferred.
Figure 4.7

Phenotypic characterization of transgenic T cells in recipient mice

Representative FACS plots showing average percent expression of Foxp3 in anti-MBP TCR Tg T cells from nmLm in control TR−, healthy (n=4) and with EAE (n=3) and TR− with EAE unprotected by DSS colitis (n=2). Healthy TR+ mice are compared to healthy TR− protected from EAE by a CD4+ T cell transfer (n=3) or with colitis after CD45RBhigh T cell transfer from wt donors (n=3).

4.3.5. Outcome of co-transfer of different T cell subsets from recipients with colitis together with transgenic T cells

Recipient RAG−/− mice received anti-MBP TCR tg T cells obtained from 4 week old unreconstituted TR− mice alone or in combination with sorted CD25+ T or CD25− T cells from the RAG−/− mice with colitis induced by CD4+CD45RBhigh T cell transfer. The latter were used as pooled donors when 80% of the original weight had been reached, at 6 weeks post transfer (Fig. 4.8 A). The CD25+ sorted population was enriched in Foxp3 (Fig 4.8 B). The CD25+ T cells failed
to protect the RAG−/− mice from EAE induced by the encephalitogenic tg T cell co-transfer. EAE was severe and ran a similar course to the EAE induced by transgenic T cells alone. In contrast, the CD25− co-transfer completely prevented EAE (Fig. 4.8 C). The CD25+ co-transfer resulted in a milder colitis (Fig. 4.8 D) and this phenomenon bore a resemblance to the failure of T cell-induced colitis in the prevention of EAE in H−TR− recipients with a similar milder and delayed onset of colitis (Fig. 4.2). Foxp3+ donor T cells controlled the severity of colitis but did not mediate EAE protection upon transfer.

**Fig. 4.8 (A-F)**

![Graph showing weight loss](image)

*Figure 4.8 A* - Weight loss of RAG−/− mice recipients of 3x10⁵ CD45RB↑↑ T cell transfer with T cell induced colitis (n=10). Initial body weights averaged 18 ± 1g

![FACS plots](image)

*Figure 4.8 B* - Representative FACS plots at 6 weeks post transfer showing CD25 and Foxp3 expression in donor CD4+ T cells, pooled from mln and nmln (n=10).
Fig. 4.8 continued

Figure 4.8 C - Cumulative EAE incidence and mean EAE score in H:\RAG−/− mice recipients of 1x10^5 tg T cells alone or in combination with 1x10^6 sorted Thy1.1+CD25+ (purity 97%) or 1x10^5 thy1.1+CD25− (purity 99%). Sorted lymphocytes isolated from pooled Ln of donors represented in Fig. 4.8 A. Results that refer to the CD25− co-transfer are representative of 2 independent transfers.

Figure 4.8 D - Weight loss and representative H&E stain of the transverse colon for each co-transfer group sacrificed for analysis at 5 weeks post transfer. Initial body weights averaged 18.4 ± 0.8 g for CD25+ and 20 ± 1.2 g for the CD25− recipients. (Original amplification - x10)
**Results**

**Fig. 4.8 continued**

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<tr>
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<tr>
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<tr>
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**Figure 4.8 E** Individual FACS plots at 5 weeks post transfer showing frequency and number of Foxp3 expression in thy1.1+ donor CD4+ populations, in mLn and nmLn.
Figure 4.8 continued

<table>
<thead>
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</tr>
<tr>
<td>Tg + CD25⁺ EAE Mild colitis</td>
<td>2 6</td>
</tr>
<tr>
<td>Tg + CD25⁻ No EAE Severe colitis</td>
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</table>

Figure 4.8 F Individual FACS plots at 5 weeks post transfer showing frequency and number of Foxp3 expression in 3H12⁺ tg CD4⁺ populations, in mLn and nmLn.

Analysis of recipients, 6 weeks post transfer, reveals that the donor CD25⁻ T cells proliferated more than the CD25⁺ T cells, suggesting that the donor Foxp3⁺ T cells included in the CD25⁺ transfer were able to control the proliferation of the non-Foxp3⁺ donor T cells but unable to control
transgenic T cell expansion with marked loss of donor derived Foxp3+ T cells in the CD25+ co-transfer, from 86% in the original transfer to a maximum of 55% in the recovered tg co-transfer (Fig. 4.8 E). There was an accompanying decrease in the frequency and number of transgenic T cells in the Ln of the CD25− T cell transfer group and the frequency and number of Foxp3+ Tg T cells in both co-transfers was similar but lower than in the transfer of tg cells alone (Fig. 4.8 F).

4.3.6. The encephalitogenic potential of tg T lymphocytes in TR- mice protected from EAE by T cell-induced colitis

In order to determine whether the transgenic T cells have a diminished encephalitogenic potential, Tg cells were sorted from TR- protected from EAE by T cell-induced colitis and transferred (either 3x10^5 or 1x10^5) to RAG−/− mice. EAE development in RAG−/− recipients was of a slower onset, non-fatal and with a much milder clinical course, when compared with the transfer of anti-MBP TCR tg T cells obtained from control young TR- that have not yet developed EAE (Fig. 4.9 A and B). In the former, 18 weeks post transfer, at a time when mice are ill with EAE, the frequency of Foxp3 in the Tg T cells reaches 60 %, not significantly different from the expression obtained in the approximately 10% of RAG−/− recipients of anti-MBP TCR tg T cells from TR- less than 4 wks old, that survive with EAE for up to 5 months after transfer allowing for the comparison with recipients of tg T cells obtained from TR- with colitis. (Fig. 4.9 C).

Fig.4.9 (A-C)

**Figure 4.9 A** - Percent EAE cumulative incidence resulting from the transfer to RAG−/− recipients (n=4) of anti-MBP TCR Tg T cells (3x10^5) sorted on the basis of the Thy1.2 marker from a pool of mLn and nmLn from TR- mice with T cell-induced colitis at 6 weeks post transfer compared to RAG−/− recipients (n=3) of anti-MBP TCR tg T cells (3x10^5) from healthy young (< 4 weeks old) TR- mice
Sorted tg T cells from TR- mice with colitis display a reduced response to peptide stimulation in vitro (Fig. 4.10).
Results

Figure 4.10

In vitro response of Tg T cells from TR– with colitis to MBP
Representative proliferation to MBP peptide (Ac1-11) measured by thymidine incorporation after 3 days in culture (results duplicated). The concentration of peptide was titrated comparing sorted anti-MBP TCR tg T cells from 3.5 week old healthy TR– mice to tg T cells sorted from one TR– mouse protected from EAE at 10 wks post colitis induction, in the presence of APC from wt donors. Purity ≥ 99% in both sorted populations. Anti-CD3 was used at 1μg/ml.

4.3.7. Cytokine production by colitogenic T cells

IFN-γ production by in vitro stimulated colitogenic T cells was observed in lymphoid organs, CNS and colon and was vastly superior to IL-17 (Fig. 4.11).

Figure 4.11

Cytokine production by colitogenic T cells
Representative FACS plots showing average percent expression of intracellular IFN-γ and IL-17 in donor T cells in one 14 week-old TR– mouse protected from EAE with T cell-induced colitis, 11 wks post transfer. Colitis was induced with sorted 3x10^5 CD4^+CD45RB^highCD25^-Thy1.1^+ T lymphocytes from wt donors.
4.3.8. Migration of tg T cells in T cell-induced colitis

There was a slight decrease in the frequency and number of transgenic T cells in the Ln of the CD25− T cell transfer group (Fig. 4.8 F) suggesting the migration of tg T cells may be altered in the presence of T cell-induced colitis. In fact, the number of tg T cells in the colon is approximately ten times higher in TR− protected from EAE by colitis than in control, still healthy TR− mice. Furthermore, the presence of colitogenic T cells in the brain matter indicates a breakdown of the BBB but, of note, their presence is associated with ten times less tg T cells when compared to the healthy TR− control (Fig. 4.12). These results suggest that diversion of T cell traffic away from the CNS may also contribute to the protection that colitis affords EAE.

![Figure 4.12](image)

**Figure 4.12**

Traffic of transgenic in protected TR− with T cell-induced colitis

Representative FACS analysis of transgenic and donor T cells in one 14 wk-old TR− mouse protected from EAE with T cell-induced colitis, 11 weeks post transfer, compared to an control 3.5 wk old TR− mouse with no signs of EAE. Colitis was induced with sorted 3x10^5 CD4+CD45RB−CD25−Thy1.1+ T lymphocytes from wt donors. The frequency (top row) and cell number (bottom row) of non-Tg (3h12−) and tg (3h12+) T cells is indicated inside plots.
4.4. Discussion

4.4.1. T cell-induced colitis inhibits spontaneous EAE

A single transfer of $1 \times 10^6$ CD4$^+$ T cells from a pool of wt donors, protected TR$^-$ mice from spontaneous EAE, up to 12 weeks of age (2.3.3.1) in accordance with previously described work (Olivares-Villagomez et al., 1998; Van de Keere and Tonegawa, 1998). The novelty in the present work is the demonstration that EAE protection in TR$^-$ mice can be mediated by the transfer of naïve CD45RB$^{high}$ CD25$^-$ T cells ($3 \times 10^5$ cells) from wt donors, but only in the presence of fast and severe colitis induction in H$^+$ TR$^-$ recipients. In H$^-$ TR$^-$ recipients, colitis induction was delayed, failing to protect the mice from spontaneous EAE. TR$^-$ mice with EAE are described to have patchy demyelinating lesions in the spinal cord, with mononuclear cell infiltrates in perivascular areas and meninges (Lafaille et al., 1994) and these were completely absent in the protected mice. In this work, EAE protection afforded by the CD25$^-$ transfer followed the rules that have been established for a fully efficacious protective transfer of CD4$^+$ T cells, namely that the transfer has to be performed in a restricted time window not to surpass a recipient age of 4 weeks (Olivares-Villagomez et al., 1998). The present finding is in agreement with a previous report, where protection from EAE was obtained by a transfer of $10^6$ CD4$^+$ T cells depleted, ex vivo, of CD25, with the difference that, in the latter, protection was achieved in the absence of colitis and in seemingly H$^-$ TR$^-$ recipients (Furtado et al., 2001).

4.4.2. Donor T lymphocytes mediate EAE protection

As protection was only seen in the presence of severe colitis of fast onset, the first question posed was, if inflammation per se was responsible for protection, in the absence of transferred T cells. However, DSS colitis, a model of innate inflammation, failed to protect from EAE, which, together with the fact that protection was abrogated after cure of colitis by donor T cell depletion, established a protective role for donor T cells. The fact that no diarrhoea was observed in the naïve T cell transfer to TR$^-$ older than 5 weeks of age is most likely due to the fulminant course of EAE as tg T cells do not inhibit T cell-induced colitis neither in a simultaneous nor in a sequential co-transfer and therefore this is unlikely to represent an inhibitory effect of EAE upon IBD.

4.4.3. Regulatory T cells accumulate selectively in inflammation

In order to understand if colitis is associated with a differential expansion or redistribution of several sub-populations of donor or tg origin, in the effector or Treg pools, a phenotypic characterization of recipient TR$^-$ mice protected from EAE by the T cell transfer revealed a considerable frequency of CD4$^+$CD25$^+$ T cells of donor origin, enriched for Foxp3 expression in a
stable fashion, from 4 weeks post transfer. In this regard, with ongoing colitis, the number of CD4+ T cells that were Foxp3+ were found to be higher in the nmLn of H− compared to H+ recipient RAG−/− mice with T cell-induced colitis, but the highest frequency, maximal at 4 weeks post transfer, was not above an average of 5%, (point 3.3.3 of the present study). Likewise, in TR− recipients, the number of donor derived Foxp3+ CD4+ T cells in lymphoid organs was found to be slightly higher in H− when compared to H+ recipients, but with higher frequencies, reaching up to 13% in both groups.

The fact that fast and severe colitis in this model only occurs in the presence of Helicobacter colonization does not allow for a conclusion on the effect of Helicobacter colonization on Treg frequency and number. Furthermore, in this model, quantitative differences in the distribution of effector T cells and Treg in lymphoid or target organs can always be a consequence of disease severity even if determined early after transfer, before diarrhoea appears. The presence of Treg has actually been demonstrated in the lamina propria in T cell-induced colitis (Leithauser et al., 2006) and Treg are known to accumulate in inflamed tissues (Piccirillo et al., 2005). In the present work, it is estimated that the protective transfer contained approximately 0.4% of Foxp3+CD45RBhiCD25− T cells, corresponding to a total 1200 T cells and 2.99×10^5 of the Foxp3− counterpart. The fold expansion of the Foxp3+ and Foxp3− populations calculated from the estimated number of Foxp3+ T cells in the transfer, revealed a major preferential accumulation of Treg in the lymphoid organs of recipient TR− mice (expansion of 100 to 200 fold), 10 times greater than that of naïve T cells. An accumulation of this magnitude was surprising and placed donor derived Treg as potential candidates responsible for EAE protection. Polyclonal regulatory CD4+CD25+Foxp3+ T cells are known to reduce the clinical signs of EAE (Kohm et al., 2002; Zhang et al., 2004; McGechy et al., 2005; Gartner et al., 2006; Tischner et al., 2006) but it remains to be ascertained if Ag specificity is an absolute requirement for regulation.

Treg themselves absolutely require interaction with MHC-peptide for expansion upon transfer to lymphopenic (Seddon and Mason, 1999; Cozzo et al., 2003) or unmanipulated wt hosts (Fisson et al., 2003) and do not lose their function upon immunization (Klein et al., 2003). As their TCR have a higher avidity for self-antigens than the other CD4+ T lymphocytes, Treg are thought to have a greater potential to undergo lymphopenia-induced proliferation than naïve T cells and the T cell population may be selectively enriched (Hsieh et al., 2004). This expansion may also result from direct activation of Treg by the TLR pathway (Caramalho et al., 2003). Furthermore, these experiments tell us that inflammatory stimuli are not hampering the
homeostatic drive with resultant expansion of Treg in the lymph nodes, contradicting previous reports (Bettelli et al., 2006).

Some of the Foxp3+ T cells could be derived from naïve T cells, a phenomenon that has been described in response to several factors such as TCR stimulation with TGF-β (Chen et al., 2003), sub-immunogenic peptide delivery (Kretschmer et al., 2005; von Boehmer, 2005) and requiring B7 co-stimulation (Liang et al., 2005). As already shown (point 3.3.4 of the present study), conversion of Foxp3− to Foxp3+ donor cells is also possible during ongoing T cell-induced colitis and therefore accumulation of Treg could be due to both expansion and conversion from naïve T cells.

The reason for the higher frequency of donor Treg in the H+[TR− vs H+ RAG−/−] recipients of CD45RBhighCD25− T cells is not clear. Together they develop T cell-induced colitis of the same severity and it is assumed that in both, the transferred naïve and Treg T cells are accumulating, driven by Helicobacter derived antigens in the gut but also by self antigens. The main difference resides in the transgenic T lymphocytes in TR− mice which may be a source of a cytokine such as IL-2, important for Treg expansion and function (Furtado et al., 2002), but this is unlikely as peripheral Tg T lymphocytes are presumed to be in a resting stage in TR− mice with no EAE (Lafaille et al., 1994).

Donor cell proliferation is inversely correlated to the degree of lymphopenia, as naïve T cells are more likely to find self-reactivity (Ernst et al., 1999). Therefore, in TR− mice, the presence of a “competing” resident tg population would actually be expected to lower the frequency and number of donor cells in TR− when compared to RAG−/− mice. The kinetic study of T cell numbers reveals that in the RAG−/− recipients, there is a time point - approximately five weeks post transfer - after which T cell number declines in contrast to TR− recipients, where no decline in cell number is detected. This was not formally tested in TR− and RAG−/− littermates and should ideally be performed in recipients that receive naïve T cell transfers simultaneously from the same pool of donors and where Foxp3 expression is analysed at the same time. The donor colitogenic population could exert its protective effect through cytokine induced inhibition of several parameters, such as effector T cell migration, activation and function, BBB permeability and CNS inflammation, or, indirectly, through the induction of Foxp3 expression in tg T lymphocytes.

4.4.4. The EAE protective population is CD25 negative
In order to establish if the protective population was from donor origin, as suspected from the EAE-inducing effect of donor T cell depletion, co-transfer experiments were performed, using
RAG−/− mice, recipients of either CD25− or CD25+ T cells obtained from RAG−/− mice with T cell-induced colitis, in co-transfer with anti-MBP TCR tg T cells obtained from unmanipulated TR− mice less than 4 weeks old. Both the CD25+ and CD25− fractions contained colitogenic T cells. The secondary transfer of donor derived CD25+ T cells failed to reveal any protection towards EAE but the following cautionary remarks are emphasized: (i) we were unable to use Foxp3gfp donor cells (due to MHC mismatch) which would have enabled the re-transfer of a pure Foxp3+ T cell population; (ii) again due to MHC incompatibility, the effect of a scurfy T cell transfer upon EAE protection could not be evaluated, either in TR− mice or in co-transfer with tg T cells to RAG−/− mice; (iii) donor derived CD25+ T cells were obtained from RAG−/− mice with T cell-induced colitis, not from TR− with T cell-induced colitis and, therefore, the correct Treg specificities for EAE protection may have been absent. The reverse situation seems to hold in that in TR− mice, EAE protective Foxp3+ tg T cells induced by peptide immunization in the presence of a strong inflammatory stimulus, fail to protect from T cell-induced colitis (S. Zelenay – personal communication).

A sub-population of the donor derived CD25+ T cells may have partially protected from T cell-induced colitis in the secondary recipients and therefore had a regulatory effect on the colitogenic population. There remains the possibility that, in the re-transfer, the CD25+Foxp3+ Treg may have been at a functional and numerical advantage in comparison to those Foxp3+ T cells in the original naïve T cell transfer, explaining why they were incapable of controlling colitis in first recipient but capable of some control upon re-transfer. Donor Helicobacter colonization may also have contributed to the development of adaptive Ag-specific Treg present on re-transfer. Loss of Foxp3 expression upon Treg transfer occurred as previously described (Uhlig et al., 2006). There was no expansion of Treg in the re-transfer of CD25− T cells unlike the original transfer, indicating that only a subset of T cells, perhaps already pre-committed, can become regulatory and expand.

Once again, severe colitis involving the CD25− T cells from the primary recipients, in co-transfer with anti-MBP TCR tg T cells, protected from EAE while milder colitis did not. Even though, as discussed, a protective effect of donor derived Foxp3+ T cells cannot be totally excluded, this result points to EAE protection mediated by CD25− Foxp3− T cells. IL-10 and TGF-β are candidate mediators of this protective mechanism.

**4.4.5. Effector tg T cells in colitis lose encephalitogenic potential**

The single transfer of anti-MBP TCR tg T cells to RAG−/− recipients causes severe EAE, which is manifest within approximately 3 weeks post transfer and which is rapidly lethal in most transfers
Discussion

(experience from our laboratory reveals that occasionally RAG−/− recipients develop severe EAE but are able to survive up to 6 months with appropriate feeding and hydration). At a time that mice are ill with EAE, the frequency of Foxp3 expressing Tg T cells can be as high as 40%. This is highly indicative that the level of Foxp3 expression in tg T cells is irrelevant for EAE protection. There is no detectable Foxp3 expression in healthy TR− and, therefore, these cells are most likely to arise by conversion from naïve tg T cells. In ill TR− there is some Foxp3 expression in the CNS and in the periphery (< 0.1%) (S. Zelenay - personal communication).

The fact that Foxp3+ tg T cells are also present in TR− unprotected from EAE by DSS induced colitis in the absence of donor T cells, together with the high frequency (up to 60%) of Foxp3+ tg T cells in the surviving RAG−/− recipients of a tg T cell transfer, suggests that these are dispensable for EAE protection. Induction of naïve tg T cells to express Foxp3 seems to be inhibited by the presence of another population of T cells as the frequency of Foxp3 in the Tg populations is much reduced in the co-transfers. Conversion may occur due to a suboptimal MBP presentation by APC much in the same way that conversion to Foxp3+ T cells may happen in donor T cells (by sub-optimal presentation of Ag) or, alternatively, by a disease induced altered presentation of myelin components, leading to MBP not being recognized as self. Alternatively Foxp3 expression may be a by-product of inflammation. In the protected TR− with colitis, Foxp3 expression by donor Treg is higher than in Tg Treg because even when a small number of polyclonal Treg is transferred into an anti-MBP TCR Tg mouse, it is more likely that the sum of self Ag that the polyclonal Treg recognize is more abundant than MBP (in a healthy TR− mouse) and, therefore, donor Treg are at an advantage for self-reactivity driven proliferation when compared to the process that drives conversion to anti-MBP TCR Tg Treg cells. In addition, donor Treg are actually transferred to the recipient mice and this may confer an advantage for the process of accumulation through expansion, compared to the process of conversion alone that we propose to occur in Tg Treg.

Transgenic T cells sorted from TR− protected from EAE by T cell-induced colitis were slightly less responsive to MBP stimulation and induced mild delayed non-fatal EAE in RAG−/− recipients. In other words, they regain encephalitogenic potential. Their effect was not tested in co-transfer to RAG−/− recipients with T cells from unmanipulated TR− mice. Most of these tg cells expressed Foxp3 long after secondary transfer but this phenomenon also occurs in those rare recipients of tg T cells obtained from unmanipulated TR− that survive for long after transfer. If Foxp3+ Tg T cells were the protective population in TR− with T cell-induced colitis one would expect that the depletion of the colitogenic population would not have abrogated EAE protection with such an immediate effect.
The inhibition of tg effector function could represent a cytokine effect from the rapidly expanding colitogenic population which, in this work, is shown to produce IFN-γ and IL-17 and to be widespread. However, EAE induction in cytokine receptor knock-out mice has produced conflicting and unexpected effects when compared to the effect of direct administration of cytokines or anti-cytokine-blocking Ab. Most of the human clinical trials were disastrous with unexpected worsening (ex. IFN-γ, anti-TNF-α) and disastrous side-effects (ex. TGF-β) and some cytokines never even entered a Phase 1 stage for EAE (ex. IL-6). Apart from the marginal effects of IFN-β, none of this knowledge on cytokines and EAE models has resulted in meaningful clinical therapy. This explains why, apart from a fresh interest in the Th17 pathway and associated cytokines, most of the work in this field, over the past decade, has centred on understanding the mechanism of action of IFN-β.

Cytokine manipulation through the transfer of cytokine deficient T cell in colitis induction or administration of anti-cytokine Ab may prove fruitless in that the colitis itself may be milder and, as such, abrogate the EAE protective effect which is observed. IFN-γ is considered to be a prime candidate for EAE inhibition but, as for EAE, there is contradictory evidence as to its effect upon colitis. IFN-γ-deficient CD4+ T cells have been shown to produce a milder form of T cell-induced colitis than wt CD4+ T cells (Bregenholt et al., 1999) and anti-IFN-γ had no effect on intestinal inflammation in IL-10−/− mice with established colitis [Kullberg, 2001 #3]. In contradiction, administration of anti-IFN-γ MAb to mice soon after T cell transfer prevented development of colitis for up to 12 weeks (Powrie et al., 1994b). The lack of EAE protection in DSS colitis may be due to the lack of a particular cytokine, such as IL-2, IL-10, IFN-γ or IL-4, expected to be produced in much lower concentrations than in T cell-induced colitis. Protection from EAE in an IL-10 dependant fashion remains a likely possibility as it has been shown to be indispensable for EAE mitigation by Treg Treg (Zhang et al., 2004; Fitzgerald et al., 2007; Selvaraj and Geiger, 2008). The administration of anti-IL-10 would be expected to aggravate colitis (Asseman et al., 2003) and nevertheless be expected to abrogate the EAE protection herein described.

4.4.6. Effector tg T cells in colitis are attracted to the inflamed colon

Chemokine gradients created following local inflammation can attract effector T lymphocytes away from the target organ as exemplified by MOG specific CD4+ T cell accumulation at BCG granuloma sites with consequent EAE amelioration (Sewell et al., 2003) and by cure of pre-diabetic mice achieved by viral infections (Christen et al., 2004). In TR− with T cell-induced colitis
there is a lower frequency of Tg T cells in the Ln and colon, which is not surprising in view of the fact that the transferred donor cells are detected. There are almost 10 and 5 times more cells in the colon and Ln, respectively indicating that apart from a loss of function, there may be an accumulation of Tg T cells in keeping with preferential migration to areas of inflammation. Ideally, the frequency of tg T cells should have been determined in deep cervical Ln where the Tg T cells are first activated by the cognate CNS Ag. The decrease in number of tg T cells in the brain of a protected TR− mouse more likely reflects absence of EAE and, even though there is a diversion of Tg T cell traffic away from the CNS, this is unlikely to be a strong enough mechanism to account for lack of EAE and inhibition of effector function.

In this work, we show that a high Foxp3 expression may be a consequence of AID and chronic inflammation and while its presence is not synonymous with dominant tolerance, it may still modulate immunopathology and partially control autoimmunity. Further work, such as the identification of chemokine, adhesion molecule and cytokine patterns should elucidate specific colitogenic T cell requirements for EAE protection.
5. CONCLUSIONS

This work has contributed to further awareness of the requirements of lymphopenia as a trigger of AID and has advanced knowledge on the biologic behaviour of Treg in response to an ongoing inflammatory immune response.

5.1. Modulation of regulatory T cells after immunosuppressive therapy

Studies on the effects of steroids upon regulatory T cells that relied on the use of dexamethasone (DEX), a more potent steroid than those used in the maintenance treatments of AID reported several stimulating effects on Treg. These included an increased expression of CD25 \textit{in vitro} (Wiegers et al., 1995), induction of regulatory potential \textit{in vivo} by culture of CD4+ T cells with DEX (Ramirez and Mason, 2000; Barrat et al., 2002) and an increased CD25 expression on SP CD4+ thymocytes after a single injection of DEX 5 mg/kg (Chen et al., 2004). An increase in circulating Treg in patients treated for multiple sclerosis (Navarro et al., 2006) and autoimmune thrombocytopenia (Ling et al., 2007) has been reported as a result of therapy with high dose methylprednisolone.

In this study, Hydrocortisone (HC), considered to be a more clinically relevant steroid, was used. HC is equivalent in immunosuppressive and anti-inflammatory effect to prednisolone, the steroid used in the maintenance treatment of AID. HC was titrated and a dose which was capable of causing a significant thymic atrophy was chosen. The effects of HC were compared to other forms of immunosuppression with CD25 depletion, cyclophosphamide (Cyp), irradiation and pertussis. This kind of screening for triggers or facilitators of AID was made possible by the use of a transgenic mouse model, with a repertoire dominated by anti-myelin basic protein T cell receptor transgenic (anti-MBP TCR Tg) T cells, which develop encephalomyelitis (EAE) in the absence of Treg.

In this work, it was demonstrated that a short course of HC does not to have a selective effect on Treg number, suppressor function or homeostatic proliferating potential and evidence is
Conclusions

provided, for the first time, for drug induced immunosuppression as a precipitating factor for EAE induction in the anti-MBP TCR Tg mouse model. When this study was started, two issues regarding potential triggers of AID remained to be solved. The first concerned the effect of selective Treg depletion and the second lymphopenia induced autoimmunity. Wild type (wt) mice remained healthy after CD25 depletion by anti-CD25 monoclonal antibody (mAb) and therefore, it remained a matter of debate whether Treg depletion alone could lead to AID, as questioned by McHugh and Shevach (2002) and Caton et al. (2004). It is now know that anti-CD25 mAb only resulted in partial Treg depletion, in contrast to the invariably fatal AID caused by complete Treg depletion in wt mice (Kim et al., 2007; Lahl et al., 2007). The present work reveals that in the anti-MBP TCR Tg mouse, a significant proportion of Foxp3+ Treg are not CD25+ and as such, not surprisingly, CD25 depletion did not induce EAE. Nevertheless, CD25 depletion was shown to augment the effect of pertussis and it was shown that selective Treg depletion caused by pertussis alone also occurs, potentially contributing to its EAE inducing capacity. On the other hand, Cyp alone precipitated EAE, more so in combination with HC, and the present results suggest that Cyp selectively depletes Treg and also affects Treg function. The question of lymphopenia induced autoimmunity seems to be inexorably linked to that of Treg depletion. By causing equivalent degrees of peripheral lymphopenia and thymic atrophy, the present work demonstrates that only those stimuli that selectively reduce Treg namely Cyp, but not HC or irradiation are capable of inducing AID.

5.2. Modulation of regulatory T cells during inflammation

The stimuli and conditions for expansion, peripheral conversion and non-self specificity of Treg are under intense research at the present time. The influence of active inflammation upon Treg function is controversial. While IL-6 is claimed to render CD4+ T cells refractory to Treg cell-mediated suppression (Pasare and Medzhitov, 2003) and to promote effector rather than Treg generation (Bettelli et al., 2006) the selective expression of TLR-4 on Treg provides a mechanism for a direct response of Treg to infection (Caramalho et al., 2003). Furthermore, foot pad peptide specific immunization together with CFA, in anti-myelin basic protein TCR transgenic mice devoid of Treg, leads to the appearance of a powerful suppressive transgenic Foxp3+ T population in the presence of severe inflammation, due to conversion from naïve T cells (Zelenay, S manuscript submitted).
We demonstrate that donor Foxp3+ T cells accumulate progressively in the TR- recipients with T cell-induced colitis with a fold accumulation which can reach up to 20 times that of the transferred Foxp3+ population. Furthermore, the re-transfer of a population enriched in Treg from mice with T cell-induced colitis to alymphoid recipients dampens the ridding immune response and limits the tissue damage, as shown by the decreased number of Foxp3- T cells and by the less severe colitis. In addition, peripheral conversion from conventional CD4+ T cells to genuine Foxp3+ Treg in an inflammatory milieu had not been rigorously demonstrated, as it required the strict exclusion of pre-existing Treg and respective precursors in the experimental model. By utilizing Foxp3gfp knockin mice, sorting of GFP negative T lymphocytes allowed for exclusion of a contaminating Foxp3+ T cell population and for the transfer of a pure CD4+CD45RBhighGFP- T cell population to RAG-/- mice. The appearance of a GFP+ population in the surviving recipient is attributed to conversion of naïve to Treg. The expanded and converted Foxp3+ T cells played an important role by decreasing mortality in the T cell-induced colitis model and therefore this work is highly suggestive that inflammation promotes Treg expansion and function. As recently reviewed (Demengeot et al., 2006) the function of Treg is essentially anti-inflammatory and therefore it is not surprising that they should expand in response to inflammation and subsequently limit the immune responses that are mediated by inflammatory reactions.

EAE protection in TR- mice can be mediated by the transfer of naïve CD45RBhigh CD25- T cells (3 x 10^5 cells) from wt donors, but only in the presence of fast and severe colitis induction in H+ TR- recipients. Colitis induction was delayed in H- TR- recipients, failing to protect the mice from spontaneous EAE. Transgenic T cells sorted from TR- protected from EAE regained encephalitogenic potential and their effect remains to be tested in co-transfer with T cells from un-manipulated TR- mice. Most of these cells expressed Foxp3 long after secondary transfer but this phenomenon also occurs in those rare recipients of Tg T cells obtained from unmanipulated TR- that survive for long after transfer and if Foxp3+ Tg T cells were the protective population in TR- with T cell-induced colitis one would have expected that the depletion of the colitogenic population would not have abrogated EAE protection with such an immediate effect. The inhibition of Tg effector function could represent a cytokine effect from the rapidly expanding colitogenic population. Protection from EAE in an IL-10 dependant fashion remains a likely possibility as it has been shown to be indispensable for EAE mitigation by Treg (Zhang et al., 2004; Fitzgerald et al., 2007; Selvaraj and Geiger, 2008). The administration of anti-IL-10 would be expected to aggravate colitis (Asseman et al., 2003) and at the same time to abrogate the EAE protection herein described.
6. CLINICAL RELEVANCE AND PERSPECTIVES

Technical advances in the methodology to define and better identify Treg, in conjunction with the recent burst of information describing polymorphisms of genes implicated in the number and function of Treg and are expected to strengthen the association of Treg to the pathogenesis and genetic susceptibility of human AID.

6.1. Consequences of immunosuppressive therapies

Because of the different surface area of humans and mice and reported *in vitro* inter-species differential sensibilities of T lymphocytes to steroids (Claman *et al*., 1971; Claman, 1972), extrapolation of the results of animal experiments to man should be done with caution and therefore we are unable to transpose the dose of HC used in this study to a safe dose of HC in man. We believe this study is as close as possible to a short course of steroids commonly used in human therapy and is, therefore of clinical relevance. The clinical translation to this work is the relative safety of short duration steroid therapy with no lasting effect on the T cell compartment of the immune system, despite significant thymic atrophy and less pronounced peripheral lymphopenia. The present work confirms the sparing effect that Dex has upon upon Treg in the thymus and periphery and the deleterious effect of Cyp and Ptx upon Foxp3+ Treg. The pronounced deleterious effect upon Treg number and function caused by combined HC + Cyp, a very commonly used therapy in the treatment of AID should serve as a warning to prescribing physicians and is in agreement with the recent realization that long term treatment of lupus nephritis with Cyp is associated with more relapses than other IS agents (Contreras *et al*., 2004; Ginzler *et al*., 2005).

This work does not exclude that intermittent or chronic steroid therapy, lasting months to years, may have deleterious effects upon Treg and contribute to autoimmunity and does not deal with the acute and chronic effects of high dosages of steroids upon the immune system and these are questions that remain to be formally tested. Furthermore, studies on Treg effects of
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Immunosuppressive agents in the anti-MBP TCR Tg mice are endowed with an easy clinical readouts and may provide the standard against which the effect of other IS agents on regulation can be studied.

6.2. Considerations for regulatory T cell immunotherapy

Animal models of IBD primarily target the gut mucosa producing a similar clinical picture characterized by loss of weight, diarrhoea and intestinal bleeding. In humans, IBD may reflect a defective innate or adaptive immune system, gut colonization by specific microbes, an abnormal response to gut microbes, an autoimmune disease or a combination of any one of these factors in a particular individual. A review of the findings in colitis models may provide several and important clues for questions pertaining to the physiology of Treg.

Current animal models which actually involve disease induction through chemicals that destroy the gut epithelium or the transfer of CD4+ T lymphocytes are able to provide clues into the aetiology of the disease and demonstrate the involvement of commensals in disease pathogenesis. On the one hand, chemical colitis, as the name proposes, involves severe mucosal injury, is primarily mediated by the innate system, is as intense in the absence of an adaptive response (Dieleman et al., 1994; Axelsson et al., 1996) and can be induced in germ free (GF) mice (Kitajima et al., 2001; Huber et al., 2004). Chemical colitis is actually reportedly more severe in GF than in conventionally kept wt mice (Kitajima et al., 2001), invoking a paradoxical protective effect of gut bacteria. Indeed, ongoing work in our laboratory has revealed that germ free mice have less CD4+Foxp3+ Treg in the mLN which together with the information that polyclonal Treg in germ free mice are not as potent suppressors in vitro, as those from conventional animals (Ostman et al., 2006), suggests that the presence of a microbial flora favors the development of Treg.

In contrast, the presence of gut bacteria is a strict requirement for colitis induction in T cell-induced colitis (Powrie et al., 1997), where disease is caused through the transfer of T lymphocytes reacting against gut commensals. In addition, colitis can occur spontaneously in germ free mice that are deficient for IL-2 (Schultz et al., 1999) and TGF-β (Boivin et al., 1997) and spontaneous colitis in IL-10 deficient mice is ameliorated by SPF conditions (Kuhn et al., 1993; Berg et al., 1996) and aggravated by experimental Helicobacter hepaticus infection (Kullberg et al., 1998). At present, we do not know which of these models best represents IBD in
humans but it is likely they complement each other rather than demonstrate several distinct aetiologies.

Treg have been found to be protective in several examples of colitis. Polyclonal Treg are protective against the induction of chemical colitis in Tg mice with defective TGF-β signalling (Huber et al., 2004), have been found to prevent (Morrissey et al., 1993; Powrie et al., 1993) and cure (Mottet et al., 2003) T cell-induced colitis. Treg are also reported to inhibit Helicobacter induced inflammation in RAG-/- mice in the absence of a T cell transfer (Maloy et al., 2003). Furthermore, CD4+CD45RBlow T cells from germ-free mice have been reported to be capable of inhibiting colitis induced by CD45RBhigh T cells in recipients that were not germ free (Annacker et al., 2000). The lack of requirement for Treg specificity in these protective transfers does not exclude that the correct natural Treg specificities were not present in the polyclonal transfer, except that the specificities for which Treg are selected in the thymus of germ free mice are only expected to represent self-specificities. One would have to argue for cross-reactivity to support a specificity requirement in this condition or for the protection resulting from naïve T cell conversion to Treg, the latter demonstrated in the present work. CD25 depletion of mice with ongoing T cell-induced colitis would be expected to further substantiate the protective effect of Treg.

In this work, we were unable to transfer disease from Helicobacter positive (H+) recipients with T cell-induced colitis to H- recipients, in SPF conditions upon secondary transfer. This is in agreement with a previously reported finding that H. hepaticus Ag specific CD4+ Th1 clones actually transfer disease to H. hepaticus-infected T cell-deficient RAG-/- hosts but not to uninfected recipients (Kullberg et al., 2003). The fact that T cell-induced colitis can be induced in H- recipients colonized with other bacteria in SPF conditions, implies that Helicobacter is not a strict requirement for DC activation and Ag presentation. The herein reported inability to induce colitis in germ free mice could be tested by concomitantly administering DC with the colitogenic T cell transfer.

These finding suggests that the immune response is not directed to self, that colitis can develop as a result of an immune response directed solely against gut bacteria and that the response to Helicobacter is dominant. These may indirectly explain why colitis in H. hepaticus infected RAG-/- animals induced by the transfer of CD4+ T cells from infected colitic IL-10-/- mice is only prevented by the co-transfer of CD45RBlow cells from H. hepaticus- infected wt mice and not from uninfected wt animals (Kullberg et al., 2002), highly suggestive of a Treg specificity requirement for colitis control. Furthermore, we provide evidence that the expansion of Treg under conditions of infection/inflammation is dramatic, dampens the immune response and
contributes to a limitation of tissue damage. A translation into clinical practice of these aspects, together with our findings, effectively means that even a very small number of Treg is probably beneficial in ongoing disease.

The issue of Treg specificity is very important for therapeutic considerations. No completely effective therapeutic strategy has been established because the etiology of IBD and AID remains largely unknown. Conventional immunosuppression and even recombinant proteins that inhibit specific inflammatory molecules (such as anti-TNF) are efficacious in remission induction but at the cost of increased infections and less tumour surveillance. The low frequency and poor growth in culture of Treg, however, makes acquiring adequate numbers of cells for adoptive immunotherapy a significant challenge, requiring isolation of Treg or conversion to Treg in vitro and subsequent expansion and re-infusion. This can be done through specific genetic manipulation, specific cytokine combinations and if a specific defect in an Ag-specific Treg is detected, the ex-vivo generation of Ag-specific Treg may be attempted through the targeting of ligands to dendritic cell such as described experimentally (Kretschmer et al., 2005) or by the use of a drug such as rapamycin which induces Treg in vivo (Battaglia et al., 2005; Battaglia et al., 2006; Keever-Taylor et al., 2007; Strauss et al., 2007; Kang et al., 2008).

For AID, a scenario is envisaged where, in the presence of autoimmunity, we use markers such as Treg number and function, HLA typing and the results of a specific screen for a known set of particular susceptibility genes (which probably include genes involved with Treg selection, survival, expansion, function and conversion) to determine the probability of AID development. In these patients, where the probability of disease is high, it would be possible to boost Treg response, if possible in a determinant Ag-specific manner in addition to Treg therapy in order to prevent disease. These strategies could be combined with treatments that would block pathogenic T cells once the disease is established and has caused end organ damage.

6.3. Perspectives

Our results, in mice, add to the large body of evidence that a reduction in Treg number or function is associated with AID, but a direct link between Treg deficiency and the most frequent AID remains to be established in humans. Many factors can affect Treg levels as exemplified by the modulation of Treg numbers with the phase of the menstrual cycle (Arruvido et al., 2007) and with aging (Haas et al., 2007). It will be important to gain insight into basic knowledge about Treg in
humans, still lacking because the following remain to be evaluated: (i) fluctuations in frequency and total number of circulating Treg in normal individuals (ii) relationship between serial levels of circulating Treg and inter-current infections/inflammations, common to daily life; (iii) serial frequency, total number of circulating Treg and suppressor function in relation to onset, relapses and remissions in several AID (iv) correlation between PB frequencies of Treg and those of lymphoid organs and target tissues and (v) Treg specificity based on the knowledge of determinant auto-antigens in disease pathogenesis. The serial study of Treg frequency and function in family members should provide many interesting answers, especially in those that develop AID. The study of the functional consequences of polymorphisms of genes involved in Treg selection and function may be the clue to the aetiology of AID in humans.

The effect of long term steroid therapy for AID upon Treg, needs to be studied with an alternative experimental layout such as the use of subcutaneous steroid implants for long term drug delivery, from 3 to 6 months in the anti-MBP TCR Tg or other models of AID such as lupus and arthritis in mice. The fact that lymphopenia only caused AID only in the context of selective Treg depletion suggests that Treg number and function should be studied in humans after documented drug related (Coles et al., 1999) or viral induced (Okada et al., 2000; Nichols et al., 2001) CD4⁺ lymphopenia, correlated to clinical follow-up with respect to AID development.

In parallel to the role of Helicobacter colonization in animal models, the present work indicates that a search for microbes which colonize human IBD patients and elicit an specific immune response is worthwhile, as for these cases, there may be a requirement for Ag specificity in Treg based therapies. Treg accumulated selectively after naïve T cell transfer but failed to reveal any protection towards EAE upon secondary transfer. Donor derived CD25⁺ T cells were nevertheless obtained from RAG⁻/⁻ mice with T cell-induced colitis, not from TR⁻ with T cell-induced colitis where, therefore, the correct Treg specificities for EAE protection may have been absent. In this respect, the backcross of Foxp3gfp mice to the B10.PL background would have enabled the transfer of a pure Foxp3⁺ T cell population derived from mice with colitis.

The realization that inflammation is an important stimulus for Treg stresses the potential therapeutic role of Treg supplementation during anti-inflammatory or immunosuppressive therapies and these are questions that remain to be formally tested. Accordingly it would be interesting to study an eventual “anti-inflammatory theory” complementary to the “hygiene theory” of AID where anti-inflammatory drugs, much frequently used in western societies, could lower the threshold of Treg and trigger AID in predisposed individuals. To test this hypothesis, epidemiological evidence should be collected and an experimental layout similar to the one used
in this study would be appropriate. The exponential increase in AID justifies further scientific
study.
7. REFERENCES


References


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Modulation of regulatory T cells in autoimmune disease


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Suppressor function of umbilical cord blood-derived CD4+CD25+ T-regulatory cells exposed to graft-versus-host disease drugs.


Modulation of regulatory T cells in autoimmune disease


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APPENDIX
