Investigating the Regulatory Cells that Mediate Recovery from CNS-Targeted Autoimmune Disease

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A Thesis Submitted for the Degree of Doctor of Philosophy

The University of Edinburgh
Declaration

I declare that this thesis has been composed by myself, describes my own work and has not been submitted in any other application for a higher degree.

Mandy McGeachy

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Abstract

Autoimmune disease, such as multiple sclerosis, results from the breakdown of immune tolerance to self. The goal of therapy is to restore this state of tolerance, and understanding how this may occur physiologically is important for improving intervention strategies. Experimental autoimmune encephalomyelitis (EAE) is a mouse model of multiple sclerosis in which disease is induced by immunisation with antigenic components of myelin. In B6 mice, disease course is monophasic and mice spontaneously enter remission, providing an ideal scenario to study the natural restoration of tolerance. IL-10-deficient mice fail to recover from EAE, and it is thought that production of IL-10 within the CNS is particularly important for recovery. Previous work from the Anderton lab had also shown B cell production of the immunoregulatory cytokine IL-10 to be vital for recovery.

The aim of this PhD was to further investigate the role of IL-10 producing B cells and CD4\(^+\) T cells during remission from EAE. While B cells clearly play an important role in recovery, and post-recovery B cells can transfer protection to naïve recipients, B cells do not localise to the CNS. Instead, IL-10 producing CD4\(^+\) cells having phenotypic markers and functional characteristics of regulatory T cells were found to accumulate in the CNS during recovery. Depletion of CD25\(^+\) cells in vivo resulted in impaired recovery, and transfer of CD4\(^+\)CD25\(^+\) cells from recovering mice protected recipients, further confirming a role for these cells. Thus, regulation through IL-10 production appears to be mediated by both B cells and CD4\(^+\)CD25\(^+\) T cells in this model.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>Ab</td>
<td>antibody</td>
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<tr>
<td>Ag</td>
<td>antigen</td>
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<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>BBB</td>
<td>blood brain barrier</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>CFA</td>
<td>complete Freund’s adjuvant</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>cutaneous T lymphocyte antigen-4</td>
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<tr>
<td>EAE</td>
<td>experimental autoimmune encephalomyelitis</td>
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<tr>
<td>DC</td>
<td>dendritic cell</td>
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<tr>
<td>FACS</td>
<td>fluorescence activated cell sorting</td>
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<td>GITR</td>
<td>glucocorticoid-induced TNF receptor</td>
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<tr>
<td>IFA</td>
<td>incomplete Freund’s adjuvant</td>
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<td>IFN</td>
<td>interferon</td>
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<td>interleukin</td>
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<tr>
<td>iv</td>
<td>intravenous</td>
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<td>LN</td>
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<td>MBP</td>
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<td>MHC</td>
<td>major histocompatibility complex</td>
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<tr>
<td>MOG</td>
<td>myelin oligodendrocyte glycoprotein</td>
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<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>MS</td>
<td>multiple sclerosis</td>
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<td>OVA</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PLP</td>
<td>proteolipid protein</td>
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<td>PTx</td>
<td>pertussis toxin</td>
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<td>rtPCR</td>
<td>reverse transcriptase polymerase chain reaction</td>
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<tr>
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<td>severe combined immunodeficiency</td>
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<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor β</td>
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<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
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<tr>
<td>Treg</td>
<td>regulatory T cell</td>
</tr>
<tr>
<td>Tr1</td>
<td>type 1 regulatory T cell</td>
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1. INTRODUCTION

1.1. Part 1: Immune tolerance

The immune system cannot predict which foreign antigens (Ag) it will encounter during a lifetime, yet is required to produce a highly specific and rapid response to invading pathogens. The adaptive immune system consists of a multitude of B cells and T cells, each bearing a randomly generated Ag receptor and capable of rapidly expanding and acquiring effector functions upon receptor engagement. The random generation of receptors with such a varied repertoire of specificities, while beneficial in terms of ability to recognise many pathogenic Ag, inevitably generates cells with reactivity against the body's own tissues, or for innocuous Ag such as proteins ingested as food. If these cells become activated they may cause harmful tissue damage. For this reason, stringent safeguards to prevent autoreactive cells entering the periphery, and to prevent initiation of inappropriate responses, have evolved in tandem with the adaptive immune system. However, these do occasionally fail resulting in autoimmune diseases (such as multiple sclerosis (MS) and type-I diabetes) or allergic reactions (such as coeliac disease and asthma).

1.2. Central T cell Tolerance

During their development, precursor T cells leave the bone marrow and enter the thymus. As they move through the thymus they are "educated" through the processes of positive and negative selection (reviewed in (1) and (2)). T cell receptors (TCR) recognise Ag indirectly, binding processed peptides presented in the context of MHC molecules on the surface of other cells. The first checkpoint in T cell development is recognition of MHC on cortical thymic epithelial cells, and only those T cells bearing TCRs capable of binding self-MHC survive; in this way the mature T cell repertoire becomes MHC-restricted. At this point T cells also change their expression of the CD4 and CD8 coreceptor molecules, going from double positive to single positive expression (3). CD8+ cells are restricted to MHC class I molecules, which
are found on the surface of most cells. CD4⁺ T cells are restricted to MHC class II molecules, which are usually only present on the surface of professional Ag presenting cells (APC) including dendritic cells (DC) and B cells (4, 5).

As the developing T cells move into the thymic medulla they are exposed to myriad self-antigens presented by epithelial cells. Thymic medullary epithelial cells possess the ability to express a variety of proteins that are otherwise highly tissue-specific (6). The transcription factor AIRE is vital for expression of some of these proteins, such as the pancreatic islet β cell Ag preproinsulin II, in the medullary epithelial cells of the thymus (7). People or mice with a mutation in AIRE develop a syndrome of spontaneous autoimmune diseases against organs including the parathyroid gland, adrenal glands, ovaries, liver, pancreas (β cells) and skin (melanin) (7). Transcripts of the myelin Ag myelin basic protein (MBP) and proteolipid protein (PLP) are also expressed in the thymus (6). Many T cells bearing TCRs with high affinity for these self peptide-MHC complexes are deleted in the thymus through apoptosis (1, 8). An alternative fate of T cells recognising self peptide with high affinity may be differentiation into regulatory T cells (9), and this will be discussed in section 1.5.

Some potentially autoreactive cells escape deletion by virtue of low avidity interactions with self peptide-MHC (8, 10), and some self peptides may not be presented in the thymus (11, 12). Also, cells recognising innocuous non-self Ag (such as food Ag) are obviously also not deleted in the thymus. Therefore, central tolerance is incomplete, and peripheral mechanisms to control the activation of T cells must be implemented to avoid harmful responses.
1.3. Peripheral T cell tolerance

1.3.1. Clonal Ignorance

Some autoreactive T cells never become activated simply because they never come into contact with their cognate Ag (clonal ignorance). Sites such as the testes, eye and CNS have tight endothelial junctions that act as a physical barrier and do not usually allow naïve lymphocyte entry (13). Therefore, T cells specific for Ag in these tissues are not normally activated, or effector cells cannot gain entry to initiate pathology, unless the barrier is broken down by inflammation (14, 15). APC in these so-called “immune privileged sites” may also actively delete T cells or induce tolerance in cells entering these sites (16).

Another form of clonal ignorance is the failure of APC to present certain epitopes of a protein Ag. During normal Ag processing, proteins are cleaved at specific enzymatic sites, so that common immunodominant epitopes tend to be presented to T cells, either for deletion in the thymus or for activation in the periphery (17). Thus, even if T cells specific for cryptic self epitopes (which are not normally generated during Ag processing) are allowed into the periphery, they will not normally be activated.

1.3.2. Deletion

The most obvious solution for dealing with autoreactive T cells in the periphery would be to delete them. T cells that bind peptide-MHC complexes with very high avidity are deleted through activation induced cell death (AICD) (18). AICD is mediated by upregulation of the pro-apoptotic molecule Fas on the surface of highly activated T cells, and Fas-deficient mice are prone to lymphoproliferative/autoimmune disease (19). Deletion of cells with high affinity TCRs may also lead to “tuning” of the T cell response, such that pathogenic cells are deleted but those with too low affinity for self to induce autoimmune disease remain
unaffected (20). While this may seem potentially dangerous, it probably also maintains TCR repertoire diversity, which is important for protection against pathogens.

1.3.3. Anergy

Not all self-reactive T cells in the periphery are deleted after their encounter with Ag. Mature T cells leave the thymus and circulate through the lymphoid system, surveying DC for cognate antigen. In order to become fully activated, naïve T cells must not only receive signals through TCR binding of peptide-MHC complexes ("signal 1"), they also require interaction with costimulatory molecules such as B7 presented on the surface of APC ("signal 2") (21, 22). Full activation results in production of interleukin (IL)-2 which acts in an autocrine fashion to allow T cell proliferation and differentiation into effector cells (23). Without the appropriate costimulation, the T cell undergoes abortive activation, leading to a state of unresponsiveness in which further TCR ligation, even in the presence of costimulation, is unable to induce proliferation (24-26). This is termed anergy.

MHC and costimulatory molecules are upregulated on the surface of APC in response to innate "danger" signals (21, 22, 27). These signals may be from host factors such as IL-6 and TNFα released during tissue damage, or through the binding of common pathogen-associated molecular patterns (PAMPs) to pattern recognition receptors on APC, for example bacterial lipopolysaccharide (LPS) binding to Toll-like receptor(TLR)-4 on DC (28). These receptors form a safeguard within the innate system by upregulating T cell activating molecules only under appropriate conditions i.e. when cell damage has occurred or foreign organisms are detected. It is thought that the continuous presentation of self-Ag in the absence of costimulation (i.e. when no danger signals have been received) is utilised by DC draining healthy tissue as an active mechanism of inducing anergy of self-reactive T cells (29, 30). Then, when these same self Ag are inevitably presented along with foreign Ag in "danger" mode, only naïve T cells recognising the foreign Ag are activated and the resultant immune response is focused on the "dangerous" Ag rather than self.
It seems counter-intuitive for the immune system to keep cells that are potentially autoreactive. Why not delete them? It may be that these anergic cells now contribute to maintaining tolerance through blocking access of naïve T cells to the same Ag, or inducing anergy or deletion in autoreactive B cells (31).

1.3.4. Immune Deviation

Polarisation of the T cell effector response may be related to the activation signals initially received by APC, and is important in determining the overall outcome of a response (32, 33). Th1 cells mainly produce IFNγ, which activates macrophages to increase killing of phagocytosed or intracellular organisms and also slows pathogen growth through its cytostatic effects. Th2 cells produce cytokines such as IL-4, IL-5, IL-10 and IL-13 to aid B cell maturation and antibody (Ab) production, and activation/recruitment of innate inflammatory cells such as eosinophils and mast cells, for the combat of extracellular pathogens. Both phenotypes cross-regulate the expansion and differentiation of the other (32, 34). Skewing of the response can be a useful mechanism of preventing or alleviating damaging responses to innocuous Ag, particularly when switching Th1 to Th2 responses,(35, 36), although both Th1 and Th2 responses can initiate autoimmune disease of different types. Immune deviation could therefore be considered a rather simplistic tolerance mechanism.

1.3.5. Regulatory T cells

It has now become clear that in addition to the above mechanisms of peripheral tolerance, harmful responses against self-Ag are actively prevented by a population of regulatory T cells (Tregs). The concept of a population of suppressor cells that prevent autoimmunity was first proposed over 30 years ago by RK Gershon (37). Two important findings in the 60s and 70s pointed towards the co-existence of suppressor cells and self-reactive (potentially pathogenic) cells in healthy animals, and that removal of the suppressor population would allow the initiation of autoimmune disease. First, it was observed that mice that were thymectomised on
day three of life (d3Tx) developed a spectrum of autoimmune diseases, and disease could be prevented by transferring normal adult CD4+ cells before day 14 (38, 39). Second, thymectomy of adult rats followed by sublethal doses of irradiation (to deplete some peripheral lymphoid cells) induced autoimmune thyroiditis that could again be prevented by transfer of normal adult CD4+ cells after irradiation (40). The field of suppressor T cell research boomed in the 70s and 80s, with some convincing evidence for the existence of both CD4+ and CD8+ suppressor cells, but fell out of favour due to a failure to identify the elusive “suppressor factors” thought to be required for function of suppressor cells (reviewed in (41)).

With the development of more sophisticated methods for identifying and sorting cell populations, the evidence for the existence of suppressor T cells became overwhelmingly convincing, and the field was rejuvenated under the title of regulatory T cells in the 1990s. Sakaguchi initially narrowed down the cell population able to prevent autoimmune disease in d3Tx mice to those CD4+ cells expressing CD5 at high levels (42). They also showed that autoimmune/wasting disease could be induced in T cell-deficient mice by transfer of splenocytes depleted of CD5hi cells (43). Mason and colleagues further refined this regulatory function to be contained within the CD45RBlo populations, using a similar approach of cell transfer into lymphopaenic recipients to induce autoimmune/wasting disease (44). Finally, Sakaguchi and colleagues showed that the CD4+ population able to suppress autoimmune cells, and lacking in d3Tx mice, constitutively expressed CD25 (IL-2Rα) and make up 5-15% of total CD4+ cells in the peripheral lymphoid system of normal mammals(45). While CD25 expression is not limited to regulatory T cells, and not all CD4+ regulatory cells express CD25 (46, 47), the identification of CD25 as a marker for Tregs was a great step forward.

The ability of CD4+CD25+ cells to regulate autoimmune responses has now been demonstrated in several models including autoimmune gastritis (45), Type-I diabetes (46, 48) and EAE(49, 50). The role of CD4+CD25+ cells in preventing responses to tumour Ag (51), infectious pathogenic Ag - a role in memory maintenance has been
suggested (52), transplant Ag (53) and allergic responses (54) has also been well documented in mice, and data on roles in human disease are now emerging. Prevention and control of autoimmune responses will be focussed on in this introduction.

Since the demonstration of CD4⁺CD25⁺ regulatory cells, several other types of regulatory cells, including CD4⁺ Tr1 and Th3 cells(55, 56), CD8⁺ T cells(57), NKT cells(58), and even B cells(59, 60), have been described. It could be argued that many cells have regulatory function within the context of the activation and control of normal immune responses, but how many of these cells have truly dedicated regulatory function is debatable. For example, Th2 cells prevent the differentiation of Th1 cells (34), and in some autoimmune settings such as EAE, where disease is induced by Th1 cells, Th2 cells could therefore have a beneficial effect simply by "regulating" Th1 activation (61). Also, some regulatory cell types can be induced by treatment of DC or altering priming conditions in vitro or in vivo (55, 62, 63). Whether these conditions occur naturally is doubtful, however these artificial manipulations can point towards the development of new therapies.

1.4. B cell tolerance

The B cell receptor (BCR) directly recognises conformational epitopes of native proteins. During development in the bone marrow, B cells are positively selected based on surface BCR expression(64), although the exact mechanisms of this selection are unclear. Immature B cells have a lower threshold for BCR signalling, and cells binding Ag with high affinity tend to be deleted at this stage (65), but this process is probably even less thorough than T cell thymic selection. Also, during the activation and differentiation of mature B cells in response to Ag, uniquely, developmental genes are reactivated to initiate genetic rearrangement of the BCR (66). The aim of this process of somatic hypermutation is to generate BCRs of increasing affinity for the Ag, but the potentially harmful consequence is the generation of BCRs (and Ab) specific for self. Therefore, stringent peripheral checks
are required to ensure B cells remain specific for the initiating Ag and that B cells specific for self Ag are not allowed to differentiate into Ab-producing cells.

In the periphery, crosslinking of the BCR by multimeric Ag generates activation signals, and BCR-bound Ag is internalised for rapid processing and presentation on MHC class II molecules (67). B cells also have receptors for "danger" signals such as LPS (28). For complete activation and maturation of the B cell response to take place (germinal centre formation, affinity maturation, class switching), CD4+ T cell help is required through the interactions of MHC-peptide:TCR, CD40:CD40L, and possibly Fas:FasL (68, 69).

B cells unable to elicit T cell help undergo apoptosis or become anergic (64). Failure to receive T cell signals is likely to occur when B cells are specific for autoAg, and the corresponding T cells have therefore been deleted or anergised. In a BCR tg model in which B cells develop in the presence of their specific soluble Ag, B cells that had not received BCR signals (due to lack of crosslinking) but presented peptide MHC complexes to T cells and therefore received signals through ligation of CD40 and Fas, underwent apoptosis (68). This is the likely outcome whenever B cells present abundant soluble self-Ag, taken up via the BCR in the absence of crosslinking, or by pinocytosis.

B cells receiving chronic stimulation via the BCR are also induced to apoptose when CD40 and Fas are ligated by CD40L and FasL on interacting T cells (68). This not only ensures downregulation of ongoing responses, but also deletes any cells responding to self Ag (i.e. Ag that is constantly present). It has also been reported that B cells specific for self Ag are excluded from B cell follicles when the Ag is chronically present, leading to their death in T cell zones (70).
It has been found that autoreactive B cell anergy may be overcome by provision of sufficient "help" from activated CD4+ T cells, in a model using B cells with transgenic TCRs specific for anti-dsDNA (71). Interestingly, in this model, addition of CD4+CD25+ regulatory T cells allowed only abortive B cell activation after Ag binding, and prevented secretion of autoAb, suggesting a role for Tregs in maintenance of autoreactive B cell anergy. In support of this, the initial reports of the effects of CD4+CD25+ cell depletion by d3Tx included B cell autoimmunity (45), and Bystry et al have reported the ability of CD4+CD25+ cells to inhibit B cell blasting in response to Ag in vitro, and to inhibit in vivo B cell autoAb production (72). However, in these studies additional effects of CD4+CD25+ cells on helper CD4+ cells may also act to prevent autoimmune B cell Ab production in vivo.

Overall then, the maintenance of B cell tolerance relies heavily on signals (or lack thereof) from CD4+ T cells. Therefore, efficient maintenance of CD4+ T cell tolerance is perhaps even more important than direct effects on B cells.

1.5. The Biology of Regulatory T cells

Over the last ten years or so, a wealth of information regarding regulatory T cell biology and functions in various immune settings has been uncovered, although many questions still remain. A more detailed description of what is currently known about these cells is therefore merited.

1.5.1. Origins and specificity of CD4+CD25+ regulatory T cells

As well as its role in deletion of self-reactive cells, the thymus has been shown to be the major generator of CD4+CD25+ cells (73, 74). Thymectomy of mice on day 3 of life (d3Tx) results in a reduction of CD4+CD25+ cells in peripheral lymphoid organs of adult mice (45, 73), demonstrating that the thymus is required for their generation, and that generation of CD4+CD25+ cells occurs fairly late in development. The autoimmune disease that develops in d3Tx mice can be prevented by transfer of
CD4⁺CD25⁺ cells from normal adult mice (73, 74), demonstrating the importance of these cells for maintenance of peripheral self tolerance.

Thymic selection of CD4⁺CD25⁺ cells is thought to be based on recognition of self Ag presented on thymic epithelial cells (75). It has been observed in mice with transgenic TCR specific for non-self Ag that CD4⁺CD25⁺ cells tend not to be generated when they are crossed onto a RAG-deficient background (74, 76). A high proportion of CD4⁺CD25⁺ cells in transgenic mice possess an endogenously rearranged TCR, suggesting that this is required for self-reactivity and differentiation into a regulatory cell in the thymus (74, 76). This has been confirmed by crossing mice expressing neo-self Ag, such as RIP-OVA mice (which express OVA in pancreatic β cells and also in thymus under the control of the rat insulin promoter) with mice bearing transgenic T cell receptors specific for OVA (DO11.10 mice). The offspring of these mice were found to have higher than normal proportions of CD4⁺CD25⁺ regulatory cells in both thymus and peripheral lymphoid organs (around 50% of peripheral CD4⁺ cells expressed CD25 and were specific for OVA)(77).

Several pieces of evidence suggest that selection to become a regulatory cell may be based on avidity for Ag(9, 76, 78). Work from Caton’s group has demonstrated that different outcomes of T cell selection could be driven by TCR affinity or level of self-Ag expression. In these studies, HA28 transgenic mice, which express the influenza virus haemaglutinin (HA) protein as a neo-self Ag in the thymus and periphery, were crossed with mouse strains having transgenic TCRs of varying affinity for the S1 epitope of HA(9). Conversely, mice bearing a single HA-specific transgenic TCR were crossed with mouse strains expressing HA under the control of different promoters leading to different levels of HA expression (79) Overall, these studies have shown that as peptideMHC:TCR avidity is increased, a combination of increased deletion of HA-specific cells and selection of a higher proportion of CD4⁺CD25⁺ regulatory cells occurs, and this process is mediated by radio-resistant thymic epithelial elements. Therefore, this model proposes that some of the CD4⁺ cells bearing TCRs with high affinity for self Ag are not deleted, but are programmed...
to become regulatory cells and released into the periphery as a crossover mechanism of central tolerance policing peripheral tolerance. This proposal has been further supported by findings in a model crossing DO11.10 mice with mice generated to express ovalbumin in all nucleated cells(76).

Whether cells selected to become CD4+CD25+ regulatory cells in the thymus differ in their TCR repertoire and specificity from CD4+CD25- cells is unclear. It would seem intuitive that they should differ to some extent, since high affinity for self may determine the cell fate, and combined usage of certain TCR-α and -β chains may predispose to self-recognition. The models described above show that a different fate for clonally-related cells is possible, depending on avidity for and level of expression of cognate Ag (9, 76). However, these studies are far from physiological. Like the naïve T cell repertoire, the TCR repertoires of peripheral CD4+CD25+ cells appears diverse (80). Recent work from Rudensky's lab has suggested that while there is some overlap in TCR alpha and beta chain usage, certain Vα chains are dominantly expressed by CD4+CD25+ cells in contrast to CD4+CD25- cells, and that this may be related to differences in affinity for self (78). Thus some T cells may be predestined to become regulatory cells.

Certain tolerance protocols, specifically low dose oral Ag administration (81, 82), intravenous injection of a low dose of soluble peptide (81), or continuous subcutaneous infusion of peptide (83) can lead to de novo induction of CD4+CD25+ cells from naïve CD4+CD25- T cells. These cells appear very similar to thymically-generated CD4+CD25+ cells in phenotype and function. Exposure to TGFβ during naïve T cell activation may also induce their differentiation into regulatory T cells with the phenotype of thymically-generated CD4+CD25+ cells(84, 85). It is therefore difficult to determine the true frequencies of thymically-generated versus peripherally-induced Tregs.
1.5.2. IL-10 producing T regulatory Type-1 cells (Tr1s)

A further population of inducible CD4\(^+\) regulators have been termed T regulatory type 1 (Tr1) cells (55, 86). Tr1 cells were initially shown to be generated by in vitro culture with Ag in the presence of IL-10, but other mechanisms of producing cells with similar characteristics have been demonstrated, including activation of naïve CD4\(^+\) cells in the presence of the active form of Vitamin D3 and dexamethasone (62), or nasal administration of peptide in vivo (63). The main defining characteristic of this group (or groups) of cells is the production of high levels of IL-10 in response to Ag Analogous with CD4\(^+\)CD25\(^+\) Tregs, Tr1 cells have been shown to inhibit colitis when cotransferred with CD45RBhi cells into Severe Combined ImmunoDeficiency (SCID) mice (86), and also to inhibit the proliferation of responder cells in vitro (although this may not require IL-10) (87).

Tr1 cells may be induced by IL-10-producing DC in vivo: work from the Mills’ group showed that DC induced to produce IL-10 by binding of filamentous haemaglutinin from Bordetella Pertussis, generated Bordetella-specific Tr1 cells (88). Natural induction of Tr1 cells by DC in vivo as a mechanism of self tolerance, although expected to occur, has not yet been definitively shown. It is thought that immature DC may induce Tr1s in vivo (55, 89).

Tr1-type (IL-10 producing) cells specific for nickel have been cloned from the blood of healthy individuals as well as skin and blood of Nickel-allergic patients (90). Non-allergic individuals appeared to have higher levels of these cells suggesting a role in prevention of allergy. Likewise, host-specific IL-10 producing cells have been observed in SCID patients that have received allogeneic bone marrow (91), and are thought to contribute to prevention of graft-versus-host disease in these patients. Veldmann et al have recently identified desmoglein 3-specific Tr1 cells in healthy people (92). Skin desmoglein 3 is the auto-Ab target in pemphigus vulgaris, and patients with this condition were found to have a relative deficiency of desmoglein 3-specific Tr1 cells (92). Low dose peptide therapy for cat dander allergy has also been associated with an increase in Ag-specific IL-10 concomitant with decreased Th1 and
Th2 cytokine production (93). Thus, Tr1 cells may play an important role in preventing certain autoimmune/allergic responses in humans.

1.5.3. Distinguishing Markers of Tregs

Thymically generated Tregs constitutively express markers such as CD25, CTLA-4 and GITR (94, 95), but these molecules are also upregulated during the activation of naïve or memory T cells. Therefore, when attempting to investigate their role in disease or inflammatory settings great care must be taken to distinguish cells that are regulatory in function from recently activated effector cells that have a similar phenotypic appearance.

One distinguishing feature is cytokine production: CD4\(^+\)CD25\(^+\) cells do not produce IL-2 or classic Th1/Th2 cytokines such as IFNγ/IL-4 upon restimulation (94, 95) (production of IL-10 and/or TGFβ is controversial and is discussed in section 1.5.4. CD4\(^+\)CD25\(^+\) cells are also classically anergic in vitro, in that they do not proliferate in response to cognate Ag in the absence of exogenous IL-2, in contrast to activated T cells which produce IL-2 and proliferate in response to cognate Ag in vitro. CD4\(^+\)CD25\(^+\) cells can inhibit IL-2 production by activated T cells(96, 97), thereby preventing their in vitro proliferation – another functional characteristic to distinguish them from activated T cells.

Very few molecules have been described that are specifically and uniquely expressed by regulatory T cells. Neuropilin-1, a receptor involved in T cell activation, was recently shown to be constitutively expressed on CD4\(^+\)CD25\(^+\) cells(98). It appears to be a distinguishing marker since expression of neuropilin-1 is not upregulated upon activation of naïve T cells(98) However, expression levels (as measured by flow cytometry) were not very high, so it remains to be established whether this will be a useful marker for identifying and sorting regulatory T cells.
Recently, the transcription factor FoxP3 was described to be required for the regulatory function of CD4⁺CD25⁺ cells (99-101) (see section 1.5.4). In humans there has been some doubt as to whether FoxP3 is also upregulated upon activation in naïve cells (102). In mice, FoxP3 expression does appear to be restricted to regulatory cells, although this included both CD4⁺CD25⁺ and CD4⁺CD25⁺CD45RB⁺ cells (101). However, FoxP3 expression does not appear to be restricted to thymically-generated regulatory cells: stimulation of naïve CD4⁺CD25⁻ cells with TGFβ has been reported to induce CD4⁺CD25⁺ regulatory cells expressing FoxP3(84, 85). Interestingly though, Tr1 cells, induced in vivo by intranasal peptide administration or by in vitro stimulation of cells with Ag in the presence of Vitamin D3 and dexamethasone, do not appear to express FoxP3(87), suggesting that Tr1 and CD4⁺CD25⁺ cells are indeed separate lineages of regulatory cells. Production of anti-FoxP3 mAb for use in mice, and GFP-FoxP3 mice will no doubt allow further rapid advances in the understanding of the biology of CD4⁺CD25⁺ cells and their clarification of their relationship to other cell lineages.

1.5.4. Regulatory Cell Functions in vivo

Intuitively, regulatory T cells cannot simply downregulate all responses, they must somehow distinguish between responses that should be prevented or stopped and those that should continue. How this is achieved is still somewhat of an enigma, but there are clues. The mechanisms of suppression by Tregs are still unclear, although several have been proposed and these are often related to molecules constitutively expressed by these cells. Some of the molecules initially thought to be important for the effector functions of Tregs are more likely to be involved in the decision whether or not to prevent a particular immune response. Therefore the factors involved in turning regulatory functions on or off as well as those mediating regulation will be discussed together.
1.5.4.1. Cellular Targets and Antigen Specificity of Suppression

CD4⁺CD25⁺ cells have been shown to regulate CD4⁺ T cells(103), CD8⁺ T cells(104), B cells(71, 72), and innate immune cells(105). The central role of CD4⁺ cells in orchestrating immune responses may mean that regulation of CD4⁺ cells is likely to be one of the most important functions of Tregs.

It is known that both CD4⁺CD25⁺ and Tr1 cells must be stimulated via the TCR for activation of suppressor function(55, 96) On the other hand, the Treg and its target do not always appear to require recognition of the same Ag, or even share MHC restriction, provided that the Treg has been recently (but not necessarily concomitantly) activated via its TCR(106). This suggests a direct T:T cell interaction rather than Treg:APC activity. Contact-mediated suppression has also been suggested by in vitro experiments in which suppression was abrogated when the Tregs and responder cells were physically separated in a transwell(96). Using MHC class I tetramers to activate CD8⁺ T cells and recently-activated CD4⁺CD25⁺ cells, Shevach et al have shown suppression in the complete absence of APC(104). However, CD4⁺CD25⁺ cells can control intestinal inflammation mediated entirely in the absence of T cells in vivo(105). Also, CD4⁺CD25⁺ cells can have direct effects on DC(107, 108). The main cellular targets of regulation will therefore most likely depend on the type of immune response, and is unlikely to be limited to one cell type.

1.5.4.1. FoxP3

A mutant strain of mice, termed “scurfy”, develop a spectrum of autoimmune diseases that is remarkably similar to the human immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX)(109) Gene analysis of scurfy mice and IPEX patients revealed that a mutation in a common gene encoding the forkhead/winged helix transcription factor FoxP3 resulted in impaired generation of CD4⁺CD25⁺ regulatory cells in the thymus(99-101). Furthermore, FoxP3 appears
to be important not just for the thymic generation of regulatory T cells but also their function. Transfection of naïve CD4⁺CD25⁺ cells with the gene encoding FoxP3 confers regulatory function on these cells, as well as the acquisition of the "regulatory phenotype": upregulation of molecules such as CD25 and CTLA-4 (99-101). It has since been found that naïve CD4⁺ cells can be induced to become CD4⁺CD25⁺ cells with regulatory function by TGFβ in vivo, and this regulatory function is associated with upregulation of FoxP3 (84, 85, 110). Thus, FoxP3 somehow determines the function of Tregs.

1.5.4.1. IL-10

The role of cytokines in the function of regulatory cells is controversial. Peripherally-induced IL-10 producing cells (collectively termed Tr1 cells) were initially characterised by their suppression of T cell responses through production of IL-10 (55, 86). However, in some cases this does not appear to hold true, at least in vitro: O'Garra and Wraith have reported that Tr1 cells induced by their methods (VitD3/dexamethasone and intranasal peptide administration) require cell-contact for in vitro suppression, not IL-10 (87). However, IL-10 production in vivo is still likely to be important for the action of these cells (111).

A similar discrepancy concerning requirements for IL-10 has been observed for CD4⁺CD25⁺ cells. In some studies, and particularly in vitro, IL-10 does not appear to play a major part in suppression. Rather, Treg:effector cell contact is crucial (96, 112). In vivo, the requirement for IL-10 varies with the model used. For autoimmune gastritis induced depletion of CD25⁺ cells by d3Tx, IL-10 is not required for prevention of disease (113). However, IL-10 does appear to be important for CD4⁺CD25⁺ cell-mediated prevention of T cell-dependent and T cell-independent colitis (105, 114, 115), as well as the suppression of EAE (50), anti-Leishmania responses (52), and allogeneic transplant rejection (116).
Another cytokine with downregulatory properties implicated in the action of some regulatory T cells is TGFβ. TGFβ mRNA can be detected in CD4⁺CD25⁺ populations from naïve mice (73), and it has been suggested that cell-bound TGFβ is involved in the cell-contact-dependent suppression observed in vitro (117). However, the importance of TGFβ for Treg function, like IL-10, remains controversial, and the observation that surface-bound TGFβ is involved in suppression in vitro has been disputed (118).

In some models, particularly type-I diabetes and colitis, TGFβ appears to be important for prevention of autoaggressive or inflammatory responses (119-121). Green et al use an inducible model of diabetes in which pancreatic β cells express CD80 and produce TNFα, thus creating an inflammatory context in which T cells may encounter Ag in the pancreas to initiate diabetes. In this model, highly potent CD4⁺CD25⁺ regulatory T cells have been observed to accumulate in the pancreatic LN before disease onset (122). Low numbers of these pancreatic LN CD4⁺CD25⁺ cells are able to transfer protection to pre-diabetic mice (122). The regulatory cells express cell surface-bound TGFβ, and the ability of CD4⁺CD25⁺ cells to suppress diabetes was directly dependent on the ability of target CD8⁺ T cells to respond to TGFβ (Green, 2003 #507).

Inflammatory colitis can be induced in lymphopaenic mice by the transfer of CD4⁺CD25⁻ cells (T-dependent), or by infection with Helicobacter hepaticus (T-independent). In both these models, protection from disease can be provided by CD4⁺CD25⁺ cells (105, 123), and in the T-dependent model of colitis CD4⁺CD25⁺ cells can cure disease that has already been established (121, 123). In these colitis models, disease prevention or cure may be mediated, at least in part, through production of TGFβ. However, these studies used blocking Ab, which may affect many cell types, and TGFβ is known to be important for normal immune regulation.
in the gut environment. A more recent study using TGFβ-1-deficient CD4⁺CD25⁺
cells found that although these could suppress proliferative responses of CD4⁺CD25⁺
cells in vitro, they could not inhibit induction of colitis in vivo (119), suggesting a
requirement for TGFβ production/expression directly by Tregs in this model.

As already discussed, TGFβ may also contribute to infectious tolerance by
instructing the development of new regulatory T cells (57, 84, 85).

1.5.4.1. Requirements for IL-2 and the IL-2R in function and
maintenance of Tregs

The constitutive expression of certain markers on Tregs suggests requirements either
for survival or for effector function. It could be suggested that CD25 on Tregs acts as
an IL-2 “sink” to absorb IL-2 and thereby reduce proliferation of the IL-2 producing
T cells. This seems unlikely, since activated T cells expressing high levels of CD25
are unable to inhibit proliferation of cocultured cells (96). It has also been shown that
Tregs directly block responder cell synthesis of IL-2 (96, 97).

While CD4⁺CD25⁺ cells do not produce IL-2 themselves, they are highly dependent
on IL-2 from other cells for survival: mice deficient in IL-2, CD25 or IL2Rβ are also
deficient in CD4⁺CD25⁺ regulatory T cells and develop spontaneous autoimmunity
(124-126). Also, administration of anti-IL-2 or anti-CD25 in vivo leads to rapid
depletion of CD4⁺CD25⁺ cells (127, 128), and increased susceptibility to
autoimmune disease (127). Likewise, STAT5 is required for IL-2 signalling and mice
in which STAT5 is over-expressed have an increased frequency of CD4⁺CD25⁺
regulatory cells (129).

Similarly to the “IL-2 sink” hypothesis, it has been suggested that co-transfer of
CD4⁺CD25⁺ cells with CD4⁺CD25⁻ cells prevents disease by competing for space in
a homeostatic manner (130). This is based on studies inducing colitis in mice with
CD4⁺CD25⁺ cells into lymphopaenic mice, which found that transferring TCR transgenic CD45RBhi T cells with the pathogenic CD4⁺CD25⁻ cells gave the same protective effect as transferring CD4⁺CD25⁺ cells. Although this may hold some truth when using lymphopaenic systems, homeostatic competition is unlikely to explain the in vitro effects of CD4⁺CD25⁺ cells or the regulatory functions observed in vivo in lymphoid-sufficient hosts. Unlike transfer of CD4⁺CD25⁺ cells into BALB/c SCID mice, depletion of CD25⁺ cells in vivo does not lead to spontaneous gastritis in lymphoid-sufficient mice (127). However, these mice are more susceptible to induction of gastritis by immunisation, suggesting that the homeostatic proliferation of CD4⁺CD25⁺ cells after transfer into lymphopaenic mice in some way serves to provide the signals necessary to activate the autoreactive cells.

1.5.4.1. “Killer” Tregs

Rather surprisingly, gene profiling of CD4⁺CD25⁺ cells showed granzyme B expression in these cells (131). This was largely ignored at the time, since granzymes are associated with CD8⁺ cytotoxic T cell function. Ley and colleagues have recently shown that human CD4⁺CD25⁺ cells upregulate granzyme A upon activation (132). These cells killed activated CD4⁺ and CD8⁺ T cells as well as monocytes and DC in a perforin/granzyme-dependent manner, suggesting a novel mechanism of regulation by CD4⁺ T cells. This mechanism would obviously be cell-contact dependent, fitting with previous in vitro observations, although cell death had not been reported in these studies (96).

1.5.4.1. CD28/CTLA-4/B7costimulatory network

CTLA-4 is a downregulatory molecule whose expression is elevated on activated T cells. CTLA-4 binds B7 molecules with high affinity, outcompeting CD28’s ability to bind the same molecules, and also signalling negatively to the T cell to inhibit further activation (22). CTLA-4 is constitutively expressed on CD4⁺CD25⁺ regulatory cells (133, 134), and it has been proposed to be necessary for suppressor function since blocking anti-CTLA-4 can abrogate suppression in some systems.
using Fab fragments to block (133, 134), but not when using whole Ab with potential cross-linking ability (103). CD4⁺CD25⁺ cells express several molecules associated with negative signalling and limitation of cell division (CTLA-4, PD-1, CD5) and it has been suggested that this could contribute to their anergic state (135). Blocking CTLA-4 could therefore act to overcome this anergy in vitro.

CD4⁺CD25⁻ cells that are deficient for B7 expression have been found to be resistant to suppression in vitro or in vivo, and are inherently more pathogenic than CD4⁺CD25⁻ cells from wildtype mice when transferred into lymphopaenic hosts (136). This suggests a role for engagement of B7 on activated T cells by CTLA-4 on CD4⁺CD25⁺, and may also partly explain the pathology seen in CTLA-4 knockout mice, which experience an overwhelming proliferative syndrome resulting in early mortality (137). Thus CTLA-4 may provide a mechanism for direct T:T cell suppression.

Conversely, Tregs may act indirectly on T cells via APC. One example of this is the downregulation of the costimulatory molecules B7-1/2 (108). A different APC-directed approach to turning off T cell responses involves tryptophan catabolism by DC. It has been known for some time that interferons produced during infection act to induce the production of indoleamine 2,3-dioxygenase (IDO), and that IDO then catabolises the degradation of tryptophan (138). This deprives the infectious organism of an essential growth factor. However, it has now become clear that tryptophan catabolism may serve additional immunoregulatory functions. Tryptophan is degraded into metabolites termed kynurenines and these induce apoptosis of various immune cells (138). Using an allogeneic islet transplantation model, Grohmann et al showed that engagement of B7 on DC by CTLA-4-Ig fusion protein stimulated IFN production by DC, resulting in synthesis of IDO and tryptophan catabolism in those DC (139). Deletional tolerance to the allogeneic grafts was thus established. CTLA-4 is constitutively expressed by CD4⁺CD25⁻ Tregs, and it has recently been shown that interactions of Tregs with DC may induce tryptophan catabolism in a manner analogous to that described for CTLA-4-Ig (107).
However, in this model there appeared to be an additional non-CTLA-4-mediated mechanism of IDO induction by activated Tregs (107).

While CD28 does not appear to be required for the suppressive function of Tregs (133), a strong signal through CD28 ligation (along with TCR stimulation) abrogates their anergic and suppressive state (96, 112). The CD28/B7 costimulatory pathway is also important for CD4⁺CD25⁺ Treg development and homeostasis. This was revealed by the surprising result that NOD mice crossed onto either a CD28⁻⁻ background, rather than being resistant to spontaneous autoimmune diabetes, developed diabetes with accelerated onset (48). The number of peripheral CD4⁺CD25⁺ cells was also reduced, and the onset of diabetes in these mice could be prevented by transfer of CD4⁺CD25⁺ regulatory T cells (48).

1.5.4.1. GITR

Gene profiling and antibody screening studies by the Shevach and Sakaguchi labs, respectively, led to the concomitant identification of the glucocorticoid-induced TNF receptor (GITR), as a molecule constitutively expressed by CD4⁺CD25⁺ cells (131, 140). Ligation of GITR abrogated suppressor functions of CD4⁺CD25⁺ cells in vitro, suggesting a role for this molecule in suppression (140). However, GITR expression is also upregulated upon activation of naïve CD4⁺ T cells, and subsequent studies have shown that ligation of GITR has positive costimulatory effects on activated T cells (141). The effects of administration of anti-GITR could therefore be attributed to increased costimulatory signals to effector cells in some models, as has been demonstrated for EAE (142). Furthermore, GITR engagement on CD4⁺CD25⁺ cells has been shown to confer resistance to suppression by CD4⁺CD25⁺ cells(143). Thus, ligation of GITR on either Tregs or activated effector T cells may act as a switch leading to abrogation of suppression.
1.5.5. Responses of Regulatory cells to Antigen: in vitro vs in vivo

The early studies using in vitro assays found CD4⁺CD25⁺ cells to be anergic, in that they failed to proliferate in response to Ag unless exogenous IL-2 or additional costimulation was provided (96, 112). It was assumed that these cells would likewise be anergic in vivo. Indeed, it has been reported that TCR transgenic CD4⁺CD25⁺ did not proliferate in vivo in response to immunisation with specific peptide in CFA, but did undergo homeostatic proliferation in a lymphopaenic environment (144). However, several studies have since shown that CD4⁺CD25⁺ cells do proliferate in response to cognate Ag in vivo (145). These studies used transgenic CD4⁺CD25⁺ cells (of different specificities) and showed that these proliferated in response to immunisation with specific Ag in adjuvant (77, 146, 147), immunisation with Ag-pulsed mature DC (148), or locally in response to Ag draining from the tissues (77, 149). A proportion of CD4⁺CD25⁺ cells also appear to slowly cycle continuously in vivo (149), presumably in response to presentation of self peptides on DC. This continual cycling may also partly explain the constitutive expression of activation markers such as CD25, and the high dependence of Tregs on IL-2.

1.5.6. Conclusions – how do Tregs fit into immune responses in vivo?

A clearer picture of how Tregs fit into the initiation and control of immune responses is now emerging. Under inflammatory conditions, such as infection, DC become activated through ligation of toll-like receptors and the presence of inflammatory cytokines (28). This results in upregulation of costimulatory molecules such as B7 and GITR-ligand, as well as enhanced expression of peptide-MHC (22, 28, 141). Interaction of CD4⁺CD25⁺ cells with these DC results in the temporary abrogation of suppressive functions and proliferation of the Tregs (presumably making use of IL-2 produced by concomitantly activated CD4⁺CD25⁻ cells) (131, 140, 148, 150). At the same time, engagement of GITRL on CD4⁺CD25⁻ cells confers resistance to suppression (143). Production of IL-6, in addition to some other unidentified cytokine(s), by DC in response to engagement of TLRs has also been reported to make responder cells resistant to suppression by CD4⁺CD25⁺ cells (151). CD4⁺CD25⁺ themselves are reported to express TLR- 4,5,7 and 8 (152). TLR-4
binds bacterial LPS, and Caramalho et al found that addition of LPS to cultures of CD4⁺CD25⁺ cells induced their proliferation, even in the absence of DC, indicating that Tregs may respond directly to inflammation, and may play a more general role than suppression of autoimmune responses.

In contrast, under steady-state conditions, suppressive functions would presumably be maintained, and activation of naïve cells would be prevented. Naïve cells responding to Ag under these conditions are more likely to recognise self-Ag or non-harmful Ag such as commensal bacteria or food Ag, and therefore it is beneficial to prevent these responses. The one potential flaw in this argument is the finding that transferred Ag-specific CD4⁺CD25⁺ cells proliferate in response to Ag draining tissues under steady-state conditions (77, 149), and the factors promoting this expansion have yet to be identified.

After expansion in vitro with Ag and IL-2 (with or without additional anti-CD28), CD4⁺CD25⁺ cells have been found to regain regulatory functions, and are in fact more potent in their suppressive capacities (45, 153, 154). In vitro stimulation of CD4⁺CD25⁺ cells for three days with LPS, followed by three days in the presence of IL-2, results in a further enhancement of suppressive potency compared to IL-2 alone (152). As the source of inflammation is cleared, it is likely that the number of activated DC will decrease and the stimuli leading to abrogation of Treg suppression will be downregulated. It would be expected that the expanded CD4⁺CD25⁺ cells would now be highly potent regulators. Thus the balance of power would shift from effector cells to regulatory cells, and the regulatory cells may contribute to the downregulation of the effector response, and perhaps more importantly the downregulation of any self-reactive cells that may have been activated as a side-effect of the inflammatory conditions. Indeed, it has been found that depletion of CD25⁺ cells in vivo leads to increased numbers of Th1 effector cells (155). Interestingly, this study used foreign Ag (ovalbumin), indicating that Tregs may control the responses to foreign Ag as well as self Ag. It has even been suggested that Tregs may prevent the complete elimination of the parasite Leishmania, and that
this has a beneficial effect by maintaining functional memory to prevent new infections (52).

1.6. Part 2: Experimental Autoimmune Encephalomyelitis as a CD4$^+$ T cell-driven autoimmune disease

Experimental autoimmune encephalomyelitis (EAE) is a widely-used murine model of multiple sclerosis (MS). The model was developed after an unfortunate side-effect of a rabies vaccine: it was observed that some people given the vaccine developed acute paralysis, with symptoms similar to MS patients. Further investigation revealed that some of the rabbit nervous tissue in which the vaccine virus had been prepared was still present in the inoculation, prompting speculation that a myelin-specific immune response had been provoked, resulting in the observed CNS inflammation and paralysis (156). This was the first suggestion that MS may in fact be an autoimmune response, and the animal model EAE was developed as a tool to investigate the pathogenesis of MS.

There is still some debate over the etiology of MS, although it is now mostly agreed that it has an autoimmune basis. This is particularly supported by the fact that the most successful therapies for MS have immunomodulatory functions (157-160). Also, myelin-reactive T cells have been identified in the lesions of MS, although these cells have also been identified in the blood of healthy individuals (161), and myelin-specific Ab are present in the CNS and blood of MS patients (158, 162). The processes that lead to the development of MS are still unclear, however studies on the pathogenesis and susceptibility to EAE have given some clues. From an immunologist's perspective, EAE provides a model with well-defined self Ags, that has proved highly useful for studying various aspects of immune tolerance.
1.7. Pathogenesis of EAE – cells types involved

To induce EAE, animals are immunised with myelin (spinal cord homogenate) or myelin components, emulsified in Complete Freund's Adjuvant (CFA). EAE can be induced in many animals, but mice provide the bulk of data for this model, due to the availability of inbred and genetically manipulated strains of this species. In mice, clinical disease manifests itself eight to ten days after immunisation, with the onset of ascending paralysis, and this correlates with perivascular infiltration of the brain and spinal cord by mononuclear cells. EAE is mediated by myelin-reactive CD4+ T cells: this has been demonstrated by the ability of purified CD4+ cells from mice with EAE to induce disease when activated and transferred into naïve mice (163, 164), and the failure to induce EAE in mice depleted of CD4+ cells (165). The most common myelin proteins used to induce disease are proteolipid protein (PLP), myelin basic protein (MBP) and myelin oligodendrocyte glycoprotein (MOG). Immunodominant T cell epitopes have been identified within each protein, and peptides containing these can also be used to induce disease (166, 167).

For this project, EAE was induced in B6 mice (H-2b) by immunising with the major encephalitic epitope of MOG: MOG(35-55) (168). MOG constitutes less than 0.1% of total myelin, but has been identified as a possible target autoAg for both T cells and B cells in MS (169, 170).

While CD4+ T cells are thought to initiate the response, macrophages clearly play a central role as effector cells in the pathogenesis of EAE, through production of cytokines such as TNFα and lymphotoxin, expression of self peptides on class II to reactivate T cells, digestion of myelin and Ab-mediated cytotoxicity (171, 172). In a rat model of EAE induced by transfer of activated encephalitogenic T cell lines, Flugel and colleagues showed that the severity of disease induced by different cell lines correlated with levels of macrophage recruitment into the CNS rather than numbers of T cells entering the CNS (173).
Activated macrophages and DC, as well as resident macrophage-type cells such as microglia, upregulate MHC class II to activate T cells in the CNS during EAE (174). This activation of T cells within the CNS is thought to be important for T cell maintenance and effector functions in the CNS, since effector cells specific for an irrelevant Ag do not accumulate in significant numbers in the CNS (173, 175), and mice in which de novo processing of myelin is prevented do not develop EAE (176). As well as presentation of myelin Ag, expression of costimulatory molecules by CNS APC appears to be important for disease propagation, since mice in which B7 was not expressed in the CNS developed mild EAE with a short duration (177). In addition to their functions in recruiting and activating T cells, activated DC migrating from the inflamed CNS are likely to perpetuate inflammation by activation of fresh T cells in draining LN, and in some models this is thought to contribute to relapses by inducing epitope spreading of the response (178).

CD8+ T cells are not thought to be absolutely required for induction of EAE, since mice depleted of CD8+ cells develop EAE after immunisation with encephalitogenic peptide, although the disease severity is decreased (179). However, CD8+ cells are present in EAE and MS lesions (180-182), and transfer of activated MOG-specific or MBP-specific CD8+ T cell lines into the relevant strain of mice can induce EAE (183, 184). Therefore it is likely that CD8+ cells are not absolutely required for, but can contribute to, pathology. This appears be mediated in part through production of IFNγ, since administration of blocking Ab against IFNγ reduced the severity of disease induced by myelin-reactive CD8+ cell lines (183).

The role of B cells and Ab in the pathogenesis of EAE (and MS) is controversial. Early work using mice made deficient for B cells by injection of depleting anti-μ Ab from birth suggested that B cells were required for induction of disease (185). However, the generation of μMT mice, genetically deficient for the immunoglobulin μ heavy chain and therefore lacking mature B cells in the peripheral circulation, showed that B cells are actually not required for induction of EAE, since these mice develop disease with normal kinetics. As described above, EAE can also be passively
induced by transfer of activated myelin-reactive CD4\(^+\) cells alone, further suggesting that B cells are not required for initial induction of disease. However, B cells and Ab with specificity for epitopes from MBP and MOG have been reported in EAE, and in some MS patients (162, 186, 187). In one clinically defined group of patients Ab may in fact be a major contributor to demyelinating pathology (187). In mice and rats with EAE, Ab against similar epitopes have also been found, and in some cases these Ab are pathogenic (188, 189). Myelin-specific Ab may have pathogenic effects through enhancement of complement deposition, or opsonisation of myelin for uptake by macrophages (187, 190). Therefore, similarly to CD8\(^+\) T cells, B cells are probably not required for EAE initiation, but myelin-reactive Ab may contribute to pathology. In the more complex clinical situation of MS, the contributions of these cells to disease may become more important in certain groups of patients.

1.8. Breakdown of tolerance in induction of autoimmune disease

1.8.1. Central tolerance

Deletion of self-reactive T cells is vital in preventing autoimmunity, as evidenced by the spectrum of autoimmune diseases that occur in people with a mutation in the thymic transcription factor AIRE(7) (see section 1.2) Another function of thymic selection that may influence an individual’s susceptibility to autoimmunity is the generation of regulatory T cells(74), see section 1.5.1.

Similarly to MS, susceptibility to EAE is MHC-dependent, with additional genetic factors involved, so different strains of mice respond differently to the various peptides/proteins, and not all strains develop disease. This strain-dependence often appears to be related to efficiency of central tolerance. In SJL mice, a splice variant of proteolipid protein(PLP), DM20, is dominantly expressed in the thymus (11). DM20 lacks the PLP(139-151) epitope present in the full transcript of PLP expressed in the CNS, and the peripheral repertoire of SJL mice has a remarkably high frequency of PLP(139-151)-specific CD4\(^+\) cells – as many as one in 20,000 CD4\(^+\)
Unsurprisingly, these mice are highly susceptible to induction of EAE with PLP(139-151), although they do not develop spontaneous EAE.

Interestingly, a recent report by Reddy et al (191) suggests that SJL mice have a relative defect in the generation of PLP(139-151)-specific CD4^+CD25^+ regulatory T cells, fitting with the observations that CD4^+CD25^+ cell selection is based on recognition of self Ag in the thymus. B10.S mice, which also have the H-2s haplotype, are resistant to EAE induction with PLP(139-151), and in the same study these mice were found to have higher frequencies of (PLP139-151)-specific CD4^+CD25^+ cells in their periphery. Although the authors state that expression of PLP is greater in the thymus of B10.S compared to SJL mice, they did not define levels of PLP(139-151) expression.

In B10.PL mice, EAE is normally induced by immunisation with the MBP peptide Ac(1-11). This peptide has extremely poor binding affinity for the H-2u MHC molecules present in B10.PL mice. It is thought that the interactions of Ac(1-11)-reactive T cells with Ac(1-11) presented in the thymus do not reach sufficient avidity for deletion and these cells therefore escape into the periphery (8).

Expression of MOG mRNA has been shown in medullary thymic epithelial cells (6). However, a comparison of the repertoire and the pathogenic potential of MOG-reactive T cells in the periphery of MOG^-/- and wildtype mice on the C57BL/6 background suggested no differences (192). Therefore central tolerance mechanisms appear ineffective for the deletion of MOG-reactive T cells.

1.8.2. CNS entry - breakdown of clonal ignorance

Clonal ignorance relies on self-reactive T cells not coming into contact with their cognate Ag. The CNS is protected by the blood-brain-barrier (BBB), a physical barrier consisting of endothelial cells with tight junctions that are thought to prevent access of immune cells into the brain and spinal cord (13). However, naïve T cells
have been observed in the CNS, in a model in which T cells are transgenic for MBP-specific TCRs (16). In this case, the MBP-specific T cells appear to have undergone tolerance induction in the CNS, while non-myelin specific cells found in the CNS were still able to respond to Ag (16). Additionally, LeFrancois' lab have reported the migration of activated/memory (non-myelin-reactive) T cells throughout the body, including the CNS, although entry into the brain was delayed in comparison to other sites such as the lungs (193). Thus, the BBB does not appear to provide complete protection from lymphocyte surveillance.

In most models of EAE, pertussis toxin (PTx) must be given around the time of immunisation for full EAE induction. PTx may serve 2 main purposes in EAE. First, it is thought to break down the blood:brain barrier to allow lymphocyte migration into the CNS (194, 195). Secondly, and perhaps more importantly, it may act as an adjuvant to activate resident APC within the CNS (196). These would then produce chemokines to recruit T cells, and also upregulate expression of MHC and costimulatory molecules to induce effector functions and retain T cells once in the CNS.

The requirement for T cell activation and recruitment by innate cells in the CNS has been demonstrated by Darabi et al (175). In this study it was shown that an intracranial injection of the microbial adjuvant CpG allowed the localised recruitment of MOG(35-55)-specific CD4^+ cells after subcutaneous immunisation with MOG(35-55) in CFA, in the absence of PTx. This suggested that localised activation of APC by an inflammatory stimulus within the CNS was sufficient to recruit and maintain activated myelin-reactive T cells, although in this model the inflammation remained localised to the site of the initial CpG inoculation. Once the initial inflammation is established, it is likely that the damage caused by T cells and mononuclear cells will be sufficient to propagate the response without additional adjuvant.
Chemokines are small soluble molecules that form gradients along which cells bearing appropriate receptors migrate (197). The chemokine monocyte chemoattractant protein-1 (MCP-1) is produced by several cell types in response to inflammatory stimuli, and MCP-1 mRNA is upregulated in CNS lesions of mice with EAE as well as in MS (198). The ligand for MCP-1, CCR2, is expressed by activated T cells and monocytes (199). Mice deficient in either MCP-1 or CCR2 are relatively resistant to EAE induction by active immunisation with encephalitogenic peptides, or by transfer of activated encephalitogenic T cells from wildtype mice (200, 201). This resistance is associated with a failure to recruit activated T cells and monocytes/macrophages to the CNS despite the “leaky” blood:brain barrier induced by administration of PTx. Therapies that target effector cell migration to the CNS, such as anti-MCP-1 or anti-VLA4, reduce the severity of EAE (202). Thus, it appears that the physical barriers protecting the CNS can be overcome during inflammation through increased expression of chemokines and T cell-stimulating molecules.

1.8.3. Molecular Mimicry and Generation of Cryptic Epitopes by Pathogens

The effects of PTx during the induction of EAE could feasibly be induced by an infectious agent in the CNS. Indeed, chronic infection with the murine neurotropic pathogen Theiler’s virus has been reported to result in the activation of myelin-reactive CD4+ cells in some mice (203, 204). Several human autoimmune disorders, including diabetes and MS, are suspected to have an infectious link. However, none has been conclusively proved; this may be due to the lag between the activation of the response and the onset of disease symptoms and diagnosis (205). Infection stimulates strong priming conditions through activation of APCs, and as already discussed may lead to presentation of Ag and epitopes not usually encountered. Under very strong stimulating conditions the high activation threshold of T cells with low affinity receptors can be reached, and tolerance based on low affinity for self may thus be overcome (206).
Another possible mechanism of tolerance breakdown caused by infectious organisms is molecular mimicry. In this case, T cells specific for Ag from the invading pathogen also cross-react to self Ag, and when activated during the infection may additionally attack the tissue(s) expressing that self Ag (207, 208). Pathogens exploit molecular mimicry to survive since cells tolerised against self may not be able to respond to homologous Ag on foreign organisms. Again, there is no clear evidence for a role for molecular mimicry in the pathogenesis of MS (205).

While T cells may be able to recognise different epitopes within a protein, expression of specific proteases in the thymus may allow dominant expression of one epitope, and therefore only T cells recognising this dominant epitope will be deleted. This has been shown to be the case for MBP (209, 210). This does not normally lead to autoimmunity, since the subdominant epitopes will not be presented by DC. However, it has been recently suggested that viruses may alter Ag processing enzymes to prevent presentation of their own viral antigenic epitopes, and in so doing may also encourage presentation of cryptic self epitopes (17, 206).

### 1.8.4. Defects of regulatory T cells

There is now much evidence for the ability of CD4⁺CD25⁺ regulatory T cells to control spontaneous autoimmune activation in various models. As already described (section 1.5.4), the crucial role of CD4⁺CD25⁺ cells has been demonstrated in human patients with IPEX, and in scurfy mice, which have a mutation in the gene for FoxP3 and therefore fail to effectively generate CD4⁺CD25⁺ regulatory T cells. This has been one of the most conclusive pieces of evidence for the central role of regulatory T cells in preventing peripheral autoimmunity in humans.

Depletion of peripheral CD25⁺ cells in mice has been shown to increase their susceptibility to autoimmunity (127), and mice deficient in CD25⁺ cells due to genetic deficiencies in molecules such as IL-2, FoxP3 or CD28 develop spontaneous autoimmunity (94).
In patients with multiple sclerosis and type-I diabetes, a decrease in the proportion and/or function of peripheral regulatory T cells has been suggested to contribute to disease (211, 212). Likewise, a proportion of NOD mice spontaneously develop type-I diabetes and other autoimmune conditions, and this strain has been reported to have a defect in thymic generation of Tregs (48, 213), although this is not the only immune defect in these mice.

In a study of juvenile idiopathic arthritis (JIA), the numbers and functional characteristics of CD4^+CD25^+ cells in the joints of patients was found to inversely correlate with severity of disease progression in some patients (214). However, another study on different types of rheumatoid arthritis found that while CD4^+CD25^+ cells with regulatory potential were enriched in inflamed joints of most patients, this did not appear to directly correlate with reduction in disease (215). Ehrenstein et al have suggested that TNFα may inhibit the ability of CD4^+CD25^+ cells to suppress effector cytokine production by T cells and monocytes in arthritis patients (216), although these studies compared CD4^+CD25^+ cells from patients before and after treatment with anti-TNFα, so the effect may be due to general inflammation rather than a direct effect of TNFα on Tregs. Defects in regulatory T cell numbers and/or function are now widely suspected, if not yet demonstrated in all diseases, to play a part in the development or progression of autoimmunity in humans.

1.9. Natural and therapeutic mechanisms of control of autoreactive cells

1.9.1. Cytokines in EAE

1.9.1.1. Th1/Th2

The role of various cytokines in EAE induction and resistance has been intensively studied. Immunisation with encephalitogenic Ag in CFA induces a Th1 response, and the cells able to transfer EAE are generally Th1 in phenotype (217). Th1 cells
produce IFNγ and TNFα: cytokines that activate macrophages to increase production of nitric oxide and other metabolites involved in pathogen killing as well as expression of MHC II molecules (33).

IL-12 has long been thought to be required for Th1 polarisation (33). Fitting with a Th1-driven disease, IL-12 deficient mice are resistant to EAE induction (218), and treatment of mice with Ab against IL-12 ameliorates EAE (219). However, IL-12p35−/− mice are susceptible to induction of EAE (220), and IL-12Rβ2−/− mice unexpectedly developed EAE with earlier onset and increased severity (221). Recent studies on IL-23 have revealed that some of the results on the importance of IL-12 in EAE may have been misinterpreted. IL-12 and IL-23 both use the IL-12p40 subunit in combination with separate second subunits (p35 for IL-12 and p19 for IL-23), and the IL-12 and IL-23 receptors also share the IL-12Rβ1 subunit (222). Thus, studies finding that mice deficient in IL-12p40 are resistant to EAE induction have not taken into account the effects of IL-23 deficiency. Cua et al dissected the roles of IL-12 and IL-23 in EAE using p19−/−, p35−/− and p40−/− mice in combination with IL-23-expressing vectors delivered systemically or into the CNS (223). In this study, IL-12 did not appear to be required for Th1 priming of MOG-reactive T cells in the LN, rather IL-23 production in the CNS (most likely by resident microglia and infiltrating macrophages) was essential for EAE induction.

Also surprising was the finding that IFNγ-knockout (IFNγ−/−) mice develop normal EAE after immunisation with myelin Ag (224). It may be that in IFNγ-deficient mice TNFα can compensate for IFNγ in functions such as activation of macrophages. Perhaps even more surprisingly, IFNγ−/− mice exhibited an impaired recovery, suggesting a more important role for IFNγ during recovery (224). This was attributed to IFNγ-mediated induction of macrophage nitric oxide synthetase, and production of nitric oxide in the periphery and in the CNS. In vitro nitric oxide was found to inhibit lymphocyte proliferation. In support of this hypothesis, a separate study reported a correlation between levels of serum nitric oxide intermediates and
resistance to reinduction of EAE in Lewis rats that had recovered from EAE (225). Induction of apoptosis by IFNγ has also been reported to limit the size or duration of immune responses (226), although this has not been directly investigated in EAE studies.

Deviation of the T cell response to a Th2 phenotype has been suggested to be responsible for resistance to EAE induction in male SJL mice (227), and injection of CNS-specific Th2-type cells (producing IL-4 and IL-10) can prevent EAE induction (227). Th2 cells specific for non-myelin Ag (keyhole limpet haemocyanin, KLH) were also found to prevent EAE induction if KLH was given at the same time. Also, IL-4 treatment ameliorated disease severity by inducing Th2 cells in an MBP-induced model of EAE (35), and IL-13-producing vector cells decreased EAE incidence and severity in a rat model (228), probably by decreasing macrophage activation. In a transgenic mouse in which most T cell receptors are specific for MBP Ac(1-11), recovery from EAE induced by Ac(1-11) was associated with a switch from a Th1 (IFNγ, TNFα) to a Th2/Th3 cytokine profile (IL-10, IL-4, TGFβ) in both LN and CNS (229).

However, MBP-specific Th2 cells can induce EAE when transferred into RAG−/− hosts (230). IL4−/− mice do not exhibit increased disease severity and undergo normal recovery (231). Finally, an attempt to therapeutically deviate the anti-myelin response towards Th2 in primates resulted in severe encephalomyelitis (232), so this approach as a therapy has to be viewed with caution.

1.9.1.1. IL-10

IL-10 has pleiotropic downregulatory effects on cells of the immune system. IL-10 receptor is expressed by all haematopoietic cells, and expression levels may be regulated by activation status, with cells such as macrophages upregulating IL-10R upon activation (233). Treatment of DC with IL-10, before or during their maturation with activation stimuli such as TNFα or LPS, rapidly decreases Ag processing and
expression of MHC and costimulatory molecules on the surface of APC, to reduce further activation of T cells (234). IL-10 appears to be required for the induction and effector stages of some regulatory T cells (Tr1 cells), although whether the T cells are directly affected or the IL-10 acts via the APC (or both) is still unclear (55). IL-10 can directly turn off cytokine production (e.g. IFNγ) in pre-activated effector T cells, as well as inhibiting the proliferation of naïve T cells under what would otherwise be priming conditions (233). Macrophage effector functions are also silenced by IL-10 (233).

IL-10 appears to play a central role in controlling severity and mediating remission from EAE, although the exact mechanisms for this remain to be clarified. IL-10−/− mice develop EAE of increased severity and fail to recover (231, 235). Both IFNγ and IL-10 are detectable in the CNS of mice with EAE, and a decrease in IFNγ concomitant with increasing IL-10 during the acute phase appears to correlate with remission (229, 236, 237).

Various interventions that utilise IL-10 can prevent EAE induction. Transgenic expression of IL-10 under control of the class II promoter is sufficient to prevent EAE induced by active immunisation or passive transfer of Th1 cells, in the absence of endogenous IL-10 production (238). In these mice, the prevention of EAE appeared to correlate with a suppression of effector T cell function rather than inhibition of Th1 cell induction. Activation of non-neuroantigen-specific Th2 cells during the induction of EAE can also give some protection, and this is likely to be mediated through production of IL-10 in the LN (61). Mathisen et al have demonstrated that PLP (139-151)-specific memory cells can be transduced to express IL-10 under control of the IL-2 promoter, and that this Ag-specific induction of IL-10 can give protection when attempting to induce EAE with PLP (139-151) (239). These cells also accelerated recovery when given after the onset of clinical symptoms. IL-10-producing transfected fibroblasts could inhibit EAE when inoculated into the brain (240). However, in this study, intracranial delivery of IL-10 in protein form or expressed by an adenoviral vector were not efficient in inhibiting
disease despite producing apparently similar levels of IL-10 to the fibroblasts (240). In contrast, Cua et al found that intracranial, but not systemic, administration of replication-deficient adenovirus IL-10-transfected vectors was effective in preventing EAE in a different mouse model of EAE (241). EAE could also be prevented by giving OVA-specific Tr1 cells systemically, but only when OVA was also injected intracranially, suggesting that CNS localisation of IL-10 production is required (62).

Systemic administration of IL-10 protein has also given conflicting results despite apparently similar protocols: two groups have reported suppression of EAE induction (242, 243), while two have reported no effect (241, 244). Overall then, there is conflicting evidence as to the ability of IL-10 to therapeutically mediate resistance to EAE, apparently depending on unidentified factors related to the model used and mode of delivery of IL-10 (245).

Taken together, the various studies looking at IL-10 in EAE suggest that IL-10 production increases with, and is required for, recovery. Similarly to other tolerance-inducing protocols using IL-10, EAE can be prevented by strategies that increase IL-10 levels during the initial induction period of EAE (61, 238), and these probably work by downregulating APC activation as well as preventing Th1 effector cytokine production. However, for full amelioration of disease, intracranial delivery of the exogenous IL-10 may be required (241), correlating with the increased IL-10 mRNA observed in the CNS during the recovery phase of EAE (236).

1.9.1.1. TGFβ

Levels of TGFβ have also been found to increase in the CNS with recovery (229). EAE can be ameliorated by administration of soluble TGFβ, while administration of blocking Ab specific for TGFβ exacerbated disease (246, 247). Induction of oral tolerance using MBP resulted in MBP-specific TGFβ-producing cells with the capacity to prevent EAE (these cells also produced IL-10) (248). TGFβ has
downregulatory effects on both T cells(249, 250) and macrophages(251), but, as is the case for IL-10, the specific in vivo targets during EAE have not been identified.

1.9.2. Regulatory T cells in EAE

There has been much excitement in recent years over the potential of both naturally occurring and experimentally induced regulatory T cells to prevent or treat autoimmune diseases. In the mouse model of induced diabetes used by Green et al, CD4⁺CD25⁺ cells expand in the pancreatic lymph nodes of mice in the early stages of developing diabetes, before onset of disease, and very small numbers of these cells are able to delay diabetes onset in naïve recipients (122). CD4⁺CD25⁺ cells with regulatory function have been found to be enriched in the joints of arthritis patients (214, 215). However, in both these studies, the accumulation of CD4⁺CD25⁺ cells did not appear to prevent or cure disease.

Relatively few studies have investigated the ability of regulatory T cells to therapeutically mediate recovery once an autoimmune response has been initiated. Two recent studies showed that CD4⁺CD25⁺ cells that had been activated and expanded in vitro could halt the progression of diabetes in the NOD mouse model, if given at the very onset of clinical signs (153, 154). These studies utilised islet-specific transgenic T cells, and it has yet to be determined whether the approach of expanding Ag-specific Tregs from a polyclonal population can be effective therapeutically. Colitis is an inflammatory model with similarities to autoimmune inflammation, since the target Ag are commensal bacteria of the gut (252). Using this model, two groups have shown that CD4⁺CD25⁺ cells transferred after inflammation is established are able to mediate resolution of inflammation and restoration of normal intestinal architecture (121, 123).

Whether Tregs can and do mediate control of established autoimmune responses under physiological conditions in vivo remains unclear. Despite the fact that the source of Ag cannot normally be cleared in an autoimmune response, many
autoimmune conditions show periods of decreased and enhanced disease signs, for example, MS patients usually initially experience periods of remission and relapses (158). This suggests that there are fairly successful attempts to regain control of the autoimmune cells, and regulatory T cells seem prime candidates to be involved.

Some time ago it was reported by Swanborg and colleagues that TGFβ-and IL-4-producing CD4+ “suppressor” cells are induced during recovery from EAE in Lewis rats (253, 254), although suppression is likely to be mediated through TGFβ rather than IL-4. These findings are analogous to the report from Weiner’s group showing enhanced TGFβ (and IL-10 and IL-4) production in the LN and CNS of MBP TCR transgenic mice during recovery (229). In Swanborg’s studies, these “suppressor” cells appear in the LN and spleen during the recovery phase and can transfer protection against active but not passive EAE induction in naive recipients for up to four months after recovery. This is in contrast to Ellerman et al, who reported the isolation of suppressor CD4+ T cells from LN cells of recovered Lewis rats: in this case the cells were able to prevent EAE induction when co-transferred with encephalitogenic MBP-specific T cells (255). In Swanborg’s studies, the suppressor cells were able to inhibit in vitro production of IFNγ, in response to MBP, but did not affect IL-2 production (256), unlike the cells described in more recent studies on regulatory T cells. In vitro inhibition of cytokine production appeared to be TGFβ-dependent (253). Interestingly, both CD4+ cells and B cells were required for protection (257). The characteristics of these cells and how they are induced have yet to be further described.

In the B10.PL/J mouse strain, EAE is induced by immunisation with MBP Ac(1-11). Sercarz et al have reported that recovery in these mice is associated with the emergence of “regulatory” CD4+ and CD8+ cells (258, 259). However, the CD4+ cells do not possess the typical characteristics that have been described for CD4+CD25+ or Tr1 regulatory T cells: they are not anergic and appear to be Th1 in phenotype (260). They recognise epitopes from the encephalitogenic TCR rather than
myelin Ag (258). Thus, it appears that stimulation of an immune response against peptides from the dominantly used TCRs (Vβ8.2) in this model of EAE may help downregulate the response, possibly through apoptosis of the encephalitogenic cells, although this has not been determined. Indeed, “TCR vaccination” with these peptides has proved effective in preventing and treating EAE in this model (261). Thus, an autoimmune response against TCRs may prove fortunate in some instances by downregulating a pathogenic response.

The high susceptibility to EAE and poor recovery of IL-10-/- mice (231) suggested classical Treg involvement, although this has yet to be shown. Barrat et al have shown that IL-10 producing Tr1 cells are capable of preventing EAE (62). They generated ovalbumin (OVA)-specific IL-10-producing Tr1 cells in vitro by culture of transgenic DO11.10 T cells with OVA in the presence of the immunosuppressive drugs Vitamin D3 and Dexamethasone. These cells were able to prevent onset of EAE when transferred into naive mice. The presence of OVA in the CNS, i.e. at the site of pathogenic effector cell action, was absolutely required for protection, suggesting downregulation of effectors by IL-10 produced in the CNS, rather than prevention of priming in the LN. It has also been demonstrated that IL-10-producing Th2 cells specific for keyhole limpet haemocyanin (KLH) could protect naïve mice against EAE if KLH was given subcutaneously (61). Thus, IL-10 producing cells are capable of affecting EAE, and since IL-10 increases in the CNS during recovery (229, 236) it appears likely that this may be a mechanism involved in mediating natural recovery.

Lafaille et al have demonstrated a role for both CD4+CD25+ and CD4+CD25- cells in preventing spontaneous autoimmune cell activation using mice with transgenic TCRs for the immunodominant epitope of myelin basic protein (MBP), Ac1-11 (47, 262, 263). These mice develop EAE spontaneously when crossed onto a RAG-/- background (100% of T cells have the transgenic receptor on this background). On a wildtype (RAG-sufficient background), a limited amount of endogenous TCR
rearrangement is known to occur, and these mice do not develop spontaneous disease (47, 262, 263). Transfer of CD4CD25+ cells from wildtype TCR transgenic mice are able to protect RAG-/- TCR transgenic mice from spontaneous disease, if given at an early age (264). Analysis of protective CD25+ cells showed that only dual-receptor cells bearing both the transgenic MBP-specific receptor and an endogenously rearranged receptor were capable of preventing disease. It is thought that the endogenously rearranged receptor is required for regulatory cell selection in the thymus, while the transgenic TCR receptor confers the correct specificity for induction of effector function after transfer.

In two separate reports, high numbers of CD4CD25+ cells from naïve syngeneic mice could decrease the severity of disease when transferred prior to EAE induction (49, 50). This protection required IL-10 production by CD4CD25+ cells (50), and appeared to be mediated within the LN since transferred cells were not observed in the CNS (49). The numbers transferred would lead to a marked increase in the frequency of CD4CD25+ cells in recipients, and it is assumed that this effectively raised the threshold for activation of naïve autoreactive T cells. Conversely, depletion of CD25+ cells prior to EAE induction resulted in increased disease severity in two reports (50, 265), but not a third (142). Taken together, these studies demonstrate a role for CD4CD25+ cells in regulating the activation of autoreactive cells, as has been demonstrated for other autoreactive T cells (266) as well as the activation of T cells specific for foreign Ag (155). However, they do not address the natural role of CD4CD25+ cells once EAE has been established.

1.9.3. “Regulatory” B cells?

Studies on the role of B cells in influencing T cell responses have not always given clear answers. There is some evidence that B cells may preferentially enhance Th2 responses through expression of ligands such as OX40(267). B cells are also capable of producing a number of cytokines: B cell production of IL-10 has been documented under Th1- and Th2-activating conditions in vitro (268). Studies using B cell deficient mice have generally reported either a decrease in the overall CD4+ T cell
response or no effect when immunising with nominal Ag or using infectious models (269-272). However, findings in EAE suggest the opposite: that B cell deficiency results in an enhanced and prolonged Th1 response with impaired recovery (59, 273), suggesting that B cells act to downregulate rather than enhance the T cell response.

Fillatreau et al used bone marrow chimaeras to further define the role of B cell IL-10 in EAE. In these chimaeras deficiency of IL-10 production was restricted to B cells by reconstituting lethally irradiated mice with a mixture of μMT and IL-10+ bone marrow (59). When EAE was induced in these chimaeras, they developed non-remitting EAE as observed in μMT mice. The normal (remitting) disease course could be restored by the transfer of wildtype (IL-10-sufficient) B cells prior to immunisation. Chimaeras in which B cells were deficient for CD40 also failed to recover from EAE, suggesting that B cell activation via CD40 is important for induction of IL-10.

The disparity in the observation on the effects of B cell deficiency may reflect differences in the type of model used, and indeed there is further evidence for B cells playing a downregulatory role in autoimmune and inflammatory disorders. Induction of IL-10-producing "regulatory" B cells has been reported in a chronic inflammatory gut model (TCRα− mice), in which prevention of disease progression was dependent on these cells arising during the acute phase of inflammation (274). In this model, the B cells expressed CD1d, a non-classical MHC I molecule required for presentation of lipid Ag to NKT cells, and it was suggested that interaction with NKT cells augmented IL-10 production, leading to downregulation of IL-1. In the EAE model, it has been reported that, while NKT cell activation (using αGalCer) could protect mice against EAE induced by MOG(35-55) in an IL-10- and IL-4-dependent manner, CD1d− mice did not develop the chronic disease seen in μMT mice (275). In a mouse model of arthritis Mauri et al have shown that production of IL-10 by splenic B cells from arthritic mice can be induced by stimulation with the immunising Ag, type II collagen (CII), and anti-CD40 (60). These in vitro stimulated cells could also protect naïve mice from arthritis induction in an IL-10 dependent manner. Analagous
to the findings in EAE (59), CD40 ligation and BCR stimulation were required for induction of IL-10, although additional unidentified T cell-mediated signals were also required for protection from arthritis (60). Therefore in situations of chronic inflammation, or prolonged antigen stimulus, the regulatory role of B cells may become important.

B cells efficiently present specific Ag to CD4$^+$ cells, principally to gain survival and differentiation signals (69). During this B:T interaction, the opportunity also arises for B cells to influence the nature of the T cell response. It is thought that Ag presentation by B cells may induce a Th2 phenotype in recently activated T cells, through binding of ligands such as ICOS:B7h or OX40:OX40L, and also lack of IL-12 (267, 276, 277). However, in our experiments, spleen and lymph node in vitro cytokine recall responses show decreased Ag-specific IFN$\gamma$ but little upregulation of IL-4 in recovered mice compared to mice with EAE ((59) and unpublished observations). Since B cells appear to regulate the ongoing response rather than preventing induction of Th1 cells in EAE, it seems more likely that IL-10 produced by B cells may directly reduce IFN$\gamma$ production in already polarised Th1 cells. The targets of B cell IL-10 have thus far not been identified.

### 1.10. Summary

Regulation of immune responses, whether preventing activation of inappropriate responses or controlling the size of useful responses, is vital for avoiding damage to host tissues. There are many ways the immune system can regulate responses and a better understanding of the specific mechanisms involved in regulating inappropriate responses, and why they sometimes fail, will allow us to develop improved therapeutic and preventative strategies for autoimmune disease. EAE is a CNS-targeted autoimmune disease, driven by Th1 cells reactive for myelin components. In some models of EAE spontaneous remission occurs, indicating successful control of pathogenic cells and inflammation. While the EAE model has been used for many
years, the mechanisms allowing recovery from CNS-targeted autoimmune disease have still not been fully identified. IL-10 clearly plays a major role in the recovery process, and B cells appear to be one of the vital sources of IL-10 during recovery, although a role for regulatory T cells has also been proposed (231).

1.11. AIMS of PROJECT

The aim of this project was to further elucidate the mechanisms behind the natural recovery in the MOG(35-55)-induced EAE model, focusing particularly on IL-10 production, with the following objectives:

- To further characterise the B cells responsible for mediating recovery via IL-10 production.
- To test the ability of lymphoid populations from recovered/recovering mice to transfer protection to naïve recipients in the MOG(35-55)-induced EAE model.
- To analyse the cell populations producing IL-10 in the CNS during recovery.
- To identify whether regulatory CD4+ cells play an additional role in recovery from EAE.
2. Materials and Methods

2.1. General reagents

2.1.1. Wash Buffer
RPMI 1640 medium containing 25mM HEPES buffer (Gibco, Life Technologies, Paisley, UK),
2mM L-Glutamine (Gibco),
100U/ml Penicillin and 100µg/ml streptomycin (Gibco),
5x10^{-5} M 2-mercaptoethanol (Gibco)

2.1.2. RPMI-5 tissue culture medium
Wash Buffer as above, with the addition of 5% heat-inactivated foetal calf serum (FCS) (Sigma, Poole, UK)

2.1.3. MACS buffer
HANKS Balanced Salt Solution (Sigma)
2% heat-inactivated FCS (Sigma),
100U/ml Penicillin and 100µg/ml Streptomycin (Gibco)

2.1.4. FACS buffer
PBS,
2% heat-inactivated FCS (Sigma),
0.05% sodium azide (Sigma)
2.2. Mice

μMT (278), IL-10 (279), OT-II (280) and Ly5.1\(^+\) congenic mice, all on the C57/B6 (H-2\(^b\)) background, as well as C57/B6 mice were bred and maintained under specific pathogen free conditions in the Institute of Immunology and Infection Research, University of Edinburgh. All mice were sex-matched within experiments, and used at 6-10 weeks of age.

2.3. Antigens

MOG(35-55) peptide (MEVGWYRSPFSRVVHLRYNGK) and OVA(323-339) peptide (hereafter referred to as pOVA) were prepared at the Advanced Biotechnology Centre, Imperial College, London, UK. Recombinant extracellular domain of mouse MOG (rMOG) was provided by Dr S. Anderton (59).

2.3.1. Production of MOG-biotin conjugate for identifying MOG-specific B cells by FACS

rMOG was biotinylated by adding 37.5μl of 1mg/ml biotin (Pierce, Cheshire, UK) (in ddH\(2\)O) to 500μl of 1mg/ml rMOG (in PBS), mixing gently, and incubating at room temperature for 30 minutes. The protein was then dialysed in PBS at 4\(^\circ\)C, changing the PBS buffer three times, to remove any excess biotin.

2.3.2. Testing of MOG-biotin as a FACS staining reagent

The MOG-biotin was tested by incubating single cell suspensions, obtained from the spleen or LN of mice with EAE or from naïve mice, with anti-B220PE and different concentrations of MOG-biotin in FACS buffer for 20-30 minutes, followed by washing and staining with SA-APC for 20 minutes. As detailed in figure legends (Figure 3-6) and text (section 3.2.4), different approaches were attempted to decrease the background staining with this conjugate.
2.3.3. Production of HEL-MOG(35-55) conjugate

The EDC kit (Pierce) was used to couple Hen Egg Lysozyme (HEL) protein (Sigma) and MOG(35-55) peptide, following manufacturer’s protocol. 10mg/ml HEL and 4mg/ml MOG(35-55) were prepared in buffer containing 0.1M 2-[N-morpholino]ethane sulfonic acid (MES) (Sigma), made up in ddH$_2$O, pH 4.7. Two-fold serial dilutions of MOG(35-55) were made, down to 0.25mg/ml, in MES buffer. 500μl of each peptide concentration was added to 200μl HEL, i.e. individual reactions contained: A: 2mg HEL + 2mg MOG(35-55)

B: 2mg HEL + 1mg MOG(35-55)

C: 2mg HEL + 0.5mg MOG(35-55)

D: 2mg HEL + 0.25mg MOG(35-55)

E: 2mg HEL + 0.125mg MOG(35-55)

1-Ethyl-3-(3-Dimethylaminopropyl)carboiimide Hydrochloride (EDC) was prepared at 10mg/ml in ddH$_2$O, and 100μl added to each reaction immediately (EDC is labile once solubilised). Samples were mixed gently and incubated for two hours at room temperature in the dark.

For purification of conjugate, 10ml Dextran de-salting columns (provided in kit) were first prepared by washing with 50ml purification buffer (50mM sodium phosphate, 150mM sodium chloride, pH7.2). The conjugate sample was then added, allowed to run through, and 0.5ml purification buffer at a time added and run through while collecting 0.5ml fractions. The absorbance (A$_{280}$) of the collected fractions was measured by spectrophotometry. Positive fractions were checked for presence of protein bands of the correct size (using HEL as positive control) on protein gels, then pooled and final conjugate concentrations calculated using the BCA protein estimation kit (Pierce). Concentrations: A: 1.28mg/ml; B: 0.66mg/ml; C: 0.7mg/ml; D: 0.42mg/ml; E: 0.7mg/ml.
2.3.4. Testing HEL-MOG(35-55) conjugate

B cells were prepared from MD4 mice, which bear transgenic BCR specific for HEL (281) (provided by Dr Andy Knight) or B6 mice by CD43-based negative selection (section 2.5.2). Purity of B cells used in this assay was greater than 97%. MOG(35-55)-reactive cell lines were provided by Claire Sweenie and used to detect presentation of MOG(35-55) by B cells in these assays. 3x10^4 T cells were cultured in triplicate with 2x10^5 B cells and a dose range of conjugate, or uncoupled HEL and MOG(35-55) in RPMI-5 tissue culture medium for three days. 0.5μCi tritiated thymidine was added for the last 18 hours of culture and assays were harvested using a liquid scintillation β-counter (Wallac).

2.4. In vivo manipulations

2.4.1. EAE induction

B6 mice were immunised in both hind legs with a total of 100μg MOG(35-55) per mouse in complete Freund’s adjuvant (CFA) containing 500μg/ml of heat-killed *Mycobacterium tuberculosis* H37Ra (Sigma). 200ng of pertussis toxin (ECACC, Dorset, UK) in 0.5ml PBS was given intraperitoneally on the same day and two days later. Mice were monitored daily for clinical signs of EAE and scored as follows, 0: no signs; 1: flaccid tail, 2: imbalance and impaired righting reflex, 3: partial hind limb paralysis, 4: complete hindlimb paralysis, 5: partial forelimb paralysis, 6: moribund or dead. Mice scored as grade 5 for two consecutive days were culled. Hydrated food was provided to cages containing mice at grade 3 or above to afford easier access to food and water.

2.4.2. Reinduction of EAE

For reinduction experiments in chapter 3, mice were immunised as above with 100μg MOG(35-55) in CFA over two sites in the flank, and 200ng/ml Pertussis toxin given on days 0 and 2. In chapter 5, mice were immunised with 100μg MOG(35-55) in
incomplete Freund's adjuvant (IFA) rather than CFA, as above. Mice were monitored and scored as above.

2.4.3. Statistics

Statistical analysis of EAE disease scores was performed using the Mann Whitney test (when the experiment contained two test groups) or the Kruskall Wallis test (when more than two experimental groups were compared). The non-parametric rank sum test was used because the measurements of EAE scores are discrete.

One flaw in the method used to compare groups in this project was that individual disease scores throughout the experiment timecourse were compared rather than cumulative disease scores (area under the curve of individual mice), and these are clearly not independent measurements. However, due to the small $n$ number in individual experiments (4-7 mice/group), and the relatively weak statistical power of rank sum tests, when several experiments shown in this thesis were analysed by comparing cumulative scores in a rank sum test they were apparently not significant. For example, figures 3-1A and 3-4, which show a clear difference in mean disease severity between groups, did not reach statistical significance when comparing area under the curve of individual mice. Increasing the size of groups would have overcome the weak statistical power of this method, but this was not economically or ethically feasible. Therefore, comparing individual disease scores by a rank sum test, while keeping greater emphasis on the biological significance rather than statistical significance of any result, was the best compromise for analysing results of experiments using EAE.

In published reports of EAE data from this lab the Mann Whitney test has been standardly used and accepted (20, 59). Analysis of results in the EAE field is generally problematic, and some labs use parametric tests for analysis of EAE (142, 191, 231), which are inappropriate since the data are discrete, or choose not to analyse their data statistically (183).
For statistical analysis of percentage of CD4\(^+\)CD25\(^+\) cells or percentage of IL-10\(^+\) cells at different time points in chapter 4 and percentage suppression by CD4\(^+\)CD25\(^+\) cells in chapter 5, an unpaired student's t test (a parametric test) was used since these are indiscrete variables.

2.4.4. Cell transfers

For all experiments in which cells were transferred into mice, the prepared cells were resuspended in Dulbecco's PBS (Gibco) and filtered to remove any clumps. Cells were injected in a final volume of 200μl intravenously into the tail vein of pre-heated mice.

2.4.5. In vivo depletion of CD25\(^+\) cells

1mg per mouse of anti-CD25 (PC61) or rat IgG1 (MAC49) was given intraperitoneally in PBS (average volume 500μl per mouse). Depletion of peripheral CD25\(^+\) cells was confirmed by taking a small (<50μl) sample of blood from the tail and staining for FACS with anti-CD4-APC and anti-CD25-FITC. Importantly, the mAb clone used for anti-CD25 FACS staining was 7D4 not PC61.

2.4.6. Production of Ab for in vivo depletion experiments

The PC61 hybridoma that produces anti-CD25 mAb (rat IgG1) was a kind gift from Dr. Fiona Powrie, University of Oxford. The MAC49 hybridoma (that produces anti-phytochrome mAb, rat IgG1) was obtained from the European Collection of Cell Culture, Wiltshire, UK and used as an isotype control Ab. Both hybridomas were grown initially in RPMI-5 medium in 10ml culture flasks (Costar) until sufficient cell density was achieved. 2.5x10\(^7\) hybridoma cells in 15ml cultivation medium were inoculated into the cultivation chamber of an Integra CL1000 (Integra Biosciences), and 1000ml nutrient medium was added to the nutrient chamber, as per instructions. On day 7, and every 3 days thereafter, 7.5ml of the cultivation medium was removed.
and replaced with fresh medium, and the entire nutrient medium was exchanged. The removed medium was centrifuged at 2000rpm for 5 minutes to remove hybridoma cells and the supernatant stored at −20°C until Ab purification.

Antibodies were purified using an Aktaprime automated chromatography system (Amersham Biosciences) with a 5ml High Trap protein G column (Amersham Biosciences). Antibody supernatant to be purified was passed through a 4micron filter. All buffers and wash solutions were passed through a 2micron filter before use in the Aktaprime. The affinity step gradient programme was used, a summary of the process is as follows: both the Aktaprime machine and column were first washed with water then PBS (“System Wash” programme). The Ab suspension was loaded onto the column (the flowthrough was collected at this stage in order to save any Ab not loaded onto the column), the column was then washed with 5x column volume PBS. Ab was then eluted with 20ml 0.1M Glycine pH5, and 3ml fractions were collected into tubes containing 1ml 9M Tris to neutralise the pH. The column and machine were then washed with water followed by 70% Ethanol for storage. Eluted Ab was dialysed into PBS and concentration calculated using the BCA Protein Assay kit (Pierce) as per manufacturer’s instructions.

2.5. Preparation and Purification of Cell Populations

2.5.1. Preparation of mononuclear cell populations from CNS

Mice were sacrificed by CO₂ asphyxiation. Immediately after death mice were perfused with 10ml PBS through the left ventricle of the heart. Spinal cords were removed by intrathecal hydrostatic pressure using cold PBS. Brains (when used) were removed by dissection. Cords and brains were cut into small pieces and digested at 37°C for 30 minutes in Wash Buffer containing 2.5mg/ml collagenase (Worthington Biochemicals, NJ) and 1mg/ml deoxyribonuclease (Sigma), followed by mechanical disaggregation to obtain a single cell suspension. Cells were washed once in 30% Percoll(Gibco) (made up with Wash Buffer), then resuspended in 30%
Percoll and underlaid with 70% Percoll. These discontinuous gradients were spun at 2000g for 20 minutes without brake (16, 176). The gradient interface was removed and cells washed thoroughly in wash buffer before sorting or stimulation.

2.5.2. B Cell Purification (MACS selection)

Single cell suspensions were obtained from LN and/or spleen by mashing. Red blood cells were lysed by incubating cells in Red Blood Cell Lysis Buffer (Sigma) for two minutes at room temperature, followed by washing in MACS Buffer three times. Cells were resuspended in MACS buffer and counted. 45μl MACS buffer and 5μl anti-CD43 microbeads (Miltenyi Biotec) were added per 10⁸ cells, and incubated for 15 minutes at 4°C, followed by washing in MACS buffer. Cells were resuspended at 10⁸ cells/ml in MACS buffer. The cell suspension was then run through a pre-prepared MACS CS depletion column, (Miltenyi Biotec) on a VarioMACS magnet (Miltenyi Biotec), and the flow-through (CD43-negative fraction containing B cells) was collected. A sample of the cells was stained with anti-B220-PCP and the purity was analysed by FACS. Purity of B cells was consistently above 80% and usually above 90%.

2.5.3. CD4⁺ Cell Purification (MACS positive selection)

Single cell suspensions were obtained from LN and/or spleen by mechanical disaggregation. Red blood cells were lysed as above followed by washing. Cells were resuspended in MACS buffer and counted. 90μl MACS buffer and 10μl anti-CD4 microbeads (Miltenyi Biotec) were added per 10⁸ cells, and incubated for 15 minutes at 4°C, followed by washing in MACS buffer. Cells were resuspended at 10⁸ cells/ml in MACS buffer. The cell suspension was then run through a pre-prepared MACS LS positive selection column (Miltenyi Biotec), and the cells retained in the column (positive fraction - CD4⁺ cells) were collected. A sample of the cells was stained with anti-CD4-FITC and the purity of the preparation analysed by FACS. Purity was consistently above 90% and usually above 95% CD4⁺ cells.
2.5.4. CD4⁺ Cell Purification (Dynal negative selection)

Single cell suspensions (as above) were resuspended at 2×10⁸ cells/ml in a cocktail of the following antibodies, produced in-house, all rat IgG: anti-CD8 (clone 53.6.72), anti-B220 (clone RAB832), anti-Mac1 (M1/70), and anti-class II (M5/114.15.2) each at 10µg/ml in MACS buffer. After incubating for 20-30 minutes on ice, cells were washed and resuspended at 10⁸ cells/ml. An equivalent volume of washed sheep anti-rat IgG M450 Dynabeads (Dynal Biotech Ltd, Wirral, UK) (concentration 10⁸ beads/ml) was added and the mixture incubated with rotation at 4°C for 20-30 minutes. The tube was then placed in a magnetic field and the supernatant (enriched for CD4⁺ cells) collected. This step was repeated once more to remove all contaminating beads. A sample of cells was stained with anti-CD4-FITC and the purity of CD4⁺ cells analysed by FACS. Purity was consistently above 70%.

2.5.5. CD4⁺CD25⁺ purification

2.5.5.1. MACS sorting

CD4⁺ cells were enriched by Dynal bead negative selection (section 2.5.4). Cells were resuspended at 10⁸ cells/ml, with 40µl/ml anti-CD25PE (Miltenyi Biotec), and incubated for 15 minutes at 4°C. After washing, 90µl MACS buffer and 10µl anti-PE microbeads (Miltenyi Biotec) per 10⁸ cells were added and the cells were incubated for a further 15 minutes at 4°C. Cells were washed and resuspended at 2×10⁸/ml and run over a MACS LS positive selection column (Miltenyi Biotec) as per manufacturer’s instructions, collecting the cells retained in the column. Purity was checked by FACS and was consistently above 75-80%.

2.5.5.1. FACS sorting

Cells were enriched for CD4⁺ cells by Dynal selection (section 2.5.4). Cells were then stained with anti-CD4-FITC (Pharmingen) and anti-CD25PE (clone 7D4, Miltenyi Biotec) in MACS buffer. Cells were then washed and sorted on a Becton Dickinson FACSTAR machine by Andrew Sanderson. Purity of CD4⁺CD25⁺ cells was consistently above 95%, and of CD4⁺CD25⁻ cells above 97%.
2.6. Ex vivo/in vitro readouts

2.6.1. Ex vivo stimulation of cells to test for cytokine production

Single cell suspensions were prepared by disaggregating LNs and spleens through gauze, and CNS cells were prepared as described above. Cells were incubated in 24 well plates at 2-5x10^6 cells/well with RPMI-5 containing 50ng/ml PMA (Sigma), 1µg/ml Ionomycin (Sigma) and 1µl golgistop (BD Pharmingen, La Jolla, USA). After 4 hours cells were washed and stained for FACS analysis. For analysis of Ag-specific cytokine production, cells were stimulated with 30µM MOG(35-55) in the presence of 1µl/ml Golgistop, or medium alone with Golgistop for 5 hours as indicated.

2.6.2. FACS analysis

Single cell suspensions were obtained as above. For staining of cell surface molecules, cells were stained with the indicated antibody (Ab) (see Table 2.1 for Ab clones and concentrations used) in FACS buffer, usually with the addition of anti-Fc receptor Ab (20µg/ml) to block non-specific uptake of Ab. For intracellular staining of cytokines and CTLA-4, cells were first stained for expression of cell surface molecules, then fixed and permeabilised using the cytofix/cytoperm kit (BD Pharmingen) according to manufacturer's instructions. All staining was performed at 4°C for 15-40 minutes (intracellular cytokine staining was always ≥ 30 minutes). Cells were then washed and analysed using a Becton Dickinson FACScan flow cytometer with CellQuest (Becton Dickinson, UK) software for collection of data, and FlowJo (Treestar, USA) software for analysis of data. Unless otherwise indicated, all antibodies were obtained from BD Pharmingen.
### Table 2.1: Antibodies used for FACS analysis

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<th>Clone</th>
<th>Fluorescent conjugate</th>
<th>Concentration Used</th>
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<td>1/100</td>
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*Purified and conjugated in-house **from Miltenyi biotec

2.6.3. Cell-based ELISAs for quantification of antigen-specific cytokine production

Single-cell suspensions of cells (number and source indicated in figure legend) were cultured in flat-bottomed 96-well microtiter plates (Becton Dickinson, Mountain
View, CA), with the indicated antigen dose(s). X-Vivo 15 serum-free medium (BioWhittaker, Maidenhead, UK) supplemented with \(5 \times 10^{-5}\) M \(\beta\)-mercaptoethanol and 2 mM L-glutamine (Gibco, Life Technologies, Paisley, UK) was used as culture medium. After 48 h of culture, 100\(\mu\)l of cells were resuspended and cytokines measured as described below, using the protocol of Beech et al.

Under sterile conditions, 96-well MaxiSorb microtiter plates (Nalge Nunc International, Roskilde, Denmark) were coated overnight at 4°C with 50\(\mu\)l per well of capture antibody (anti-IL-2, (clone JES-1A12), anti-IL-4 (11B11), anti-IL-10 (JES5-2A5) or anti-IFN\(\gamma\) (R4-6A2), all from BD Pharmingen) diluted to 2\(\mu\)g/ml in carbonate/bicarbonate buffer (0.05 M, pH 9.6). After washing twice in PBS/Tween (0.1%), plates were blocked at 37°C for 1 h with 200\(\mu\)l per well of a sterile PBS/BSA (1%) solution. After two washes in PBS/Tween followed by two further washes in PBS, cytokine standards diluted in PBS/BSA were added at 100\(\mu\)l per well in duplicate (IL-2 and IL-4 top concentration: 1000 pg/ml, IL-10 and IFN\(\gamma\) top concentration: 100 ng/ml) and two-fold dilutions performed to give a standard curve for each plate. Samples of each culture were resuspended and added at 100\(\mu\)l per well in duplicate. Samples and standards were then incubated at 37°C in a humidified atmosphere of 5% CO\(_2\) for 20-24 h. After this period, plates were washed four times in PBS/Tween and incubated with 100\(\mu\)l per well biotinylated anti-cytokine detecting antibody (anti-IL-2 (clone E56-5H4), anti-IL-4 (BVD6-24G2), anti-IL-10 (SXC-1) or anti-IFN\(\gamma\) (XMG1.2), all BD Pharmingen) diluted to 5 ng/ml for IL-2 and IL-4 and 100 ng/ml for IL-10 and IFN\(\gamma\), in PBS/BSA for one hour at room temperature. Plates were washed a further six times before incubation with a 1/1000 dilution (final concentration \(\geq 2\mu\)g/ml) of ExtrAvidin peroxidase (Sigma) in PBS/BSA at 100\(\mu\)l per well for 30 min. Plates were washed six times before addition of 100\(\mu\)l per well of Tetramethylbenzidine (TMB; Sigma) substrate (prepared by adding 100\(\mu\)l of 10 mg/ml TMB in DMSO to 9.9 ml of phosphate citrate buffer pH 5 and 3\(\mu\)l of hydrogen peroxide) followed by 100\(\mu\)l of 2M sulphuric acid to stop the reaction. At this point,
absorbance values were read at 450 nm using a Multiskan plate reader (Labsystems, Basingstoke, UK).

2.6.4. ELISAs to measure MOG-specific Ab in serum
Using a protocol optimised by the Gray lab, flat-bottomed 96-well MaxiSorb microtiter plates (Nalge Nunc International, Roskilde, Denmark) were incubated with 50μl/well of MOG (35-55) or rMOG (50μg/ml diluted in 0.1M bicarbonate buffer pH 9.6) overnight at 4°C (plates were wrapped in clingfilm to prevent evaporation). Plates were washed three times in PBS. Non-specific antibody binding was blocked by adding 200μl/well of 1% BSA in PBS for one hour at 37°C. After a further three washes in PBS, serum samples (50μl) were added in duplicate. Plates were incubated for one-two hours at 37°C then washed three times with PBS. 50μl alkaline phosphatase (AP) -conjugated anti-Ig secondary antibody (diluted 1/500) was added to each well and plates were incubated for two hours at room temperature. Plates were washed four times in PBS and developed by adding PNPP (one tablet dissolved in 5ml ELISA buffer: 400ml ddH20, 24.5mg MgCl2.6H2O and 48ml diethanolamine, stored wrapped in foil to prevent degradation by light). Plates were read at 450nm using the Multiskan ELISA plate reader (Labsystems) once wells had turned yellow.

When peptide was added in for experiments to measure blocking of antibody binding to rMOG (Figure 3-7), the indicated concentrations of peptide or PBS were mixed with the serum at the time it was added to the ELISA plate.

2.6.5. Suppression assays
For assays to test suppression by CD4+CD25+ cells from μMT versus B6 mice (Figure 5-4), assays were set up similarly to those described in the literature (96). 2x10⁴ CD4+CD25+ responder cells were cultured with 10⁵ irradiated B6 splenocytes as APC in flat-bottomed 96-well plates (See Appendix Figure A-2) for test of optimal APC numbers, μMT splenocytes proved inefficient APC in one experiment
so were not used). MACS-sorted CD4⁺CD25⁺ cells were titrated in at numbers indicated on graph axes. The purity of MACS-sorted cells was between 70-85%, but always comparable within each experiment. Cultures were stimulated with 0.5µg/ml anti-CD3, clone 145.2C11 (this concentration was chosen as the optimal concentration for stimulation of CD4⁺CD25⁻ cells without preventing suppression by CD4⁺CD25⁺ cells, see Appendix figure A-1 for dose response). Cells were cultured at 37°C for 72 hours, with tritiated thymidine added for the last 18 hours of culture before assays were harvested.

For the suppression assays comparing CD4⁺CD25⁺ cells from the LN of mice with EAE and naïve mice (Figure 5-2), conditions were further optimised in separate experiments using TCR transgenic cells by Dr Leigh Stephens. The main changes were to culture the cells in round-bottomed 96 well plates to optimise cell contacts, and to extend the culture time to 96 hours, which proved to be helpful when investigating antigen-specific responses of small numbers of cells. CD4⁺CD25⁺ cells were selected by MACS-sorting from mice 16 days after immunisation with MOG in CFA with or without the addition of pertussis toxin (the results of assays using cells from these two conditions were similar). In all assays, 1.6x10⁵ irradiated splenocytes from naïve mice were added as APC. Between 10⁴ and 4x10⁴ CD4⁺CD25⁻ responder cells were added and CD4⁺CD25⁺ cells were titrated in. The source and number of responder cells and CD4⁺CD25⁺ cells used for each assay are indicated in the figure legends.

To test suppression by CNS CD4⁺CD25⁺ cells (Figure 5-1), mononuclear cells were prepared and CD4⁺CD25⁺ cells purified by FACS sorting (see section 2.5.5) and titrated into assays containing 10⁴ CD4⁺CD25⁻ cells purified from LN of naïve mice were used as responder cells. 1.5x10⁵ irradiated splenocytes from naïve mice were used as APC. Cells were stimulated in round-bottomed 96 well plates with 2.5µg/ml anti-CD3 (clone 145.2C11) in RPMI-5 tissue culture medium for 96 hours. 0.5µCi tritiated thymidine (Amersham Intl) was added for the final 18 hours of culture before assays were harvested.
2.6.6. Proliferation responses of CD4^+CD25^+ cells (Figure 4-10)

CD4^+CD25^+ cells were purified by MACS sorting from LN or CNS of mice with EAE. In conditions similar to the above suppression assays, 2x10^4 cells were cultured with 1.6x10^5 APC, and the antigen and IL-2 concentrations indicated in figure legend, in round-bottomed wells for 96 hours.

2.6.7. Foxp3 rtPCRs

CD4^+ cells were enriched from spinal cords and inguinal LN of mice 20 days after induction of EAE and mRNA extracted using the Micro-FastTrack 2.0 Kit (Invitrogen Life Technologies, UK). cDNA was made using the Stratascript First-Strand Synthesis System (Stratagene, UK) Foxp3 PCR was performed using the following primers (MWG Biotech, Milton Keynes, UK), designed by Angie Harris: 

5'-CCTGGCCCACCTGGGATCAA-3' (spanning exons 3 and 4 to avoid genomic DNA amplification); 5'-TTCTCACAACCAGGCCACTTG-3'.

As a control β-actin PCR was also performed

(Primers: 5'-TGGAATCTGTGGCATCCATGAA-3',
5'-TAAAACGCAGCCTCAGTAACAGTC-3').

2.7. Cloning

Attempt 1:

A method adapted by Dr Steve Anderton from the protocol described by Taylor, Thomas and Mills (283) was used. B6 congenic Ly5.1^+ mice were used to allow eventual tracking of the cells in vivo. On day 18 after EAE induction, CD4^+CD25^+ cells from LN and CD4^+ cells from CNS were purified and plated out at limiting
dilutions ranging from 30 to 600 cells/well/plate, with \(3.5 \times 10^5\) irradiated splenocytes as APC, 10\(\mu\)M MOG(35-55) and 100u/ml IL-2. After 1 week wells were checked for the presence of growing T cells by microscopy, and positive wells were restimulated weekly with APC, 4\(\mu\)M MOG(35-55) and 100u/ml IL-2 until cells had expanded sufficiently to be tested for reactivity to MOG(35-55). Wells from plates in which less than 20% of wells scored positive for growing cells were considered clonal. All these wells were restimulated weekly in 24 well plates with 3\(\times\)10\(^6\) APC, 2\(\mu\)M MOG(35-55) and 100u/ml IL-2.

**Attempt 2:**

Cells were prepared and plated out as described above, except that 200pg/ml IL-10 (R&D Systems, Abingdon, UK) (88) was added for the first two rounds of stimulation, and 100ng/ml IL-15 (R&D Systems) was added for every restimulation (as well as for the initial stimulation). After 9 weeks, the concentration of IL-2 added for restimulation was increased to 1000u/ml.
3. B cells as Regulatory Cells Mediating Recovery from EAE

3.1. INTRODUCTION

B cell deficient mice and chimeric mice in which B cells are unable to produce IL-10 fail to recover from EAE (59, 273). It has also been reported that rats in remission from EAE are resistant to further induction of EAE, and that this may be mediated by a combination of T cells and B cells (257). In this chapter, the susceptibility of recovered B6 mice to the reinduction of EAE is examined. Possible mechanisms behind both recovery and resistance are investigated, focussing on the role of B cells.

3.2. RESULTS

3.2.1. Mice that have recently recovered from EAE are resistant to reinduction of disease

Induction of EAE in B6 mice using the MOG(35-55) peptide in this lab results in a monophasic course of disease, and relapses are very seldom observed after remission. It has been known for some time that Lewis rats that have recovered from EAE induced with MBP are resistant to rechallenge with MBP(284). To test this in our model, B6 mice that had recovered from EAE were rechallenged with a second immunisation with MOG(35-55). Most of the mice developed EAE with an earlier onset after reinduction, indicating a memory response. However, the disease severity in most of these mice was mild and the duration of disease shortened when compared to mice immunised for the first time (Figure 3-1a). This resistance to reinduction was only effective when mice were rechallenged at early points after recovery (1-3 weeks). When mice were rechallenged 6 to 8 weeks after recovery from primary disease, they were found to be fully susceptible, and developed EAE of similar severity and duration to the primary disease. (Fig 3-1b).
Resistance to reinduction of disease after recovery from EAE suggests two possible scenarios. First, during recovery pathogenic T cells may be deleted or made anergic and therefore unable to respond to a second challenge. Although in vitro LN and spleen recall responses are depressed after recovery compared to early points in the disease, they are not completely abolished (59), suggesting that MOG(35-55)-reactive Th1 effectors are still present. Also, mice that did develop EAE after rechallenge had an earlier onset of disease (1 to 2 days before controls, Figure 3-1) consistent with a memory response that was then rapidly regulated. Therefore, an active mechanism of controlling pathogenic cell re-activation and/or priming of fresh CD4+ cells after rechallenge can be proposed. Since this is likely to be the same mechanism employed to control autoaggressive T cells and mediate recovery, resistance to reinduction provided a useful starting point for investigating the recovery process.

3.2.2. MOG(35-55)-reactive CD4+ T cells with pathogenic potential are still present in mice after recovery from EAE

To determine whether the CD4+ T cell compartment in EAE-recovered mice contained cells with either pathogenic or regulatory potential, CD4+ cells from the pooled spleen and inguinal LN of mice 30 days after EAE induction were transferred into naïve recipients. One day later, EAE was induced in recipients, as well as control mice that had not received cells, and disease course monitored. The disease incidence and severity was consistently slightly higher in mice receiving CD4+ cells compared to controls (Fig 2). Also, disease onset was slightly accelerated in recipients of post-EAE CD4+ cells (1 to 2 days earlier than controls). However, in 2 out of 3 experiments the differences between CD4+ recipients and controls did not reach statistical significance. Although this difference was admittedly not marked, earlier onset and increased disease in recipients suggested the presence of pathogenic memory cells in the CD4+ compartment of recently recovered mice, and that the effects of these cells was dominant over any regulatory cells that may have been present in the spleen/LN populations that were used.
To further examine the contribution of transferred post-recovery CD4+ cells to disease in recipients, cells were tracked in vivo after transfer. For this purpose, EAE was induced in B6 congenic mice bearing the Ly5.1 allele of CD45 (present on all leukocytes). On day 30 after immunisation (when all mice had recovered), Ly5.1+ CD4+ cells were transferred into wildtype B6 mice (which have only the Ly5.2 allele) and EAE was induced 1 day later. Inguinal LN, spleen and spinal cord were harvested at the peak of disease (days 13-15 after induction), and pooled cells from each organ analysed by FACS for surface expression of Ly5.1 and CD4, and intracellular cytokine levels after stimulation with PMA and ionomycin.

Thirteen to fifteen days after induction of EAE, Ly5.1+ CD4+ cells could be detected by FACS in the inguinal LN, spleens and spinal cords of recipient mice (Figure 3-3a). Compared to LN and spleen, Ly5.1+ cells were greatly enriched in the CNS, constituting 27.93% ± 4.47% (mean ± std deviation) of total CD4+ cells in 3 separate experiments, while only reaching 2.03% ± 1.04% in inguinal LN and 3.5% ± 1.32% in spleens of the same animals (Table 3-1a). A proportion of these Ly5.1+ cells produced IFNγ after ex vivo restimulation with PMA and ionomycin, consistent with expansion of Th1 effectors (Figure 3-3b). The proportion of Ly5.1+ cells producing IFNγ in 2 experiments was greater in the CNS (mean ± SD: 28.5% ± 14.9%) than in inguinal LN (8.25% ± 4.6%) (Table 3-1b), consistent with enrichment of effector cells in CNS rather than LN, on day 15. However, although the percentage of CD4+ cells that were Ly5.1+ was around 8-fold lower in spleen, the proportion of Ly5.1+ cells producing IFNγ was similar in CNS and spleen (38% ± 5.66%) (Table 3-1b). Although for this set of experiments transfer of naïve CD4+ Ly5.1+ cells was not done, in later experiments (where CD4+CD25+ cells were transferred), naïve Ly5.1+ CD4+ cells were not found to accumulate in the CNS of recipients after EAE induction (Fig 3c). The percentage of Ly5.1+ cells in the CNS is lower in this experiment, but this can be explained by a lower number of cells transferred – only 2x10^5 compared to 3x10^6 for whole CD4+ cell transfers.
Without a full time course it is difficult to draw conclusions regarding numbers of cells in lymphoid sites after transfer. In other transfer systems, using transgenic T cells, the peak of expansion of memory cells occurs around day 5, followed by a contraction phase (285). In a recent study the proportion of PLP(139-151)-specific CD4+ cells in the LN of SJL mice after EAE induction with PLP(139-151) was analysed by tetramer staining (286). Tetramers are multimeric complexes of MHC and peptide incorporating fluorescent dye (287). This allows direct identification by flow cytometry of T cells bearing TCRs that specifically bind those MHC peptide complexes, i.e. Ag-specific T cells, within a lymphoid population without the requirement for transfer of transgenic TCR cells. Consistent with other studies examining in vivo responses to various Ag using tetramers, the peak of expansion of PLP(139-151)-specific CD4+ cells in the LN occurred between days 6 and 8 (PLP(139-151)-specific cells represented 0.9-1.5% of total CD4+ cells), followed by a rapid decline to background levels between days 16 and 20 (0.5% of total CD4+ cells) (286). So, by day 14 after EAE induction the proportion of MOG(35-55)-specific cells would be expected to have returned to low levels in the LN and spleen.

In one experiment in which Ly5.1+ cells were analysed on days 8 and 15 after immunisation with MOG(35-55), the proportion of cells on day 8 was higher than day 15 (percentage of CD4+ cells that were Ly5.1+ in inguinal LN on day 8 was 9.4% compared to 3.1% on day 15; and in spleen Ly5.1+ cells made up 6.9% of total CD4+ cells compared to 5% on day 15), suggesting expansion of MOG(35-55)-specific cells. Given that the transferred cells were from a non-transgenic mouse and taken at a late timepoint in the disease course, the frequency of any expanded MOG-reactive cells would only be a small proportion of Ly5.1+ cells labelled. A method for staining MOG(35-55)-specific cells, such as tetramer staining for FACS, would be highly useful for singling out this small population, particularly in LN and spleen. A further control group of non-immunised post-recovery transfer recipients would have been useful for determining the starting proportion of Ly5.1+ cells, but due to restraints in mouse numbers this was not done.
These results confirm that potentially pathogenic cells remain in the spleen/LN of mice recently recovered from EAE, arguing against deletion of autoreactive cells as the mechanism behind recovery. After transfer into naïve recipients at least a proportion of these cells can be expanded by antigen challenge and contribute to CNS inflammation, further suggesting that resistance to reinduction of EAE is not due to deletion or anergy of pathogenic MOG(35-55)-reactive cells, but rather involves active regulation of these pathogenic cells in recovered mice.

3.2.3. Post-recovery B cells are able to transfer resistance to induction of EAE

Given that B cells are required for recovery from EAE(59, 273), B cells present in recovered mice were tested for their ability to regulate activation of MOG(35-55)-reactive T cells in vivo. B cells were purified from pooled spleens and inguinal LN of mice on day 30 post-immunisation and transferred into naïve recipients one day before EAE induction.

In contrast to CD4+ cells, post-recovery B cells were able to transfer some resistance to induction of EAE (Figure 3-4). The level of protection achieved varied between experiments, but disease severity of recipients of B cells was consistently lower than untransferred controls in four separate experiments. An important control of naïve B cell transfer was only done in one experiment (see Figure 4-2), and in this experiment was not protective compared to post-EAE B cells. Transfer of whole splenocytes from naïve mice also gave no protection (data not shown).

One might expect that the efficacy of post-EAE B cells in reducing disease may be enhanced by co-transfer of post-EAE CD4+ T cells which would more efficiently provide early "help" to activate these B cells. Indeed, it has been reported that a combination of B cells and T cells from rats that have recovered from EAE can protect recipients from induction of EAE, but neither cell population was effective in isolation (257). However, in 4 separate experiments in which whole post-recovery
splenocytes were transferred, disease was enhanced in 2 experiments and decreased in 2 experiments (Fig 3-5), therefore co-transfer of CD4+ cells does not seem to be required for B cell transfer of resistance to induction of EAE.

3.2.4. Antigen specificity of B cells during recovery?
The above data as well as published data (59) suggests that post-recovery B cells, but not B cells from mice at the peak of disease or from naïve mice, are able to transfer resistance to induction of EAE. This suggests expansion of protective antigen-specific B cells during the course of EAE, and particularly the recovery phase. In order to be able to further phenotype these cells, attempts were made to enumerate numbers of MOG-reactive B cells and determine their fine specificity.

i) Detection of MOG-specific B cells by FACS
In order to detect antigen-specific B cells by FACS it is necessary to label the BCR in a specific manner. One way of doing this is to fluorescently label the antigen recognised by the BCR, allow antigen binding by B cells, then look for labelled cells by FACS. For this purpose, recombinant MOG protein (rMOG) was biotinylated. LN cells from mice on day 28 as well as LN cells from naïve mice were stained using MOG-biotin followed by streptavidin-APC. Cells were also labelled with antibody against the B cell marker B220. The background staining of MOG-biotin positive cells in naïve cell preparations was unacceptably high, and there were no differences discernable between cells from naïve and MOG(35-55)-immunised mice (Figure 3-6a). To minimise high background due to non-specific staining, several approaches were taken: a dose range of rMOG-biotin was tested; dead cells and macrophages were excluded from FACS analysis by staining with propidium iodide and F480-PcP and excluding positive cells; all staining and blocking was done using FACS buffer containing 10% FCS to reduce non-specific "stickiness" of cells for protein. However these approaches did not improve the background staining and it remained impossible to distinguish MOG-specific B cells (Figure 3-6c).
ii) Detection of MOG-specific antibody in serum of mice with EAE by ELISA

Since it was not possible to detect MOG-specific B cells directly, the presence of MOG-specific Ab in serum was used as a measure of whether MOG-specific B cells were being activated during EAE. Serum was collected at time of sacrifice on days 14 and 30 post-EAE induction. Serum IgG1, IgG2a and total IgG binding to recombinant MOG protein (rMOG) were tested. In six separate experiments, around half the mice were found to have detectable serum antibodies against rMOG on day 30, while in one experiment only 2 out of 10 mice had weak anti-rMOG responses on day 14 (Table 3-2/fig 3-7a). As negative controls sera from naïve mice were used and showed no reactivity to rMOG (Figure 3-7a). 28 of 49 mouse sera tested had anti-rMOG responses of the IgG1 isotype, while only 12 of 44 mice had IgG2a responses (Table 3-2), surprisingly suggesting a Th2-dominated response. However, in the absence of MOG-specific antibody standards it was not possible to quantify antibody concentrations, and comparing relative isotype responses was not possible since there may have been differences in sensitivity between the ELISAs.

By conventional ELISA, anti-MOG(35-55) responses appeared to be very low. This could be due to poor binding of MOG(35-55) to ELISA plates, or changes in structure when bound to the plate causing decreased antibody binding efficiency. Therefore, an indirect blocking study was undertaken: ELISA plates were coated with rMOG as before, then different concentrations of MOG(35-55) were added in with the serum during incubation on the plate. As can be seen in Figure 3-7b, addition of MOG(35-55) blocked binding of serum antibodies to rMOG in a dose-dependent manner. This blocking was observed in 8 individual positive mouse sera (Figure 3-7c), as well as pooled positive sera from 10 different mice (data not shown), strongly suggesting the presence of B cells specific for an epitope(s) within MOG(35-55).

To further define specificity of antibody, a truncated peptide, MOG(35-50), was tested in the blocking assay. Interestingly, removal of the amino acids at position 51-55 almost completely abrogated blocking, with only slight effects at high peptide
concentrations compared to MOG(35-55) (Figure 3-7b), showing that the p50-55 portion is required for BCR binding.

3.2.5. Resistance to induction of EAE transferred by post-recovery serum – is B cell protection mediated by antibody?

Given that anti-MOG(35-55) antibody was detectable in around half of the mice tested on day 30 of EAE, the question arose could B cell protection in fact be mediated by antibody? It has been reported in a rat model of EAE that serum from rats in remission from MBP-induced EAE can protect recipients (288, 289). To test whether serum could protect in our B6 mouse model, two experiments were performed. First, 150μl of pooled sera from mice at day 30 or day 14 of EAE (made up to 200μl with PBS), or PBS alone, were transferred on days −1, +1, 4 and 6, and EAE induced on day 0. In this experiment, a significant reduction of disease severity was observed in mice receiving day 30 serum but not day 14 serum (Figure 3-8a). Since day 14 sera were found to be almost completely lacking in anti-rMOG responses compared to day 30 (Table 3-2), the experiment was further refined to transfer pooled day 30 sera which had tested either positive or negative by ELISA for anti-rMOG antibodies. 150 μl sera were transferred on days −1, +1 and 3. In this experiment, a small reduction in maximum disease scores was observed in both groups that received serum compared to controls, but there was no difference in the protection afforded by anti-rMOG positive versus negative sera (fig 3-8b). Collection of sufficient sera to perform these experiments was very restrictive in determining how many repeats and variations could be done, Interpretation of the results is also complicated – see discussion (section 3.3). Therefore, while it appears that serum transfer is able to confer some protection on recipients, this line of investigation was not continued further.

3.2.6. Do post-recovery B cells inhibit in vitro T cell responses?

As reported(59), anti-MOG(35-55) T cell responses in vitro are consistently lower on day 30 (recovery phase) than on day 10 (effector phase) in wildtype B6 mice. However in μMT mice, these responses are not depressed, correlating with impaired
recovery. Given the observations that transferred post-recovery CD4\(^+\) cells could be activated after immunisation and that transferred post-recovery B cells inhibit EAE induction, it was possible that B cells present in the in vitro cultures could be regulating proliferation and cytokine production of MOG(35-55) reactive cells. This could be mediated by IL-10 production by antigen-presenting B cells, or inhibition of antigen presentation due to BCR binding of peptide. To test this, MOG(35-55)-specific cytokine production by CD4\(^+\) cells from mice 30 days after EAE induction (d30 EAE) was tested in the presence of different APC: purified B cells or CD4-depleted splenocytes from the same (d30 EAE) mice, or from naïve mice.

In an initial experiment, stimulating the CD4\(^+\) T cells in the presence of naïve APC or naïve B cells did appear to enhance IL-2 and IFN\(\gamma\) production in comparison to cells stimulated in the presence of d30 EAE APC (Figure 3-9a). In contrast, IL-10 and IL-4 were marginally decreased in the presence of naïve APC or naïve B cells, although the overall levels of production of these cytokines, particularly IL-10, was low. This experiment was repeated twice using B cells from naïve mice or d30 EAE mice, but the results were not consistent with the first experiment (not shown). The experiment was further refined by culturing d30 EAE CD4\(^+\) cells with MOG(35-55) in the presence of splenic CD11c\(^+\) DCs (from naïve mice) and titrating in d30 EAE B cells. However, while a small decrease in IL-2 was observed with increasing numbers of B cells, IFN\(\gamma\) was increased under the same conditions (Figure 3-9b). Therefore, it does not appear that B cells significantly inhibit cytokine responses in these in vitro assays.

3.2.7. Requirements for B cell antigen presentation during recovery from EAE – making an antigen to specifically target B cells in vivo

IL-10 production by B cells has been shown to be important for EAE remission in the B6 model (59). In the same paper it was shown that the presence of IL-10 sufficient B cells with restricted specificity for hen egg lysozyme (HEL), and therefore unable to take up and present CNS antigens via their BCR, were unable to promote recovery. This was the case even when mice were additionally immunised
with HEL at the time of EAE induction (SMA - personal communication). This result suggests that mere activation of B cells is insufficient for mediating recovery. Rather, direct B cell interactions with pathogenic T cells via presentation of MHC-peptide complexes may be required.

To further refine this model, it is desirable to be able to activate B cells in vivo while concurrently immunising with CNS antigen either separately or targeted to these B cells via BCR. Production of a conjugate of HEL and MOG(35-55) was therefore attempted. Chemical coupling of the HEL protein and MOG(35-55) protein was chosen because this is a far quicker process than designing and producing a genetic construct of the conjugate. Coupling of the MOG(35-55) peptide rather than MOG protein avoided the difficulties associated with the poor solubility of MOG protein, particularly at the pH required for optimal chemical coupling. The EDC coupling kit from Pierce was chosen because it is particularly recommended for coupling haptens or peptides to carrier proteins by the manufacturers. When added to a mixture of peptide and protein, EDC initially reacts with carboxyl groups to form amine-reactive intermediates, which then react with amine groups to form a link between the two compounds.

It was a concern that using too high a concentration of MOG(35-55) peptide during the coupling procedure would lead to blocking of HEL uptake by B cells, and conversely that too low a peptide concentration would lead to insufficient peptide presentation to activate T cells. Therefore, a range of 5 peptide concentrations was used, with a fixed concentration of HEL. The conjugates were then tested in vitro. It is known that B cell presentation of antigens taken up via BCR-binding is accelerated and more efficient compared to non-specific uptake by fluid phase pinocytosis, so that much lower concentrations of antigen are required to activate T cells (67). All B cells from MD4 mice bear a transgenic receptor specific for HEL (281). Therefore, presentation of MOG(35-55) after HEL-MOG(35-55) conjugate uptake by MD4 B cells, was compared to presentation by wildtype B6 B cells. Antigen presentation
was measured by proliferation of MOG(35-55)-reactive CD4⁺ cell lines in 3 day cultures.

The expected result was observed in a preliminary experiment: T cell stimulation by MD4 B cells occurred at much lower concentrations of conjugate, with 100 to 10000 fold increases in concentration required for equal stimulation by B6 B cells (fig 3-10). The minimum concentration of conjugate required for antigen presentation was lowest for the conjugate made with the highest peptide concentration, and the concentrations required increased stepwise correlating with decreasing peptide concentrations.

These results would appear to confirm that the conjugate is able to target presentation of MOG(35-55) to HEL-specific B cells in vitro, and that presence of a HEL-specific BCR is required for presentation of lower concentrations of conjugate, as would be expected to occur in vivo. However, when the control results were analysed serious doubt was cast on this conclusion. For controls, free MOG(35-55) peptide was added in conjunction with, but separately to, HEL. This should have excluded any advantage gained by antigen-specific BCR-mediated uptake and presentation, resulting in equal stimulation of T cells in those cultures. This was not the case: the MD4 cells again induced greater proliferation at lower peptide concentrations than B6 B cells, and the levels of stimulation observed closely correlated with conjugate-driven stimulation by the respective B cell populations (Figure 3-10). This could be explained by an up-regulation of class II molecules on the surface of MD4 B cells being activated via BCR (67). However, similar results were observed when peptide alone was added – MD4 B cells stimulated T cells better than B6 B cells. It has also been reported that naïve MD4 mice have a high background level of serum HEL-specific antibody (281), suggesting that their B cells may have a higher activation status than wildtype B6 B cells, although this was not investigated.
While further validation is required, this approach is potentially useful for setting up a system for targeting antigen to specific B cells in vivo to separate requirements for B cell activation and antigen presentation from B cell activation alone. One major drawback with the protocol used in this project was the amount of conjugate generated per reaction, this would have to be scaled up for use in vivo. Also, the conjugate would have to be tested in vivo for ability to induce EAE at different concentrations in the presence or absence of HEL-specific B cells, since it is only possible to estimate the amount of peptide present.

3.3. DISCUSSION

It has been known for some time now that after recovery from EAE induced by immunisation with MBP, Lewis rats are resistant to further induction of disease(284). This resistance only occurs after active induction of disease by immunisation with encephalitogenic peptide - EAE induced passively by transfer of in vitro-activated encephalitogenic T cells does not result in resistance to further disease after recovery(290). The resistance or susceptibility of other strains of rats or mice has not been extensively characterised, although it is known that SJL mice are not resistant to rechallenge after recovery(291), and interestingly this strain has a relapsing-remitting disease course(178).

B6 mice were resistant to reinduction after primary active immunisation with MOG(35-55) (Figure 3-1). Interestingly, this resistance was not very long-lived, and by 7 weeks after recovery (11 weeks after primary immunisation), mice were once again fully susceptible to induction of EAE. This is in contrast to findings in the Lewis rat model, where lifelong resistance develops(284). Whether mice were reinduced 2 weeks or 7 weeks post-recovery the average day of onset of clinical signs was 1 to 2 days earlier than control mice immunised for the first time, consistent with a memory response. In addition, transfer of CD4$^+$ cells from post-recovery mice into naïve mice followed by immunisation with MOG(35-55) resulted in activation of these cells and accumulation of Ly5.1$^+$ Th1 effectors in the CNS of
recipients, contributing to a slightly enhanced severity of disease. These results suggest an active mechanism(s) of regulation that comes into force during recovery and continues to protect for several weeks.

The role of B cells in EAE pathogenesis and recovery is controversial. B cell deficient mice on the B10.PL background fail to recover from EAE induced by the MBP peptide Ac(1-11) (273). We have corroborated this result in the B6 model of EAE (59, 292). Our results in B cell IL-10−/− chimaeras additionally showed B cell IL-10-production to be very important for recovery from EAE (59). In contrast, Cross et al have described a role for B cells in the pathogenesis of EAE in B6 mice (189, 292). They found that μMT mice on the B6 background were resistant to EAE induction by immunisation with whole MOG protein, but not MOG(35-55), despite apparent activation of MOG-reactive Th1 cells (292). The resistance to MOG-induced EAE could be overcome by transfer of B cells from MOG-immunised mice or serum from MOG-immunised but not MOG(35-55)-immunised wildtype mice (189), and this was attributed to pathogenic effects of CNS-specific antibody in the CNS. However, these studies used recombinant human MOG protein for EAE induction, and when we immunised with recombinant mouse MOG full EAE could be induced in either B cell sufficient or deficient mice (59). Oliver et al have described similar discrepancies in a study directly comparing the ability of human and rodent MOG to induce EAE (293).

In this chapter, the presence of MOG-specific serum antibody as well as the ability of transfers of day 30 EAE B cells or serum from recovered mice to protect in the B6 MOG(35-55)-induced EAE model have been investigated. B cells from recovered mice were protective when transferred 1 day prior to immunisation, suggesting a role for B cells in resistance to reinduction. In the published study from our lab it was found that transfer of B cells from wildtype mice on day 14 of EAE into B cell IL-10−/− chimaeras was not protective (but did allow the mice to recover) (59), suggesting preferential expansion of "regulatory" B cells during the recovery phase. The only caveat to this finding is the fact that the method used for B cell purification was the
commonly-used negative selection based on lack of expression of CD43 on B cells. However, CD43 is upregulated on activation of B cells (294), and it could be proposed that selecting B cells in this way at the peak of disease may have in fact removed the very cells which would have given protection – activated MOG-reactive B cells – while by day 30 these cells are once again in a resting state and have downregulated CD43.

Transfers of whole splenocytes from recovered mice were also done, but these gave very variable results, with a decrease in disease severity observed in half the experiments but an increase in disease severity observed in the other half. Therefore, unlike reported results in rats (that used a mixture of purified CD4+ and B cells) (257), in B6 mice B cells were most consistently able to protect when transferred without T cells. This is most likely due to the transfer of pathogenic T cells concomitant with T cells “helpful” to B cells (these may in fact be the same cells) when transferring whole cell populations. It seems unlikely that naïve B cells would offer any protection from EAE upon transfer, given that transferred cells only make up around 1% of total B cells in recipients (see Figure 4-2). However, without having done this control it cannot be ruled out. Transfer of CD4+CD25+ cells from naïve mice has been shown to give some protection from EAE induction (49), but there are important differences: the frequency of endogenous CD4+CD25+ cells present in naïve recipients is only 5-15% of total CD4+ cells, and the numbers required to give protection (2x10^6 cells) would lead to an approximate doubling of the total number of CD4+CD25+ cells in the recipient. Also, CD4+CD25+ cells are thought to be positively selected on high affinity for self-antigen (9), while B cells would be deleted under these conditions (21), therefore the proportion of self-Ag reactive cells would be expected to be far lower in the transferred cohort of naïve B cells than CD4+CD25+ cells.

The mechanism of B cell-mediated resistance to induction of EAE is unclear. This lab has shown B cell IL-10 to be important for recovery from EAE, and that B cells can be induced to produce IL-10 in vitro by stimulation with MOG(35-55) in the presence of anti-CD40 (59). Suppression of T cell and APC activation by IL-10 is a
well characterised function of certain types of regulatory T cell (55). Since memory CD4⁺ cells with pathogenic potential appear to be present in the spleens of recovered mice (Figures 3-2 and 3-3), it was intriguing to speculate that B cells may be producing IL-10 to suppress T cell cytokine production and proliferation in the in vitro assays performed on day 30 of EAE. However, while an increase in Th1 cytokines was initially observed when day 30 EAE B cells were replaced with naïve B cells (Figure 3-9), the effect was small and inconsistent. This may not be a sensitive enough method for measuring effects of IL-10 production by B cells on T cells, since in an in vitro assay most B cells are likely to present peptide, while only a relatively small proportion of those B cells may be producing IL-10. In vivo, this small-scale production would be locally targeted to T cells recognising peptide:MHC on MOG-specific B cells, so the effects would be greater. MOG(35-55)-specific Ab in sera of recovered mice suggests that a proportion of post-recovery B cells that were transferred into naïve recipients before EAE induction would have BCRs specific for MOG(35-55), and may therefore present this peptide early after immunisation, without the requirement for release of MOG from the CNS during inflammation.

An alternative role for IL-10 is on the B cells themselves: while IL-10 has mostly downregulatory effects on immune cells, it is in fact an important growth factor for B cells, with particular functions in promoting Ab production (233). A protective role for serum antibody has been described in the Lewis rat model of EAE induced by MBP (289). In this model, serum from recovered rats suppressed disease in recipients when transferred either before or early after immunisation with MBP. Also, the severity of disease in non-transferred rats after immunisation with MBP was found to inversely correlate with levels of serum MBP-specific antibody. In a further study, a panel of antibodies with specificities for different MBP epitopes was found to have effects varying from highly pathogenic, to no effect, to protective, after transfer (188). Very early studies in this rat model also suggested that transfer of B cells from recovered rats could protect recipients - at the time limited sorting techniques were available, so the protective fraction of cells was shown to be nylon...
wool adherent cells, of which 80% were Ig\(^+\) - and later it was found that co-transfer of both MBP-specific B cells and T cells from recovered rats was necessary for protection (257, 288). However it must be said that this group mainly favours expansion of a TGFβ-producing suppressor T cell as the mechanism of resistance to reinduction (254).

The role of MOG-specific antibodies in EAE and MS is still unclear. It has been reported that many MS patients have MOG-specific antibody in their serum (162), and plasma exchange can have a beneficial effect on disease in some patients (286). Anti-MOG Ab has also been found in the lesions of active MS (186).

Anti-MOG Ab are likely to contribute to pathology by opsonisation of myelin, leading to complement deposition and accelerated clearance of myelin debris by macrophages (187). A study in marmosets showed that antibodies recognising linear epitopes from MOG were essentially non-pathogenic, presumably due to inability to effectively bind native protein in the CNS, while antibodies recognising conformational (discontinuous) epitopes were able to initiate demyelination in the CNS (295). Interestingly, mice on the H-2b background appear unable to generate antibodies against discontinuous epitopes of MOG, only producing antibodies against linear epitopes (296). This finding could explain the differences in requirements for B cells for induction of EAE observed when B6 mice are immunised with MOG from humans or rodents (hMOG or rMOG) (293). After immunisation with rMOG, pathogenic antibodies cannot be produced, so disease is mainly T cell-dependent and induction of EAE is not affected by the absence of B cells (59). However, immunisation with hMOG stimulates production of cross-reactive antibodies specific for discontinuous epitopes of MOG (i.e.pathogenic), while the T cell response is reduced due to a single amino acid difference in the T cell epitope between mMUG and hMOG (187, 293, 297). Therefore, when immunising with hMOG, disease is highly B cell dependent, and can only be induced in B cell deficient mice by co-transfer of serum containing antibodies generated by immunisation with hMOG (187).
In the experiments described in this chapter, serum from recovered mice was able to transfer some resistance to EAE induction, although these studies were not extensive. It was interesting that specificity of MOG-binding antibody in recovered mice appeared to be completely directed against the MOG(35-55) epitope. Due to differences in the way BCRs and TCRs recognise antigen – BCRs tend to recognise epitopes formed by tertiary folding of the protein, while TCRs bind small peptides (primary structure) - generated by processing of protein and presented on MHC molecules by APC, it is generally thought that for most proteins the dominant BCR and TCR epitopes will be different, and that BCR will not recognise peptide epitopes. However, as has already been discussed, antibodies with specificity for linear peptide epitopes from CNS antigens are commonly observed in both EAE and MS (162, 187), and it has been reported that the dominant B cell epitope within MOG does in fact span MOG(35-55) (298).

The location of Ab epitopes in relation to TCR epitopes within a protein can have important consequences for subsequent antigen presentation whether by B cells after BCR-mediated uptake or APC taking up opsonised Ag. Both enhancement and inhibition of presentation of epitopes close to or within the BCR binding domain has been observed, and this appears to be highly dependant on the individual epitope (299). Therefore, it could be that transfer of serum containing MOG(35-55)-specific antibodies prevents activation of T cells after immunisation with MOG(35-55) by binding to the peptide and blocking its presentation. It may also be possible that MOG(35-55)-specific B cells contribute to reduced activation of pathogenic T cells by binding and internalising MOG(35-55) without subsequent presentation on MHCII.

Around half the mice tested had MOG-specific Ab in their sera on day 30 after immunisation, and very few had MOG-specific Ab at the peak of disease (Table 3-2). It cannot therefore be said that serum antibody as detected by ELISA correlated with recovery, since there were no observable differences in disease severity or remission rates in mice with detectable antibody compared to those without serum antibody.
(data not shown). However, the most conclusive way to rule out any role for antibody would be to use mice in which B cells are unable to secrete antibody but do have membrane immunoglobulin (BCR), so can internalise and present antigen specifically. The effects on disease course of transfer of post-recovery serum into μMT mice with EAE would also be informative. However, additional effects of serum cytokines or other factors when transferring serum from recovered mice cannot be ruled out.

In conclusion, this chapter has shown that B6 mice are resistant to rechallenge with MOG(35-55) shortly after recovery from a primary bout of EAE induced by immunisation with MOG(35-55). This resistance to rechallenge can be mimicked in naïve mice by transfer of B cells from mice that have recently recovered from EAE, but not total CD4+ cells from these recovered mice. Approximately half of recovered mice had detectable levels of anti-MOG antibody in their serum, and serum from recovered mice also had some protective effect when given around the time of EAE induction. However, these studies are inconclusive in terms of defining whether B cells regulate EAE through production of IL-10 during B:T cell interactions, or through effects of antibody binding to MOG(35-55). More refined methods are necessary for elucidating the requirements for B cell antigen presentation and direct interactions with MOG-reactive T cells during recovery from/resistance to EAE, such as use of MOG-specific BCR transgenic mice in conjunction with MOG-reactive TCR transgenics, or use of antigen targeted to B cells in vivo.
**Figure 3-1**: Mice are resistant to reinduction of EAE early after recovery.

Closed symbols: EAE was induced by immunisation with MOG(35-55) in CFA and PTx on day 0. Mice were rechallenged with MOG(35-55) on day 43 (A - 10 mice) or 74 (B - 4 mice) after first immunisation. Open symbols: As controls, 5 mice were immunised for the first time with MOG(35-55) and PTx on the same day as the reinductions. Disease burden in mice reinduced on day 43 (A) was significantly lower than controls (p<0.05), while disease burden in mice reinduced on day 74 (B) was not statistically different from controls. A is representative of 3 separate experiments in which mice were reinduced within 2 weeks post-recovery; B is representative of 2 separate experiments in which mice were reinduced 6-7 weeks post-recovery.
Fig 3-2: Transfer of post-recovery CD4\(^+\) cells exacerbates disease. CD4\(^+\) cells were purified from pooled spleens and inguinal LN of mice recovered from EAE (day 30 after immunisation). 3x10\(^6\) CD4\(^+\) cells were transferred and EAE was induced in recipients (5 mice) one day later. 5 mice which did not receive a transfer were also immunised as controls. In this experiment (representative of 3 similar experiments), disease severity was not significantly greater than controls (P<0.0594).
Figure 3-3: Tracking transferred CD4+ cells at the peak of disease. A/B: CD4+ cells were purified from spleens of Ly5.1+ B6 congenic mice on day 30 post-EAE induction. Cells were transferred into wt B6 mice (Ly5.2+) and EAE was induced 1 day later. At the peak of disease (day 15), inguinal LN, spleens and spinal cords harvested after perfusion with PBS, single-cell suspensions were stimulated with PMA/ionomycin, and stained for FACS(ICC). FACS plots show percentage of CD4+ cells which were Ly5.1+ and IFNγ+, gated on CD4+ cells, and represent 2-3 mice/plot. C: 2x10^5 CD4+CD25+ Ly5.1+ post-EAE or naïve cells were transferred one day prior to EAE induction. The percentage of CD4+ cells which are Ly5.1+ in CNS on day 14 is shown for one mouse/FACS plot, representative of 2 mice/group.
Table 3-1a Ly5.1* cells as % of total CD4+ cells

<table>
<thead>
<tr>
<th>Day post-immunisation</th>
<th>LN</th>
<th>spleen</th>
<th>CNS</th>
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<tbody>
<tr>
<td>13</td>
<td>1.2</td>
<td>2.5</td>
<td>30</td>
</tr>
<tr>
<td>14</td>
<td>1.7</td>
<td>3</td>
<td>22.8</td>
</tr>
<tr>
<td>15</td>
<td>3.2</td>
<td>5</td>
<td>31</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>2.03 ± 1.04</td>
<td>3.5 ± 1.32</td>
<td>27.9 ± 4.47</td>
</tr>
</tbody>
</table>

Table 3-1b: IFNγ+ cells as % of total Ly5.1*CD4+ cells

<table>
<thead>
<tr>
<th>Day post-immunisation</th>
<th>LN</th>
<th>spleen</th>
<th>CNS</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>11.5</td>
<td>42</td>
<td>18</td>
</tr>
<tr>
<td>14</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>15</td>
<td>5</td>
<td>34</td>
<td>39</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>8.25 ± 4.6</td>
<td>38 ± 5.66</td>
<td>28.5 ± 14.85</td>
</tr>
</tbody>
</table>

Table 3-1: CD4* cells were purified from Ly5.1* mice 30 days after EAE induction and transferred into naïve Ly5.2* recipients. One day later, EAE was induced and at the peak of disease (days 13-15), inguinal LN, spleen, and CNS were harvested, cells were stimulated with PMA/ionomycin, and the total percentage of Ly5.1* cells (1a) and proportion of Ly5.1* cells producing IFNγ (1b) analysed by ICCS (1a). Results from 3 separate experiments are shown.
Figure 3-4: Post-recovery EAE B cells can transfer resistance to induction of EAE, more consistently than whole splenocytes. B cells were purified by removal of CD43+ cells from spleens of day 30 recovered EAE mice and transferred (3x10^6 cells/mouse) one day prior to induction of EAE. Control mice did not receive any cells prior to EAE induction. The data shown are for 5 mice/group and representative of 4 similar experiments. In this experiment, B cells gave significant protection to recipients (p<0.041).
Figure 3-5: Transfer of post-recovery splenocytes gives inconsistent effects on EAE induction. Single cell suspensions were obtained from spleens of mice on day 30 post-EAE induction, 3-5x10^6 cells transferred, and EAE induced 1 day later. These results are representative of a total of 4 experiments. The difference between group scores in A was not significant (p<0.22). In B the whole cell transfer gave significantly increased disease (p<0.01).
Figure 3-6: Attempts to identify MOG-specific B cells ex-vivo by FACS
Spleens and inguinal LN were harvested from naïve mice or mice on day 28 post-EAE induction. Single cell suspensions were made and cells stained with anti-B220PE, and rMOG-biotin at the dilution shown followed by streptavidin-APC. A shows two separate experiments testing different concentrations of rMOG-biotin in B cells were stained with B220PE and rMOG-biotin followed by streptavidin-APC, and additionally stained with F4-80-PcP and propidium iodide: all FL3⁺ cells (macrophages and dead cells) were excluded from the analysis.
Figure 3-7: Serum antibody responses and specificity to rMOG and MOG(35-55)

A: Pooled serum from mice on day 30 of EAE (10 mice) or naïve mice (2 mice) was incubated on ELISA plates coated with rMOG with the indicated concentration of soluble MOG(35-55) peptide, or OVA(323-339) as control. Binding of MOG-specific Ab was then detected in the usual way. B: As for A, except only sera from EAE mice was used, and an additional test group was added, in which MOG(35-50) was added at the time of serum incubation. C: Individual mouse sera from day 30 of one EAE experiment, showing effects on binding to MOG of adding PBS or MOG(35-55) to serum during incubation on the plate.
Table 3-2: numbers of mice with MOG-specific IgG1 and IgG2a on day 30 and day 14 post-EAE induction. Serum was harvested from mice on days 14 or 30 after induction of EAE and tested for presence of rMOG-specific antibody by ELISA. Shown is the number of individual mice testing positive for Ab out of total mice tested. Not shown: Sera from all naive mice tested was negative for MOG-specific Ab.

<table>
<thead>
<tr>
<th></th>
<th>IgG1</th>
<th>IgG2a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 30 EAE sera</td>
<td>28/49</td>
<td>12/44</td>
</tr>
<tr>
<td>Day 14 EAE sera</td>
<td>2/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>
Figure 3-8: Effects of serum transfer on recipient EAE disease course A: Sera were pooled from mice at day 30 (recovered) or day 14 (peak disease) of EAE. 150μl of sera were injected iv into mice on days -1, +1, 4 and 6, with EAE induced on day 0. Significant difference between control and recipients of day 30 sera (P<0.0135). No significant difference between controls and recipients of day 14 sera. B: Day 30 sera which had tested positive for antibodies against rMOG were pooled, as were negative sera. 150μl sera was injected iv on days -1, +1 and 3, with EAE induced on day 0. There was a significant difference between controls and recipients of negative sera (P<0.0391), but no significant differences between controls and positive sera recipients, or recipients of positive versus negative sera.
Figure 3-9: Do post-recovery EAE B cells inhibit MOG(35-55) CD4+ cell responses in vitro? A: 10^5 day post-EAE (day 30) CD4+ cells were incubated with 4x10^5 irradiated CD4-depleted splenocytes (APC) or B cells, both from the same day 30 mice ("EAE") or from naïve mice in the presence of 10μM MOG(35-55) (filled bars), or medium only (open bars). Cytokines were measured in duplicate by ELISA. B: 10^5 post-EAE CD4+ T cells were cultured with 5x10^3 CD11c+ splenic DCs from naïve mice and the indicated number of post-EAE B cells (not irradiated) per well in the presence of 10μM MOG(35-55) (filled bars), or medium only (open bars). Cytokines were measured by ELISA from duplicate wells.
Figure 3-10: Testing of HEL-MOG(35-55) conjugates. MD4 (closed symbols) and B6 (open symbols) B cells were purified by MACS negative selection using anti—CD43 beads (>97% purity) and 2x10^5 B cells cultured with 3x10^4 MOG(35-55)-reactive T cells in 96 well plates for 72 hours with the indicated concentration of conjugate. Tritiated thymidine was added for the last 18 hours of culture. Conjugates A-E were made with decreasing concentrations of MOG(35-55), starting with the highest in A down to the lowest in E (see materials and methods). As controls for non-specific presentation of peptide, cells were incubated with a fixed concentration of HEL (1μM) and a dose range of MOG(35-55). Controls not shown: B cells alone with conjugate, no antigen controls – in each case proliferation was not above background.
4. Phenotyping of IL-10 producing cells in the CNS during Recovery Reveals Regulatory T cells, not B cells

4.1. Introduction

As described in chapter 3, B cells appear to be playing a role in the active resistance to reinduction of EAE after recovery, and it is already known that B cells are normally required for recovery from EAE (273). IL-10 is vital for recovery from EAE, since mice which are unable to produce IL-10 fail to control disease (231). Also, levels of IL-10 mRNA increase in the CNS during recovery from EAE in rats (236). In vivo expression of IL-10 by transfected replication-deficient adenovirus prevents EAE induction in naïve mice, accelerated remission in mice with EAE and prevents relapses when given in a relapsing-remitting model of EAE (241). However, this exogenous production of IL-10 was only effective when targeted directly to the CNS and had no effect when the engineered virus was given systemically (241). Similarly, OVA-reactive transgenic cells stimulated in vitro to become IL-10-producing regulatory T cells could prevent induction of EAE, but only when ovalbumin was injected into the brain, presumably to localise Tregs (and therefore IL-10 production) to the CNS (62). Given these reports, and the finding that B cell IL-10 is important for recovery from EAE (59), the obvious next step was to investigate the location of B cells after transfer, and analyse which cell populations produce IL-10 in the CNS during the normal recovery from EAE.
4.2. RESULTS

4.2.1. Few B cells are present in CNS at any point during normal recovery from EAE

In order for B cells to directly downregulate CNS inflammation via IL-10 they would have to migrate to the CNS. Therefore, at different time points during EAE lymphoid cells in the CNS were stained for the presence of B cells, using Ab against two separate cell-surface markers: B220 and CD19. Very few B cells were observed in the CNS at any time point (Figure 4-1), with the proportion of total CNS mononuclear cells staining positive for CD19 or B220 never rising above 10%, and frequently falling below 1% (Figure 4-1b). The proportion of B cells observed in the CNS varied in a non-time dependent manner, i.e. there was no apparent increase in B cell frequency as mice recovered (Figure 4-1b). For B220+ cells, many of the cells appeared to express this marker at low levels compared to B220+ cells in the spleen or LN (Figure 4-1a). This was suggestive of the recently described plasmacytoid DC (300), and in support of this hypothesis, a high proportion of these B220lo cells were found to co-express CD11c and CD8 (Figure 4-1c). Thus, very few B cells were present in the CNS.

4.2.2. Post-recovery B cells do not accumulate in the CNS of transfer recipients after EAE induction

As described in chapter 3, transfer of post-recovery B cells results in some resistance to induction of EAE in recipients. To determine whether post-recovery B cells mediate these effects in the LN/spleen or in the CNS, Ly5.1+ B6 congenic B cells from recovered mice were transferred into B6 recipients (Ly5.2+) and EAE was induced. 14 days after immunisation the inguinal LN, spleen and CNS of these mice were examined by FACS for the presence of Ly5.1+ B cells. In contrast to transfer of post-recovery CD4+ cells, transferred post-recovery B cells were mainly found in lymphoid sites, and were not present above background levels in the CNS (Figure 4-2). There were no obvious increases in the proportion of Ly5.1+ B cells in LN or
CNS of mice receiving post-recovery compared to naïve B cells (Figure 4-2). However, as discussed in chapter 3, the lack of markers for MOG-specific B cells within the polyclonal Ly5.1+ population makes measurement of in vivo expansion in LN difficult.

4.2.3. IL-10 production can be detected in cells from the CNS of recovering mice

Since IL-10 mRNA is known to increase in the CNS during recovery (236), the production of IL-10 protein by mononuclear cells in the CNS was investigated by FACS (Figure 4-3). IL-10-producing cells were detected in the CNS, constituting up to 5% of total mononuclear cells in different experiments. The few B cells present in the CNS did not produce significant levels of IL-10. Likewise, CD11c+ dendritic cells did not appear be major contributors to IL-10 production in this assay. Interestingly, over half of the IL-10-producing cells were CD4+, and this was confirmed in further experiments. Some of the IL-10 produced appeared to be from CD8+ cells, although this has not been confirmed.

The CNS was greatly enriched for cytokine-producing CD4+ cells compared to LN – 25-50% of CD4+ cells produced IFNγ in the CNS in comparison to less than 5% of CD4+ cells in LN, and negligible IL-10 was detected in LN at any time (see Figure 4-7c) - fitting with accumulation of effector cells in the CNS. Although the proportion of CD4+ cells in the CNS producing IL-10 ex vivo was small in comparison to those producing IFNγ (25-50% IFNγ+ compared to 2-13% IL-10+), when comparing several experiments in which IL-10 production was measured, there was an overall increase in proportion of IL-10+ CD4+ cells over time, correlating with recovery (Figure 4-4a/b). It was interesting to note that the proportion of CD4+ cells producing IFNγ did not appear to decrease as mice recovered (Figure 4-4c), although stimulation with PMA and ionomycin only indicates cytokine-producing potential, not whether the cytokines detected were actually being produced in vivo.
4.2.4. Analysis of production of IFNγ and IL-4 by IL-10 producing CD4+ cells in CNS of mice with EAE.

Production of IL-10 is characteristic of some Treg cells. However, both Th1 and Th2 cells can also produce IL-10 in conjunction with IFNγ or IL-4, respectively (233). Ex-vivo production of IL-10 by CD4+ cells from the CNS of mice with EAE was therefore analysed in conjunction with IFNγ and IL-4. A large proportion of CD4+ cells produced IFNγ (this typically ranged from 25 to 50% in different experiments), and some of these cells were double-positive for IL-10 (Fig 4-5a). The proportion of IFNγ+ cells also staining positive for IL-10 production varied between experiments. However, there was always a population of CD4+ cells that exclusively produced IL-10. IL-4 production was detected at low levels in a small proportion of CNS CD4+ cells (Fig 4-5b). Double staining for IL-10 and IL-4 production in the same cell was not possible at the time due to the antibodies having the same fluorescent dyes, but the percentage of IL-4+ cells was lower than the percentage of IL-10+ cells, suggesting that at least half, if not all, of the IL-10 producers did not produce IL-4.

4.2.5. Expression of CD25 by IL-10- and IFNγ-producing cells in the CNS suggests two distinct populations

Production of IL-10 and IFNγ by separate cell populations suggested the presence of both Tregs and pathogenic T cells. CD25 is constitutively expressed by some Treg populations (45), therefore expression of CD25 by cytokine-producing CD4+ cells in the CNS was examined. There was a clear contrast: IL-10+ cells were CD25+, while IFNγ+ cells were CD25- (Figure 4-6a). Cells positive for both IL-10 and IFNγ tended to show a mixed phenotype, with at least half being CD25- while some expressed CD25 at intermediate or high levels (Figure 4-6b). IL-2-producing cells were also negative for expression of CD25 (Figure 4-6c). This result supports the hypothesis that IFNγ-producing cells are pathogenic Th1 effectors, while IL-10-producing cells may have a regulatory function.
4.2.6. The number of CD4⁺CD25⁺ cells in the CNS increases during EAE, correlating with recovery

Tregs have been shown to expand/accumulate in draining lymph nodes after immunisation or in response to tissue inflammation (145), as well as at the site of inflammation (123). Since the proportion of CD4⁺IL-10⁺ cells was observed to increase in the CNS during recovery, and these cells expressed CD25, the proportion and number of total CD4⁺CD25⁺ cells in the inguinal LN, spleen and CNS at different time points during EAE were analysed. In the LN and spleen, the percentage of CD4⁺ cells that expressed CD25 remained fairly constant throughout the disease course, with no marked increases in numbers of CD25⁺ cells (Figure 4-7a). However, there was a marked increase in the percentage of CD4⁺ cells expressing CD25 in the CNS as EAE progressed from days 13 through to day 20 (Figure 4-7a). The total number of CD4⁺CD25⁺ cells also increased over time (Figure 4-7b), although the cell counts from the CNS are variable between preparations and therefore cell numbers are really estimates. This increase in CD4⁺CD25⁺ cells correlated with progression of the recovery phase of disease (the peak of disease is usually reached around day 14). Approximately one third of CNS CD4⁺CD25⁺ cells consistently produced IL-10 after ex vivo stimulation, while the proportion of LN CD4⁺CD25⁺ cells that produced IL-10 after ex vivo stimulation was low (Figure 4-7c).

4.2.7. CD4⁺CD25⁺ cells in the CNS phenotypically resemble naturally-occurring CD4⁺CD25⁺ cells

Although no unique cell surface marker has yet been described for Tregs, the so-called naturally-occurring CD4⁺CD25⁺ cells are known to constitutively express several T cell activation markers in addition to CD25 (94). The CD4⁺CD25⁺ and CD4⁺CD25⁻ populations in LN and CNS were compared for their expression of GITR, CTLA-4, CD44, CD69 and αEβ7 (Figure 4-8). CD4⁺CD25⁺ cells in the CNS displayed a similar phenotypic profile to CD4⁺CD25⁺ cells in the LN of both naïve mice and those with EAE: they were GITR⁺, CTLA-4⁺ and CD44⁺. In contrast, CD4⁺CD25⁻ CNS cells expressed GITR and CTLA-4 at low levels, albeit slightly
higher than LN CD4^+CD25^- cells. The majority of CD4^+ cells in the CNS were CD44^hi, confirming the presence of mainly antigen-experienced/activated cells at the site of inflammation. The proportion of both CD25^+ and CD25^- cells expressing CD69 was also greater in the CNS than LN, suggesting recent activation within the CNS, and indeed CD69^+ cells appeared to be the major cytokine producers ex-vivo (data not shown). Expression of the integrin αβ7 has recently been found to correlate with a more potently suppressive subpopulation of CD4^+CD25^- cells(301, 302) that preferentially migrates to sites of inflammation(301). It was therefore interesting to note that CD4^+CD25^- cells in the CNS of mice with EAE were enriched for αβ7-expression.

The levels of expression of CTLA-4 and GITR were higher on CD4^+ cells (both CD25^+ and CD25^-) from the CNS compared to LN of naïve mice or those with EAE (Figure 4-8), and this was particularly evident on CD25^- cells, again suggesting a high activation status of cells in the CNS. It is interesting to note that naïve and EAE LN were phenotypically similar apart from a slight increase in the proportion of CD25^- cells which were CD44^- and CD69^-hi, and an increased proportion of CD25^- cells which were CD69^-hi, correlating with activation of only a proportion of cells in the draining LN of immunised mice, while CNS cells appear highly enriched for activated cells. In summary, CNS CD4^+CD25^- Tregs, and appear to be highly activated compared to LN cells.

4.2.8. FoxP3 expression

CD4^+CD25^- cells express the transcription factor FoxP3, and this appears to be important for their function (99, 100). In mice this expression appears to be limited to regulatory cells (although not exclusively to CD25^- regulatory cells) and has not so far been found to be upregulated significantly during activation of naïve T cells. It was not possible to obtain mRNA in sufficient quantities from the small numbers of CD4^+CD25^- cells purified from the CNS in these studies, so rtPCR was performed using whole CD4^+ enriched cells. CD4^+ cells from both inguinal LN and CNS were positive for FoxP3 expression (Figure 4-9), indicating that at least some of the cells
in the CNS are likely to be FoxP3-expressing regulatory T cells. One of the major limitations of the rtPCR approach is the failure to identify what proportion of a cell population express the gene in question, and the production of mAb to identify FoxP3 by FACS, and also mice that have GFP expression linked to FoxP3 expression, should be major steps forward in analysing the homogeneity of CD25+ Treg populations.

4.2.9. Ag-specificity of CD25+ cells: in vitro proliferative responses and ex vivo analysis of cytokine production in response to MOG(35-55)

The specificity of CD4+CD25+ regulatory T cells involved in preventing autoimmunity is a subject of great debate, and in most cases has not yet been defined unless TCR transgenic systems are used. The best approach to identify MOG(35-55)-specific CD4+ cells would be to use class II Aβ-MOG(35-55) tetramers or liposomes to label individual cells for FACS, but these tools were not yet available for use in this system. Therefore, two alternative approaches were attempted: measurement of proliferation and cytokine production by CD4+CD25+ cells in response to MOG(35-55).

i) proliferation assays

It is now well established that CD4+CD25+ Tregs are anergic in vitro, in that they fail to proliferate to antigenic stimulus in the absence of exogenous IL-2. CD4+CD25+ cells purified from the LN and CNS of mice with EAE were therefore cultured with APC and MOG(35-55) in the presence or absence of exogenous IL-2, or with IL-2 alone. As a further control, cells were cultured with anti-CD3 with or without IL-2. The results in the presence of anti-CD3 confirmed that CD4+CD25+ cells in the CNS were anergic – they only responded when exogenous IL-2 was added (Fig4-10a).

The results of LN cells in the presence of MOG(35-55) were less clear-cut: CD4+CD25+ cells failed to proliferate when peptide alone was added, but did
proliferate when exogenous IL-2 was present (fig 4-10b/c). However, the proliferation of CD4^+CD25^+ cells in response to MOG(35-55) in the presence of IL-2 was similar to responses to IL-2 alone in some of the assays performed. This high background of proliferation in the presence of IL-2 without antigen may make any additional responses to MOG(35-55) insignificant. Due to the small numbers of cells obtained from the CNS, these assays also suffered from low proliferation counts. Therefore it was not possible to conclusively determine the level of antigen-specific responses by this method, although the presence of MOG(35-55)-reactive CD4^+CD25^+ T cells in the LN is suggested.

ii) cytokine production ex vivo

As described, ex vivo stimulation with the non-specific stimulants PMA and ionomycin was used to determine cytokine-producing potential of CD4^+ cells in the CNS. This method was refined to determine the proportion of cells which produce cytokine in response to MOG(35-55) ex vivo. Since IL-10 production by Tregs has not been described to require addition of IL-2, these assays were performed by culturing whole CNS or LN cells with or without MOG(35-55). The addition of splenocytes as APCs was found to have no affect on CNS cell cytokine production so was not included.

IFNγ production in response to MOG(35-55) was readily detectable by FACS and significantly higher than controls cultured for the same length of time without antigen. (Figure 4-11a). The proportion of cells producing IFNγ was 3-fold lower after stimulation with MOG(35-55) than with PMA and ionomycin. It is difficult to compare these stimuli directly, but it does appear that MOG(35-55)-reactive cells may only make up a proportion of the Th1 cells in the CNS.

IL-10 production by CD4^+ CNS cells was not above background in response to MOG(35-55), although there was a suggestion of increased IL-10 in some mice (Fig 4-11b). In the experiments in which this assay was tested the IL-10 response to PMA
and ionomycin was quite low, and it may have been more fruitful to test IL-10 in response to MOG(35-55) at later time points when IL-10 production increases (i.e. day 20 rather than day 16).

4.3. DISCUSSION

Investigating the location of B cells during normal recovery from EAE and after transfer of post-recovery B cells was the first step to identify the location of action of B cells. It was apparent that B cells are not major contributors to the cellular infiltrate into the CNS at any point during EAE in B6 mice (Figure 4-1), never being observed at greater than 10% of total mononuclear cells (and as already described, many of the B220+ cells observed are likely to be DCs not B cells). Downregulation of B cell surface markers by B cells in the CNS is unlikely to explain the lack of B cells, since two separate surface markers were used to identify them (CD19 and B220). After transfer, post-EAE Ly5.1+ B cells localised to the LN and spleen, but did not accumulate in the CNS at either the peak of disease or during recovery (Figure 4-2), further confirming that B cell entry into the CNS does not occur to a great extent in this model. IL-10 production was not observed from the few B cells that were present in the CNS. However, lack of IL-10 production by transferred post-recovery B cells under the same stimulation conditions (data not shown) raises the possibility that the conditions used were not sensitive enough for detecting IL-10 production by B cells.

Taken together, these results suggest that B cells principally act in the draining LN and/or spleen, but not CNS, to contribute to recovery from EAE induced with MOG(35-55) in B6 mice. During T cell activation, a proportion of T cells enter B cell follicles where they may engage with B cells expressing their cognate Ag. These interactions promote the maturation of the B cell response through formation of germinal centres and class switching in response to cytokines(69). At this stage, B cells may also influence the interacting T cells through expression of costimulatory molecules and production of cytokines. It is likely therefore that B cell IL-10 may act in a highly localised manner to downregulate specific T cells. It is also possible that
B cell IL-10 has a more indirect effect through non-specific down-regulation of APC within the lymphoid organ. As discussed already, IL-10 is an important growth factor for B cells(233), therefore effects of IL-10 on B cells themselves to promote some other activity of B cells (such as Ab production or expression of costimulatory molecules) cannot be ruled out as yet.

As an aside, it was interesting to note the presence of cells with the phenotype of plasmacytoid dendritic cells (pDCs), i.e. cells expressing low levels of B220, CD8 and CD11c. This subpopulation of DCs has recently been under intense investigation, and similar to other DC subsets may have roles in Th1/Th2 polarisation, in type-1 IFN related inflammatory diseases in humans, and in regulatory T cell induction(303-306).

IL-10 mRNA transcripts increase in the CNS during recovery(236), and targeting exogenous production of IL-10 to the CNS can prevent or ameliorate EAE(241). The lack of B cells in the CNS therefore suggested that another cell population(s) may promote recovery via IL-10 production in the CNS. Further investigation revealed CD4$^+$ cells to be a major IL-10-producing population, and the proportion of IL-10 producing cells appeared to be generally higher during the later recovery phase (day 19 on) than at the peak of disease, fitting with the reported increase in total IL-10 mRNA during recovery. These IL-10-producing CD4$^+$ cells expressed CD25$^+$, and other activation markers, and appeared to be a phenotypically distinct population from IFN$\gamma$-producing CD4$^+$ cells in the CNS (Figures 4-3 to 4-8).

It could be argued that the CD4$^+$CD25$^+$ cells in the CNS were simply the most recently activated effector cells, particularly since they also exhibited high expression of other activation markers including GITR and CTLA-4, in contrast to CD4$^+$CD25$^-$ cells which were low for these markers (Figure 4-8). However, the CD4$^+$CD25$^-$ cells were also CD44$^{hi}$ and a proportion were CD69$^{hi}$, indicating that CD4$^+$CD25$^-$ cells in the CNS are indeed antigen-experienced cells, and some have
recently seen antigen - probably in the CNS. A high proportion of CD4⁺CD25⁺ cells also produced IFNγ ex vivo, suggestive of Th1 effectors, while CD4⁺CD25⁺ cells were only observed to produce IL-10 (Figure 4-6). Therefore, the hypothesis that IL-10-producing CD4⁺ cells were regulatory T cells was supported by their phenotype.

While it is surprising that the IFNγ-producing cells are negative for expression of activation markers such as CD25, a similar dichotomy of expression of cell surface markers on effector and regulatory T cells has been reported in the synovial fluid from the joints of patients with JIA (214), and in the subcutaneous lesion site during Leishmania infection (52), both situations in which the initiating Ag are also still abundant. CD25 upregulation by effector cells in the CNS has been reported by Flugel et al, using GFP-transfected MBP-specific cell lines in rats (307) and also by Bischoff et al, using tetramers to stain PLP(139-151)-specific T cells in SJL mice with EAE (286). However, in the cell line transfer studies, CD25 upregulation by MBP-specific cells was an early event after entry into the CNS, while in this project, CD25 expression in relation to cytokines was only investigated from day 13 on, while disease onset begins around day 8. Also, in the Bischoff study (286) no increase in the percentage of CD25⁺ PLP-specific cells in the CNS over the course of disease was observed, and cytokine production by CD25⁺ or CD25⁻ cells was not investigated. SJL mice develop a relapsing-remitting course of EAE, and do not become resistant to rechallenge after recovery (291). It has recently been reported that SJL mice may have an relative defect in the generation of PLP(139-151)-specific CD4⁺CD25⁺ cells(191). Therefore it appears that the mechanisms of recovery from EAE, in terms of regulation by CD4⁺CD25⁺ cells, may be different between SJL and B6 mice.

It was interesting to note that a proportion of CD8⁺ cells from the CNS also produced IL-10 ex vivo (Figure 4-3). IL-10 production is not thought to be a major mechanism of regulation by CD8⁺ cells. While CD8⁺ cells with a regulatory phenotype have been reported to protect from EAE (57), these cells were induced in vitro by stimulation in the presence of TGFβ. CD8⁺ cells that produce TGFβ and prevent
EAE have also been induced in rats during induction of oral tolerance (308). A recent report using B6 mice found that mice depleted of CD8\(^+\) cells prior to induction of EAE developed disease of increased severity, suggesting some form of regulation by CD8\(^+\) cells (265), although the mechanism was not investigated.

As described in Chapter 1, regulatory T cells can be broadly categorised into "naturally-occurring" CD4\(^-\)CD25\(^+\) cells which are generated in the thymus, and peripherally-induced regulatory cells. The phenotype of the CD4\(^-\)CD25\(^+\) cells in the CNS was most consistent with the thymically-generated CD4\(^-\)CD25\(^+\) Tregs (94). This implies that pre-existing CD4\(^-\)CD25\(^+\) Tregs were expanded by Ag presented either as a result of the initial immunisation or after damage to the CNS by DC migrating from the inflamed brain and spinal cord. Proliferation of CD4\(^-\)CD25\(^+\) cells in the inflamed gut has been reported in a model of colitis (105), so expansion of Tregs in the CNS itself cannot be ruled out. The increase in the proportion of CD4\(^-\) cells expressing CD25 during recovery could either be due to continued/preferential recruitment of Tregs over Teffectors to the CNS, or due to enhanced survival of Tregs compared to Teffectors. The increase in numbers of CD4\(^-\)CD25\(^+\) cells in the CNS, although not a reliable measure, suggests the former, but rates of recruitment or death in the CNS have not been investigated.

Initial reports of CD4\(^-\)CD25\(^+\) cells described them as classically anergic, i.e. failure to proliferate to antigenic stimulus in vitro in the absence of exogenous IL-2 (106, 112), and it was assumed that CD4\(^-\)CD25\(^+\) cells would similarly fail to expand to immunisation in vivo, as has been observed for tolerant T cells (285). However, several studies have now shown that CD4\(^-\)CD25\(^+\) cells do proliferate in response to immunisation with antigen in adjuvant or antigen-pulsed mature DCs, and also that antigen-specific Tregs accumulate in vivo in sites draining that antigen (145). Therefore, it is feasible, if not likely, that CD4\(^-\)CD25\(^+\) cells are expanded after the immunisation with self-Ag used to induce EAE, and/or in response to self-Ag presented on DC activated by CNS inflammation.
In a mouse model of diabetes induced by expression of inflammatory molecules on pancreatic β cells, a localised accumulation of CD4⁺CD25⁺ cells was found to occur in the pancreatic LN (122). Likewise, in a mouse model of IBD (in which colitis is induced by transfer of CD4⁺CD25⁻ cells and can be prevented by co-transfer of CD4⁺CD25⁺ cells) there is a localised accumulation of CD4⁺CD25⁺ cells in the MLN compared to other LN, as well as entry into the intestine itself (105). In this project, an accumulation of CD4⁺CD25⁺ cells was not observed in the inguinal LN or spleen as mice recovered from EAE.

The specificity of CD4⁺CD25⁺ cells in the CNS is an intriguing question. It is known that the major encephalitogenic epitope in MOG is MOG(35-55) (168), and since this is the peptide used for induction of EAE one might predict that most of the CD4⁺ cells would be specific for this epitope. However, it is known that during inflammation, "bystander" effector cells that are not specific for the tissue, can be recruited by the local inflammatory environment, although it is generally thought that Ag recognition is required in order for cells to remain and perform effector functions in the site (175, 309). Focussing on CD4⁺CD25⁺ cells, in several models expansion has been found to be localised to the LN draining the target organ or site of immunisation, i.e. CD4⁺CD25⁺ expansion appears to be Ag-specific, or at least driven by localised inflammation (77, 145, 148). Prevention of diabetes in NOD mice by transfer of CD4⁺CD25⁺ cells also appears to require a relatively high frequency of CD4⁺CD25⁺ cells with specificity for pancreatic islet Ag, since protection was provided by CD4⁺CD25⁺ cells with transgenic TCRs specific for pancreatic antigens, but not by cells with polyclonal TCRs (153, 154).

The attempts to determine Ag-specificity of CD4⁺CD25⁺ cells in this project were hampered by high background proliferation of cells in the presence of IL-2 alone (figure 4-10), and low frequencies of IL-10-producing cells (Figure 4-11) after ex vivo stimulation. Without more refined tools such as MOG(35-55)-A⁺ tetramers, it has so far been impossible to conclusively determine the proportion of CD4⁺CD25⁺ cells with specificity for MOG(35-55).
Taken together, the results of this chapter indicate that CD4\(^+\) cells but not B cells are able to produce IL-10 in the CNS during recovery from EAE. These CD4\(^+\)IL-10\(^+\) cells express CD25, in contrast to IFN\(\gamma\)\(^+\) cells, and further analysis of CD4\(^+\)CD25\(^+\) cells in the CNS revealed that they phenotypically resemble CD4\(^+\)CD25\(^+\) regulatory cells.
Figure 4-1: Few B cells are present in the CNS of B6 mice with EAE. Spinal cords and inguinal LN were removed from mice and cells stained for FACS analysis of expression of either B220 or CD19 as indicated. Fig A shows typical FACS staining of cells from mice on day 14 post-EAE induction (4 mice/plot). B: Combined results from several experiments in which the percentage of B cells in the CNS (4-6 mice pooled for each point) was measured on the day indicated after EAE induction. Mean % B cells on days 13/14 = 4.44% ± 0.64% (n=12) and on days 19-21 = 4.46% ± 1.04% (n=7), there was no statistical difference in % B cells between these time points. C: CD11c and CD8 expression by B220+ cells in CNS on day 20 post EAE induction.
Figure 4-2: Transferred post-recovery B cells do not accumulate in the CNS after EAE induction. B cells were purified by depletion of CD43⁺ cells from pooled spleen and inguinal LN of Ly5.1⁺ mice on day 30 post-EAE induction, and from spleens of naïve mice. 3x10⁶ B cells were transferred into B6 recipients and EAE was induced the following day. 14 days later inguinal LN and CNS were harvested and cells stained for FACS. For LN, each plot represents a single mouse and the mean percentage of Ly5.1⁺ B cells (± SD) for three mice is shown above each plot. For CNS, each plot represents 3 mice and staining for Ly5.1⁺ cells was not above background.
Figure 4-3: IL-10-producing cells can be detected in the CNS, and CD4+ cells appear to be major producers of IL-10. Spinal cords were removed from 4 mice 28 days after EAE induction and mononuclear cells were stimulated with PMA and ionomycin. Cells were stained for surface markers B220, CD11c, CD8 or CD4 followed by intracellular staining of IL-10.
Figure 4.4: The proportion of IL-10-producing, but not IFNγ-producing, CD4⁺ cells increases over the course of EAE. A: percentage of CD4⁺ cells positive for IL-10 after 4 hours ex vivo stimulation with PMA/ionomycin at peak (days 13-16) or recovery phase (19-28 days after EAE induction), from several experiments. B shows the same data (solid lines) overlaid with a representative EAE plot (dotted line). C: percentage of CD4⁺ cells positive for IFNγ in the same experiments.
Fig 4-5: Comparison of IL-10, IFNγ and IL-4 production by CD4+ cells in CNS mononuclear cells were isolated from 4 mice 28 days after EAE induction and stimulated with PMA/ionomycin. Cells were then stained for surface expression of CD4 and intracellular IL-10, IL-4 or IFNγ as indicated. Right-hand panels show isotype control staining for cytokine stains.
Figure 4-6: CD25 expression by cytokine-producing cells in the CNS Spinal cords were harvested from 4 mice 20 days after EAE induction and mononuclear cells were stimulated for 4 hours with PMA/ionomycin then stained for surface expression of CD25 and CD4, and intracellular IL-10, IFNγ and IL-2 as indicated. For typical isotype control staining see Fig 4-5.
Fig 4-7: The proportion of CD25⁺ cells in the CNS, but not LN or spleen, increases during recovery A/B: On days 12, 16 and 20 post-EAE induction, inguinal LN, spleen and CNS were harvested, cells stimulated with pma/ionomycin and stained for FACS (ICCS). A: The percentage of CD4⁺ cells expressing CD25 in these sites is overlaid with the mean EAE scores of the same mice. Results shown are pooled data from 2 representative experiments in which 4-5 mice/group were analysed at each timepoint. The percentage of CD25⁺ cells in CNS is significantly higher on day 20 than on day 13 (p<0.0096). B shows mean number of cells obtained from pooled spinal cords from 4 mice/day in the same 2 experiments. C shows typical IL-10 production profile by CD4⁺ cells in inguinal LN and CNS on day 20 post-immunisation.
Figure 4-8: CD4+CD25+ cells in CNS phenotypically resemble naturally-occurring Tregs. CNS and inguinal LN cells from 4 mice on day 20 post-EAE induction as well as LN from 3 naïve mice were stained for FACS. Plots shown are gating on CD4+ cells and thin line shows isotype control stain. MFI for GITR and CTLA-4 staining and percentage hi for CD44, CD69 and αEβ7 staining are shown in top right corner of histogram plots.
Figure 4-9: FoxP3 expression can be detected in CD4+ cells from CNS and LN of mice with EAE 20 days after EAE induction. CD4+ cells were enriched from CNS and LN of 5 mice. rPCR was performed to detect FoxP3 and β-actin. This was done with the help of Steph Palmer.
Figure 4-10: Proliferative responses of CD4+CD25+ cells to MOG(35-55) and/or IL-2. A: 2x10⁴ purified CNS cells from mice on day 16 after EAE induction were stimulated with 1µg/ml anti-CD3 with/without 100u/ml IL-2. B/C: 2x10⁴ CD4⁺CD25⁺ cells were purified from the inguinal LN of mice 16 (B) or 18 (C) days after EAE induction, and stimulated with 30µM (A) or 20µM (B) MOG(35-55) and IL-2 as indicated in graph legend. In A and B cells were purified by MACS (purity >75%), in C cells were purified by FACS sorting (purity > 97%).
Figure 4-11: Ex vivo cytokine production by CNS CD4+ cells in response to MOG(35-55) or PMA and ionomycin. 16 days after EAE induction, spinal cords were harvested from 4 mice and mononuclear cells from single mice stimulated with 10μM MOG(35-55), PMA/ionomycin, or medium alone before ICCS. The PMA (isotype control) group shows the background staining with cytokine isotype control Ab of cells stimulated with PMA and ionomycin. These results are representative of two separate experiments.
5. Functional Characteristics and in vivo Importance of CD4+CD25+ cells During Recovery from EAE

5.1. Introduction

Concentration of IL-10 production within the CD4⁺CD25⁺ T cell compartment in the CNS suggested that a regulatory CD4⁺ T cell population may be recruited to the CNS during recovery. CD4⁺CD25⁺ cells have been shown to downregulate the activation of CD4⁺ and CD8⁺ T cells (94, 104) as well as B cells (71, 72) and cells of the innate immune system (105). Most studies have looked at their roles in preventing these responses rather than controlling active disease. However, cure of colitis (121, 123) and early-onset diabetes (153, 154) by transfer of CD4⁺CD25⁺ cells has been demonstrated. CD4⁺CD25⁺ cells with protective function have been found to accumulate in the pancreatic LN in a murine model of diabetes (122), although these cells ultimately did not prevent diabetes. Also, human studies in rheumatoid arthritis have shown the accumulation of regulatory T cells at the site of inflammation (the joint), and these cells do not appear to mediate resolution of disease (214, 215), although in one study there was a correlation between Tregs and disease progression(214). Therefore, the demonstration of regulatory T cells playing a role in controlling an ongoing autoreactive response, without the use of transgenic cells or artificial manipulation, was a very exciting prospect, but the functional importance of CD4⁺CD25⁺ cells in the CNS remained to be demonstrated.

5.2. RESULTS

5.2.1. Attempts to clone MOG(35-55)-specific CD4⁺CD25⁺ cells from the CNS

Obtaining sufficient cells from the CNS to perform functional experiments is difficult without using high numbers of donor mice. For human therapeutic applications, Tregs would ideally be purified from peripheral blood of the patient, manipulated in vitro and then returned. Cloning of these cells would allow the selection of cells with known Ag-specificity and function, reducing the risk of
expanding and returning pathogenic cells to exacerbate disease. For this project, cloning of cells from the CNS would allow more concise identification and functional characterisation of regulatory cells in terms of cell surface markers and requirements for IL-10 production for regulation in EAE.

For cloning, B6 congenic Ly5.1+ mice were used to allow eventual tracking of the cells in vivo. CD4\(^+\)CD25\(^+\) cells from LN and CD4\(^+\) cells from CNS were purified from mice 18 days after EAE induction and cloned by limiting dilution in the presence of IL-2 (see section 2.7 for details). 32 LN CD4\(^+\)CD25\(^+\) clones and 75 CNS CD4\(^+\) clones were initially restimulated. By 4 weeks this had decreased to only 6 LN clones and 33 CNS clones still growing. By 2 months all of the LN clones had died, but 12 CNS clones were still growing. These continued to grow in culture and 11 eventually reached sufficient numbers to be tested between 2 and 4 months after initial cloning. Appendix Table A-1 summarises the results of this cloning attempt.

Clones were tested for responsiveness to MOG(35-55) in the presence or absence of IL-2, and for MOG(35-55)-specific IL-10 and IFN\(\gamma\) production (summarised in Table A-2, Appendix). 8 of the 11 clones tested proliferated in a dose-dependent manner to MOG(35-55), but none were anergic. All clones that proliferated to MOG(35-55) also produced IFN\(\gamma\) in a MOG(35-55)-specific manner. 5 of the 8 clones also produced IL-10, in varying amounts. Since these clones from the CNS have a Th1 phenotype, they would be predicted to be pathogenic, although this has not yet been tested in vivo.

It was apparent from this initial cloning attempt that the CD4\(^+\)CD25\(^+\) cells died more quickly in vitro than the whole CD4\(^+\) cells, and given the phenotype of surviving clones they were most likely generated from CD25\(^+\) cells. There are very few published reports on cloning of Tregs ex vivo. In one report, IL-10 was added to the initial rounds of stimulation (88). IL-15 is also known to be a survival factor for some memory T cells (310), and can augment human Tr1 expansion in vitro (311).
CD4^+CD25^+ cells were purified from the LN of mice early in the recovery phase of EAE and plated out for cloning as before, except IL-10 was added for the first two rounds of stimulation, and IL-15 was included in each restimulation. This approach did appear to enhance the survival of clonal cells, and by nine weeks 13 out of 46 wells initially scored as positive still contained living lymphocytes (see Table A-3 for summary). However, despite apparently enhanced survival, the cells failed to expand significantly upon restimulation.

In vitro expansion of CD4^+CD25^+ cells has recently been reported (153), with up to 200 fold expansion in two weeks achieved by stimulating purified CD4^+CD25^+ cells with anti-CD3 and anti-CD28 bound to beads, with the addition of 2000U/ml recombinant human IL-2. This type of approach, to either initially expand cells before cloning or directly clone CD4^+CD25^+ cells from the CNS, followed by testing for MOG(35-55)-reactivity, although more laborious may ultimately prove more successful than the attempts described here. This may also have the advantage of cloning out regulatory cells that may have specificity for other CNS Ag, since cells would initially be expanded polyclonally.

5.2.2. In vitro assays to measure suppressive properties of LN and CNS cells from EAE mice

One of the commonly used functional assays to test for regulatory cells is the in vitro suppression assay. This involves culturing responder cells – in these experiments CD4^+CD25^+ cells were used – with APC and either Ag or anti-CD3, in the presence or absence of CD4^+CD25^+ cells. Responder cell proliferation should be suppressed in the presence of regulatory T cells.

The suppression assay conditions were first optimised for conditions such as concentration of anti-CD3 used and number of APC per responder cells (see Appendix Figure A-1 and A-2). Further optimisation was done by Dr Leigh Stephens using TCR transgenic cells (see section 2.5.5). For assays using cells from all
peripheral LN and spleen of naïve mice, the number of CD4⁺CD25⁺ cells obtainable was reasonably high (around one million/mouse). However, when using cells from draining inguinal LN or CNS of immunised mice it was necessary to use as few cells as possible per well. The number of APC proved to be particularly important for facilitating suppression – a T cell:APC ratio of approximately 1:1 gave best results, and raising APC numbers eventually abrogated suppression, presumably due to spatial separation of responder and CD4⁺CD25⁺ cells in the well (cell contact appears to be important for in vitro suppression by CD4⁺CD25⁺ cells). Different methods of purifying cells were also attempted, as described in section 2.5.5. A combination of CD4 magnetic enrichment followed by FACS sorting gave consistently higher purities and reasonable yields.

5.2.3. CNS CD4⁺CD25⁺ T cells suppress in vitro proliferation

In two separate experiments, CD4⁺CD25⁺ cells obtained from the CNS of mice in the recovery phase of EAE (day 20 after EAE induction) were able to suppress the proliferation of naïve CD4⁺CD25⁻ cells in response to anti-CD3 (Fig 5-1). Importantly, this confirms that these are indeed functional regulatory cells. It was also interesting to note that CNS CD4⁺CD25⁺ cells appeared to be more potent than naïve CD4⁺CD25⁻ cells, giving greater inhibition of CD25⁻ cells and suppressing at a lower number of CD25⁻ cells. Likewise, the suppression by EAE LN CD4⁺CD25⁺ cells appeared more potent than naïve CD4⁺CD25⁺ cells, as has been observed in other experiments – see below. EAE LN CD25⁻ cells also appeared more potent than EAE CNS CD25⁻ cells, but this may reflect differences in the purity of the cells used in the experiment: the CNS cells were only 85% CD4⁺CD25⁺, while the EAE LN cells were 98% CD4⁺CD25⁺.

5.2.4. Testing In vitro Suppression by LN CD4⁺CD25⁺ cells

Tregs are thought to suppress in a non-Ag-restricted but specific manner, in that the responder TCR and Treg TCR may recognise different Ag (106), however, the Tregs must first themselves be activated by cognate peptide:MHC (106). If MOG(35-55)-reactive CD4⁺CD25⁺ cells are expanded during EAE, one would predict that
CD4^+CD25^+ cells from the draining LN of mice in the early recovery phase would therefore be able to suppress responder T cells in a MOG(35-55)-specific manner. CD4^+CD25^+ cells were purified from the inguinal LN of mice 16 days after EAE induction, the time point when CD25^+ cells are beginning to accumulate in the CNS (Figure 4-7). These LN CD4^+CD25^+ cells were tested for their ability to suppress the proliferative response of CD4^+CD25^- cells from the same LN in the presence of MOG(35-55). To address their specificity, their ability to suppress OT-II CD4^+ cells (which are specific for an unrelated Ag pOVA) was tested in the presence of either pOVA alone or pOVA in conjunction with MOG(35-55).

5.2.5. CD4^+CD25^+ cells from inguinal LN of mice with EAE suppress in vitro, but do not appear to require restimulation with Ag

In three separate experiments CD4^+CD25^+ cells, but not CD4^+CD25^- cells, from day 16 EAE mice were able to suppress the proliferative response of CD4^+CD25^- cells (from the same mice) to MOG(35-55) (Figure 5-2a). The counts per minute (cpm) were low for these assays, probably due to the low cell numbers used and the relatively long time after immunisation, but background proliferation was also correspondingly low. The same CD4^+CD25^+ cells also suppressed responses of OT-II CD4^+ cells to pOVA in the presence of MOG(35-55) (Fig 5-2b). Surprisingly though, in these assays OT-II CD4^+ cell responses were also suppressed when pOVA alone was added (Fig 5-2b). This suggests that CD4^+CD25^+ cells mediating suppression did not need to be activated by Ag in vitro.

5.2.6. Suppression of Ag-specific Responses by Naïve CD4^+CD25^+ cells

It has been shown that Tregs that have been recently activated are able to suppress in vitro without further TCR ligation (106). Thus, it was possible that the EAE LN cells were already in a sufficiently activated state ex vivo to mediate suppression without further activation. Therefore, CD4^+CD25^+ cells from non-immunised mice were included as a negative control. Perhaps even more surprisingly, naïve CD4^+CD25^+ cells were also able to suppress both MOG-specific and OVA-specific responses in
an apparently completely Ag non-specific manner (Figure 5-2c,d). It could be argued that suppression of OT-II cells was somehow an effect due to using transgenic cells, for example the OT-II mice may not have been fully backcrossed onto the B6 background allowing presentation of minor histocompatibility Ag to the Tregs. However, the suppression of MOG-specific CD4⁺CD25⁺ cell proliferation by naïve CD4⁺CD25⁺ T cells from completely syngeneic mice ruled this possibility out.

5.2.7. Draining LN CD4⁺CD25⁺ T cells may have enhanced suppressive capability

It was noticeable that in all conditions where both EAE and naïve CD4⁺CD25⁺ cells suppressed responder cells (responses to MOG(35-55), pOVA or anti-CD3 Figure 5-2), the cells from mice with EAE gave consistently better suppression than naïve cells, and this was particularly evident at lower Treg:responder ratios. Also, suppression of OT-II CD4⁺ cells by EAE-derived CD4⁺CD25⁺ cells appeared to be enhanced when MOG(35-55) was present in addition to pOVA (Figure 5-2b). Within each experiment, the purity of CD4⁺CD25⁺ cells from the different sources was similar (90-98%).

In summary, results of suppression assays using LN cells suggest that CD4⁺CD25⁺ cells from mice in the early recovery phase of EAE may have enhanced suppressive capability compared to CD4⁺CD25⁺ cells from naïve mice. However, suppression of OVA-specific responses in the absence of MOG(35-55) suggests this suppression may not require specific activation of these Tregs in vitro. Suppression of peptide-specific responses by CD4⁺CD25⁺ cells from naïve mice in the same assays confuses interpretation of any of the results, so no real conclusions can be drawn regarding expansion of MOG(35-55)-reactive CD4⁺CD25⁺ cells during EAE.

5.2.8. Are CD4⁺CD25⁺ defective in B cell-deficient mice?

It has been reported that μMT mice have a reduced frequency of CD4⁺CD25⁺ cells in the periphery (312). This was confirmed in the mice in our animal house (data not
shown), including those with EAE (Fig 5-3). However, although μMTs had a consistently lower frequency of peripheral CD4⁺CD25⁺ cells, this was still within the levels generally considered to be normal (5-15% of total CD4⁺ cells (94)). It was interesting to note that although the frequency of CD4⁺CD25⁺ cells was reduced in peripheral lymphoid organs of μMT mice, the frequency in the CNS was similar to that in B6 mice and likewise increased during recovery (Fig 5-3). Hence the failure of μMT mice to recover cannot be attributed entirely to a failure to expand or recruit sufficient numbers of CD4⁺CD25⁺ cells into the CNS.

Suppressive capacities of CD4⁺CD25⁺ cells from μMT versus B6 mice were compared in vitro. CD4⁺CD25⁺ cells from μMT mice were consistently less suppressive than those from wildtype B6 mice, whether responder cells were from B6 or μMT mice in these assays (Figure 5-4). When comparing results from three separate experiments, the μMT CD25⁺ cells gave significantly less inhibition of responder cell populations at ratios of 0.5:1 or 0.25:1 of CD25⁺:CD25⁺. It was interesting that at the lowest ratio of Treg to responder (0.25:1), the B6 CD4⁺CD25⁺ cells were also significantly less able to suppress μMT CD4⁺CD25⁺ cells than CD4⁺CD25⁺ cells from wildtype mice (Figure 5-3c). Hence, it does appear that CD4⁺CD25⁺ cells from μMT mice are inherently less potent than CD4⁺CD25⁺ cells from wildtype mice.

NOD mice have a lower frequency of peripheral CD4⁺CD25⁺ cells (around 5% of total CD4⁺ cells (48)), which may contribute to their enhanced susceptibility to autoimmune disease although this is controversial (313). An increased incidence of autoimmunity has not been reported in B cell deficient mice, and in fact development of diabetes in NOD mice is B cell dependent (314). Also, in the B cell IL-10-deficient chimaera model, recovery from EAE is restored in chimaeric mice that receive B cells from IL-10 sufficient mice (59). This suggests that B cell IL-10 is not required for peripheral CD4⁺CD25⁺ maintenance in this model, since transfer of IL-10 sufficient B cells immediately prior to EAE induction is unlikely to allow enough
time for restoration of a major defect in levels of peripheral CD4⁺CD25⁺ cells. This
does not rule out a role for B cell IL-10 in somehow expanding CD4⁺CD25⁺ cells
after EAE induction. While the proportion of peripheral CD4⁺CD25⁺ cells has not
been assessed in naïve IL-10-deficient B cell chimaeras, during EAE the proportion
of CD4⁺CD25⁺ cells in LN and CNS appeared similar to wt-B cell chimaeras (data
not shown).

5.2.9. Depletion of CD25⁺ cells prior to induction of EAE exacerbates
disease and delays recovery

The data presented so far suggest that regulatory T cells accumulate in the CNS
during recovery, but their importance in vivo had yet to be established. Therefore, in
vivo depletion of CD25⁺ cells was performed to determine whether CD4⁺CD25⁺
cells are actually required for recovery. Use of the anti-CD25 monoclonal antibody
PC61 for the depletion of CD25⁺ cells in vivo has now been widely reported.
Administration of 1mg PC61 intraperitoneally three days before induction of EAE
allowed enough time for over 90% CD25⁺ cell depletion (Figure 5-5). Depletion of
CD25⁺ cells was found to persist for at least two weeks before a gradual increase in
the percentage of peripheral CD25⁺ cells was seen, presumably due to thymic
generation of CD4⁺CD25⁺ cells, and levels returned to normal by four weeks after
administration of PC61 (Figure 5-5).

Mice treated with PC61 three days prior to EAE induction were found to develop
disease of increased severity compared to isotype-treated controls (Fig 5-6). Importantly, the recovery of PC61-treated mice was consistently impaired in several
experiments (Fig 5-6). It was notable that towards the end of several experiments
(day 25-35), some mice were showing signs of recovery, i.e. a decrease in clinical
score by at least one grade. The length of experiment has not been extended beyond
35 days, but it would be predicted that eventually some, if not all, mice would make
a full recovery.
5.2.10. The proportion of CD4\(^+\)CD25\(^+\) cells gradually increases after depletion, and this may be accelerated in mice with EAE

As shown in Fig 5-5, administration of PC61 in vivo does not lead to permanent depletion of CD25\(^+\) cells. Therefore, the delay in recovery could be explained by the time taken for the gradual repopulation of peripheral CD4\(^+\)CD25\(^+\) cells from the thymus, so that recruitment of Tregs to the CNS is delayed but not abolished by PC61 treatment. The proportion of CD4\(^+\)CD25\(^+\) cells in the LN, spleen and CNS of mice either given PC61 or isotype-control Ab was measured over the time-course of EAE.

When comparing PC61-treated or untreated mice with EAE, in three experiments the proportion of CD4\(^+\)CD25\(^+\) cells in the CNS at the peak of disease (day14/15) was found to be 2 to 3 fold lower in PC61-treated mice than controls (Figure 5-7). However, on days 21 and 28, the CD4\(^+\)CD25\(^+\) cells in the CNS of PC61-treated mice had reached similar levels to controls (Fig 5-7). Results in the LN and spleen were not as clear – in two experiments the proportions of CD4\(^+\)CD25\(^+\) cells in the LN of PC61-treated mice with EAE were similar to controls on day 15 (Fig 5-7b), while in a third experiment the PC61-treated mice had markedly lower frequencies of CD25\(^+\) cells (not shown). By day 28, the CD4\(^+\)CD25\(^+\) levels were the same in all experiments (not shown), as expected since levels of CD25\(^+\) cells had returned to normal in non-immunised mice by this time point (Figure 5-5).

There was some evidence that CD4\(^+\)CD25\(^+\) cells may be recruited/expanded more quickly in mice treated with PC61 then immunised, compared to mice given PC61 but not immunised. In two experiments, the mean proportion of CD4\(^+\)CD25\(^+\) cells in the (draining) inguinal LN on day 15 was 2 to 4 fold higher in PC61-treated mice with EAE compared to PC61-treated alone (Figure 5-7b). However, the groups were too small (2-3 mice/group) to achieve statistical significance. In the spleens of these mice, there was a similar increase in CD4\(^+\)CD25\(^+\) cells of PC61-treated and immunised mice in one experiment, but no increase in the other, consistent with a localised rather than systemic effect (not shown).
Depletion of CD25^+ cells in vivo prior to immunisation has been shown to enhance the level of T cell activation (particularly numbers of cytokine-producing effector cells) (155), and it could be argued that the increase in frequency of LN CD4^+CD25^+ cells could be due to an increased frequency of activated T cells rather than Tregs. This would be conclusively answered by performing in vitro suppression assays using CD4^+CD25^+ cells from these mice, although this has not yet been done. Levels of IL-10 production were low in all sites in these experiments (data not shown). However, cytokine production profiles of CD4^+ cells argue against the CD25^+ cells being activated/effector cells: IL-2 production after ex vivo stimulation of cells from PC61-treated mice with EAE was mainly from CD4^+CD25^- cells (Figure 5-8a), and this was also the case for IL-2 and IFN\gamma production by CD4^+ cells from the CNS (Figure 5-8b).

Taken together, these results suggest a delayed, but not abolished, accumulation of CD4^+CD25^+ cells in the CNS of PC61-treated mice with EAE may contribute to delayed recovery. Also, the eventual accumulation of CD4^+CD25^+ cells in the CNS of PC61-treated mice appears to be preceded by an accelerated recruitment or expansion of fresh CD4^+CD25^- thymic emigrants in the draining LN of some mice.

5.2.11. Administration of PC61 during EAE has variable effects on disease outcome

As stated already, depletion of CD4^+CD25^+ cells has effects on activation of naïve T cells that are difficult to distinguish from the effects of reduced availability of Tregs for expansion and recruitment to the CNS. Also, as discussed above, repopulation by CD4^+CD25^+ cells does gradually occur in PC61-treated mice and appears to correlate with a gradual recovery in some of these mice. PC61 was therefore given on different days after the induction of EAE in an attempt to target the recovery phase after the activation of effector cells has taken place.
1mg PC61 was given intraperitoneally on days 8, 15 or 20 after induction of EAE. In two out of three experiments, depletion of CD25+ cells on day 8 after immunisation resulted in significantly milder disease compared to untreated controls (Figure 5-9a), although in a third experiment disease was significantly enhanced (not shown). In one experiment disease was more severe in PC61-treated mice. Likewise, CD25+ cell depletion at the peak of disease (days 14-16) significantly accelerated recovery in two out of three experiments, and had no effect in one (Figure 5-9b). Treatment on day 20 had little effect (Figure 5-9c), although most mice are already recovering by this stage so the effect of treatment on day 20 after EAE induction is more difficult to judge. Overall then, depletion of CD4+CD25+ cells had the surprising effect of reducing disease severity in most experiments.

5.2.12. Transfer of low numbers of CD4+CD25+ cells from recovering mice protects naïve recipients against EAE induction

Transfer of high numbers of CD4+CD25+ cells purified from the peripheral LN of naïve mice reduces the severity of EAE in recipients ((49, 50) and Figure 5-10a). Activation of Tregs in vitro is thought to increase their potency (106, 153, 154). Therefore, one would predict that CD4+CD25+ cells that have accumulated in the CNS during inflammation should be highly potent, may have increased frequencies of MOG(35-55)-reactive cells, and should be able to reduce or prevent EAE severity in recipients at low cell numbers.

CD4+CD25+ cells from the CNS of recovering mice were FACS sorted and transferred into naïve recipients either on the same day as, or one day after, immunisation to induce EAE. In each case, 1-3x10⁴ CNS CD4+CD25+ cells/mouse significantly reduced disease severity in recipients, while the same number of naïve LN CD4+CD25+ cells had no effect (Figure 5-10b). Although the protection achieved was not complete, the number of cells transferred was very low, and it may be that transferring slightly higher numbers would give greater protection. Overall, CNS CD4+CD25+ cells were able to transfer equivalent protection at 100-fold lower numbers than naïve CD4+CD25+ cells. Therefore, CD4+CD25+ cells in the CNS of
recovering mice do indeed appear to be highly potent. It also seems likely that this CD25⁺ cell population is enriched for CNS-specific cells.

CNS-specific Tregs are likely to be activated and expanded in lymph nodes prior to migration to the CNS (where they may undergo further expansion). Without using transgenic cells it is difficult to determine where CD4⁺CD25⁺ cells are expanded. There are two likely options: first, CD4⁺CD25⁺ cells may be activated and expanded in the inguinal LN, that drain the subcutaneous immunisation site, in response to MOG(35-55) and other (non-CNS related) Ag. Alternatively, or additionally, CD4⁺CD25⁺ cells may be expanded by CNS Ag presented on activated DC migrating from the inflamed CNS during EAE. CD4⁺CD25⁺ cells sorted from the inguinal LN of recovering mice were also able to protect at low frequencies (1-4x10⁴ CD4⁺CD25⁺ cells) (Figure 5-11) However, the effect was less consistent than with CNS cells, with a reduction in disease severity achieved in only 3 out of 6 experiments. This may reflect differences in the proportion or activation state of the relevant “protective” cells in the CNS compared to LN, since the CNS is enriched for IL-10-secreting cells compared to LN, and CD4⁺CD25⁺ cells in the CNS phenotypically appear more activated (Chapter 4). CD4⁺CD25⁺ cells from the cervical LN were not tested for protective ability.

5.2.13. Depletion of CD25⁺ cells after recovery restores susceptibility to reinduction of EAE

As shown in Fig3-1, for a time after recovery from EAE mice are relatively resistant to induction of a second bout of disease, suggesting that the mechanisms involved in recovery are still dominant immediately afterwards. Post-recovery splenic B cells appear able to transfer resistance to induction of EAE, suggesting they may contribute to active resistance to reinduction. Likewise, transfer of CD4⁺CD25⁺ cells from recovering mice conferred protection on recipients. To investigate whether CD4⁺CD25⁺ cells are required for post-recovery resistance to EAE induction, CD25⁺ cells were depleted in vivo 30 days after primary EAE induction (after most mice had recovered). Mice were then rechallenged 3 days later with MOG(35-55) in IFA
and pertussis toxin. As expected, isotype-treated mice remained resistant to reinduction (Figure 5-12). However, mice depleted of CD25+ cells developed EAE of similar severity to their primary EAE (Figure 5-12), although unlike naïve mice treated with PC61, rechallenged mice did not show a markedly impaired recovery. Hence, CD4+CD25+ cells play a role in the active resistance to reinduction of disease.

5.3. DISCUSSION:
The presence of a large proportion of CD4+ cells having phenotypic characteristics of regulatory T cells and IL-10-producing capacities strongly suggested a role for regulatory T cells acting in the CNS to control inflammation. The results presented in this chapter show that these CD4+CD25+ cells from the CNS possess the functional characteristics of regulatory T cells, in that they are able to suppress naïve T cell proliferation in vitro and transfer protection against induction of EAE to recipients. Also, the requirements for CD4+CD25+ cells in recovery and resistance to reinduction of EAE were demonstrated by the effect of depletion of CD25+ cells in vivo.

Demonstration of suppressive capability of CNS CD4+CD25+ cells in vitro (Figure 5-1) was important in establishing that these cells did not merely represent a stage in effector cell differentiation (although cytokine production profiles suggested this was unlikely). Due to the constraints in cell numbers obtainable, this was only done with anti-CD3 as stimulus, but the ability of CD4+CD25+ cells obtained from the CNS to inhibit the proliferation of naïve CD4+CD25- cells showed that these were indeed regulatory cells. It also appeared that they were more potent than naïve CD4+CD25+ cells, since they gave stronger inhibition of responder cells, particularly at lower Treg:responder ratios.
The results of the suppression assays using CD4⁺CD25⁺ cells purified from the LN of either mice with EAE or naïve mice (Figure 5-2) were intriguing and confusing. Suppression of peptide-specific responses by polyclonal CD4⁺CD25⁺ cells from mice that have not been exposed to that Ag was a surprising result. Several studies have reported the requirement for interaction with cognate Ag by CD4⁺CD25⁺ cells for suppression to occur, including in assays very similar to those described in this project: polyclonal CD4⁺CD25⁺ cells from syngeneic Balb/c mice are unable to suppress the proliferation of DO11.10 CD4⁺ cells in response to pOVA alone, but do suppress a response to anti-CD3 (106, 153). This requirement for Ag-specific activation can be met if the CD4⁺CD25⁺ cells are pre-activated with Ag immediately before addition to the cultures, and pre-activation also increases the potency of CD4⁺CD25⁺ cells (106). This could offer an explanation for the non-specific suppression by CD4⁺CD25⁺ cells from mice with EAE, since it could be argued they have been activated in vivo. There were indications that CD4⁺CD25⁺ cells from the LN of EAE mice were indeed more potent than those from naïve mice, giving consistently greater suppression at lower Treg:Tresponder ratios. However, extensive pre-activation of regulatory cells is unlikely to explain the suppression observed by CD4⁺CD25⁺ cells from naïve mice housed in a specific pathogen free facility.

The reduced severity of EAE in vivo after transfer of low numbers of CD4⁺CD25⁺ cells from the CNS (Figure 5-10), and to a variable extent inguinal LN (figure 5-11), suggests two possibilities. Since recent activation of Tregs increases their potency (153, 154), it could be that CD4⁺CD25⁺ cells from mice with active disease are enriched for activated cells, and therefore have an equivalent efficacy to high number of CD4⁺CD25⁺ cells from naïve mice. The expression profiles of CD4⁺CD25⁺ cells from CNS compared to naïve LN certainly support this hypothesis, since CNS cells had higher expression levels of most of the markers analysed. CD4⁺CD25⁺ cells in the LN of EAE mice were more similar to naïve mice, with only slight increases in the proportion of cells expressing markers such as CD69. Also, the CNS was highly enriched for cytokine-producing cells compared to LN. This difference in the activation status of CD4⁺CD25⁺ cells in the LN and CNS of mice with EAE may also
go some way to explaining the disparity in the levels of protection afforded by CD4⁺CD25⁺ transfers: CNS CD4⁺CD25⁺ cells gave consistent protection, while LN CD4⁺CD25⁺ cells protected in only half the experiments.

The mechanism of action of CD4⁺CD25⁺ regulatory T cells in this model remains unclear. However, IL-10 seems a likely candidate based on the proportion of CD4⁺CD25⁺ cells in the CNS observed to produce IL-10 ex vivo, and the known importance of IL-10 for recovery in this model (231). One way to determine whether IL-10 production plays an important role in protection mediated by CNS CD4⁺CD25⁺ cells would be to transfer these cells from IL-10 knockout mice. This was attempted, but unfortunately disease incidence in this experiment was extremely poor so no conclusions could be drawn. However, it was observed that the proportions and phenotype of CD4⁺CD25⁺ cells in the CNS of IL-10⁻/⁻ mice did not differ greatly from those in B6 mice on day 16 after EAE induction (data not shown).

In vivo depletion studies further confirmed the importance of CD4⁺CD25⁺ cells during recovery from EAE. Administration of PC61 three days prior to induction of EAE gave sufficient time for depletion of CD25⁺ cells (Figure 5-5), and also for removal of much of the Ab from the system (315) so that effector T cells should be unaffected. Depletion of CD25⁺ cells prior to induction of EAE is likely to result in an increased number of effector pathogenic T cells, and indeed the severity of disease was increased in CD25-depleted mice. In addition to this effect, the rate of recovery was markedly slower than would normally be expected even for untreated mice with a high maximum disease score, suggesting that removal of Tregs had impaired the recovery process as well. The effects of CD25⁺ cell depletion in vivo prior to induction of EAE have been reported by three groups so far, with conflicting results. Montero et al (265) and Zhang et al (50) reported enhanced severity of disease in B6 mice and SJL mice, respectively, and in SJL mice a significant increase in mortality was observed (265). Both these groups used PC61 for CD25 depletion, In contrast, Kohm et al reported no effect of CD25⁺ cell depletion, using 7D4 Ab in
SJL mice (142). Importantly, none of these studies looked at the effect of CD25 depletion on the recovery phase of EAE.

The accelerated recovery of mice treated with PC61 after the induction of EAE was perhaps surprising (Figure 5-9). While the peak of disease occurs around 14 days after immunisation, the peak of effector T cell expansion is more likely to occur around 7 to 10 days after immunisation (286), so treatment with PC61 8 days after immunisation could deplete or prevent further expansion of pathogenic T cells as well as Tregs, resulting in reduced severity of disease. In EAE provoked by immunisation of SJL with PLP(139-151), specific CD4+ cells were tracked in vivo using Aβ-PLP(139-151) tetramers (286). In this model, the peak of expansion of PLP(139-151)-specific cells in the draining LN occurred on day 8 after immunisation, at which point 80% of these cells expressed CD25. The proportion of PLP(139-151)-specific CD4+ cells expressing CD25 then gradually declined, to reach normal levels between day 16 and 20. Likewise, on days 16 and 20 the data from this report suggest that the majority of effector Th1 cells do not express CD25. However, since MOG(35-55)-specific CD4+ cells cannot yet be tracked in B6 mice, the expression of CD25 by pathogenic cells in the LN cannot be excluded. If newly-generated effector T cells are depleted in addition to regulatory T cells, the overall effect may be a reduction in pathology, since the source of continued inflammation is cut off. Indeed, a humanised anti-CD25 mAb has been licensed for use in the prevention of renal transplant rejection (in combination with other immunosuppressive agents) (316), and in a small clinical trial had a beneficial effect in ten patients with multiple sclerosis (317).

CD25+ cells depletion by PC61 treatment is transient, and by 4 weeks the numbers of peripheral CD4+CD25+ cells have returned to near normal, probably mainly due to thymic generation of new Tregs. It was interesting to observe that in some mice, immunisation with MOG(35-55) to induce EAE appeared to drive a more rapid increase in the proportion of CD4+CD25+ cells, particularly in the draining LN (Figure 5-7). Cytokine production profiles of these cells suggested that they were not
effectors (Figure 5-8), and the initial increase in LN CD4^CD25^ cells was followed by an increase in CD4^CD25^ cells in the CNS so that these reached similar levels to untreated controls more quickly than expected. Given the data showing expansion of Tregs in response to activated DC and immunisation, this is the most likely explanation for this observed increase in CD4^CD25^ cells after immunisation, and the effect may be heightened by a reduction in homeostatic pressure by PC61 treatment. This result indirectly supports the hypothesis that CD4^CD25^ cells are initially expanded in LN then migrate into the CNS.

It is surprising that the proportions of CD4^CD25^ cells in the CNS appeared to reach similar levels between PC61-treated and control mice. However, in these experiments the number of inflammatory cells, such as macrophages, was not enumerated. In experiments where a four hour ex vivo stimulation was performed, a film of cells, presumably macrophages, were observed to adhere to the culture plates after removal of cells for FACS analysis. Therefore, although the proportion of total CD4^ cells is directly comparable between groups, the proportion of total cells that were CD4^CD25^ cells may not be comparable. It has been shown that disease severity correlates more closely with levels of macrophage recruitment than T cells in rats (173), and it could therefore be argued that although similar levels of Tregs were obtained by 20 days post EAE induction, the levels of inflammation that have to be controlled may be higher in PC61-treated mice, which do develop increased severity of disease.

In vivo depletion of CD25^ cells after recovery was in some ways more informative than depletion prior to induction of EAE, since any other (non-CD4^CD25^) regulatory mechanisms contributing to recovery and the active resistance to reinduction should remain unaffected by this treatment. Indeed, while CD25^ cell depletion resulted in increased susceptibility to reinduction, confirming the role of Tregs in recovery, the rate of recovery in those mice was consistently more rapid than in mice treated with PC61 and immunised for the first time (Figure 5-11). Thus, it appears that there may be additional mechanisms in force to reduce the severity of
a second bout of EAE – such as B cells. The reason for the gradual loss of this protection remains unclear. It may be that the relevant CD4⁺CD25⁺ cells lose their potency and/or undergo attrition, as CNS Ag is no longer presented after resolution of CNS inflammation, and the balance shifts back to the susceptible state found in naïve mice.

Taken together, these results further confirm that CD4⁺CD25⁺ cells with regulatory function accumulate in the CNS and play an active and central role in mediating recovery from EAE that remains in effect in the time period immediately after recovery.
Figure 5-1: CD4⁺CD25⁺ cells isolated from the CNS of mice recovering from EAE are suppressive in vitro. CNS and inguinal LN were harvested and pooled from 12 mice 20 days after induction of EAE, and all peripheral LN from one naïve mouse. For LN samples CD4⁺ cells were first enriched by Dynal bead negative selection. CD4⁺CD25⁺ cells were purified by FACS sorting to purity greater than 95% for LN and 85% for CNS. Naïve CD4⁺CD25⁻ cells were used as responder cells at 10⁴ cells/well, with 10⁵ irradiated splenocytes as APC and the indicated number of CD4⁺CD25⁺ cells. Cells were stimulated with 2.5μg/ml anti-CD3 for 96 hours. One result of two similar experiments is shown.
Figure 5-2A-B: Testing in vitro suppression by CD4+CD25+ cells from LN of mice with EAE and naïve controls. Inguinal and para-aortic LN were pooled from mice on day 16 after EAE induction, or all peripheral LN from naïve OT-11 mice were pooled. CD4+CD25+ cells were purified by Dynal bead and MACS sorting. Source of suppressor and responder cells is indicated in schematic next to each graph. In A, 4x10^4 CD4+CD25+ cells were used. In B, 10^4 CD4+CD25+ OT-II cells were used. 1.6x10^5 irradiated APC were added to all wells.
Figure 5-2(C-E): Testing in vitro suppression by CD4+CD25+ cells from LN of mice with EAE and naïve controls. Inguinal and para-aortic LN were pooled from mice on day 16 after EAE induction, and all peripheral LN were pooled from naïve naïve B6 or OTII mice. CD4+CD25+ cells were purified using Dynal beads and MACS sorting. Cell source and Ag used are indicated in schematic next to each graph. In C, 4x10^4 EAE d16 CD4+CD25+ cells were used. In D, 10^4 CD4+CD25+ OTII cells were used. In E, 4x10^4 CD4+CD25+ cells were used. 1.6x10^5 irradiated APC were added to all wells. A-E are results from 3-4 similar experiments.
Figure 5-3: Proportion of CD4^+CD25^+ cells is reduced in peripheral LN/spleen of μMT mice. EAE was induced in μMT and B6 mice, and on days 12 and 16 post-induction inguinal LN, spleen and CNS were removed, pooled, and the proportion of CD4^+CD25^+ cells analysed by FACS (4 mice/group).
Figure 5-4: CD4⁺CD25⁺ cells from μMT appear to be defective in vitro. CD4⁺CD25⁺ cells were purified from peripheral LN of naïve B6 and μMT mice by MACS selection. 2x10⁴ CD4⁺CD25⁺ responder cells/well from either B6 or μMT, 7.5x10⁵ irradiated B6 APC/well and the indicated numbers and of B6-derived or μMT-derived CD4⁺CD25⁺ cells/well added. Cells were stimulated with 0.5mg/ml for 72 hours. Results shown are pooled from three separate experiments, in which similar purities of B6 and μMT CD4⁺CD25⁺ cells were obtained. A-C show percentage inhibition of CD4⁺CD25⁺ cells from B6 or μMT mice at different Treg:effector ratios. D shows combined data on the percentage inhibition of B6 cells at different Treg ratios.
Figure 5-5: Administration of PC61 effectively depletes CD25+ cells in vivo. 1mg PC61 or isotype control Ab (MAC49, rat IgG1) was given intraperitoneally on day 0. Mice were bled on days indicated and percentage CD4+CD25+ cells analysed by FACS (using anti-CD25 clone 7D4). Results shown are for 1-2 mice/group, representative of 3 experiments.
Figure 5-6: Depletion of CD25+ cells 3 days prior to EAE induction results in exacerbated disease severity and delayed recovery. 1mg PC61 or isotype control Ab was given ip on day −3. Depletion of peripheral blood CD25+ was checked by FACS on day of immunisation and was greater than 85% in all mice. EAE was induced on day 0 and mice scored daily for clinical EAE signs. The disease burden in PC61-treated mice was significantly greater than controls (p<0.001). This result is representative of 5 experiments in which CD25+ cells were depleted prior to EAE induction.
Figure 5-7: The percentage of CD4+CD25+ cells rises in CNS and LN after PC61 administration and EAE induction. 1mg PC61 was given 3 days before EAE induction ("PC61+EAE"), "EAE" groups received isotype control Ab before EAE induction, "PC61" received PC61 but EAE was not induced. On the days indicated, inguinal LN and CNS were harvested and cells stained for FACS analysis of CD4+CD25+ cells. Results in A show mean percentage CD4+CD25+ cells in CNS from 2-3 mice, and in B, percentage CD4+CD25+ cells in LN from individual mice are shown, and are representative of three separate experiments using 2-3 mice/group.
Figure 5-8: CD4+CD25- cells not CD4+CD25+ from LN and CNS are the producers of IL-2 and IFNγ ex vivo. PC61 was given 3 days prior to EAE induction. 14 days later, inguinal LN and CNS were harvested and cells stimulated with PMA and ionomycin in the presence of golgistop for 4 hours, followed by FACS staining of cell-surface CD4 and CD25 and intracellular staining of IL-2 and IFNγ. Results shown are mean of 2-3 mice/group (stained separately), and representative of 2 separate experiments.
Figure 5-9: Treatment with PC61 after EAE induction ameliorates disease. EAE was induced on day 0 and mice scored daily for clinical signs of EAE. 1mg PC61 was given intraperitoneally on the day indicated (A: day 8, B: day 16, C: day 20), controls remained untreated. Each result is representative of 2-3 experiments in which 5 mice/ group were used.
Figure 5-10: CD4*CD25+ cells from the CNS decrease EAE at low numbers compared to CD4*CD25+ cells from naïve mice. CD4*CD25+ cells were sorted from the peripheral LN of naïve mice, or from the CNS of mice on day 20 post EAE induction. In A, 2x10^6 or 10^4 naïve CD4*CD25+ cells were transferred and EAE induced 1 day later. In the only experiment in which this was done (5 mice/group), 2x10^6 cells gave significant reduction in EAE severity (p<0.001). In B, 1-3x10^4 CD4*CD25+ cells from CNS or naïve LN were transferred and EAE induced. The plot shows combined results from 2 experiments (total 10-11 mice/group) in which cells were transferred on the day of immunisation or on day 1. CNS CD4*CD25+ cells gave a significant reduction in severity of EAE (p<0.019).
Figure 5-11: Transfer of CD4*CD25* cells from LN of mice with EAE gives variable protection. 1-4x10^4 CD4*CD25* cells from the inguinal LN of mice on days 16 or 20 post EAE induction were purified and transferred into naïve recipients on days –1, 0 or 1 of EAE induction. The results shown are 2 experiments representative of 6 separate experiments in which 4-7 mice/group were used.
Figure 5-12: Depletion of CD25^+ cells after recovery restores susceptibility to reinduction of EAE. EAE was induced in 11 mice on day 0 by immunisation with MOG(35-55) in CFA and pertussis toxin given on days 0 and 2. On day 30, mice were split into two groups according to EAE scores so that each group had comparable mean EAE scores in the first 30 days after induction. 6 mice received 1mg PC61 and 5 mice received 1mg isotype control Ab ip on day 30. Depletion of CD25^+ cells from peripheral blood was checked and mice were immunised with MOG(35-55) in IFA on day 33, pertussis toxin was given on days 33 and 35. PC61-treated mice had significantly higher disease burdens from day 35 (p<0.011). This result is representative of 3 separate experiments.

Figure 5-12: Depletion of CD25^+ cells after recovery restores susceptibility to reinduction of EAE. EAE was induced in 11 mice on day 0 by immunisation with MOG(35-55) in CFA and pertussis toxin given on days 0 and 2. On day 30, mice were split into two groups according to EAE scores so that each group had comparable mean EAE scores in the first 30 days after induction. 6 mice received 1mg PC61 and 5 mice received 1mg isotype control Ab ip on day 30. Depletion of CD25^+ cells from peripheral blood was checked and mice were immunised with MOG(35-55) in IFA on day 33, pertussis toxin was given on days 33 and 35. PC61-treated mice had significantly higher disease burdens from day 35 (p<0.011). This result is representative of 3 separate experiments.
6. DISCUSSION

6.1. Regulation of T cell mediated CNS-targeted autoimmune disease by IL-10

Regulation of immune responses is vital for maintaining health. This regulation may take the form of reducing collateral damage by turning off responses after an invading pathogen has been removed, or preventing the activation of harmful responses against self or innocuous foreign antigens. This project addressed the mechanisms of regulation of an induced autoimmune response against the autoantigen MOG after inflammation had been established in the CNS. Previous studies have reported a requirement for IL-10 in recovery from EAE (231, 235), and an increase in IL-10 and Th2 cytokines in the CNS is associated with recovery (229, 237). B cells have been identified as an important source of IL-10 in EAE (59). This project has further shown that B cells do not appear to migrate to the CNS in significant numbers, suggesting that their main site of action is the LN. Rather, CD4⁺ T cells were identified as the major lymphoid source of IL-10 in the CNS. These cells possessed phenotypic and functional characteristics of the well-described CD4⁺CD25⁺ regulatory T cells. In vivo CD25⁺ cell depletion studies further confirmed the importance of these cells in the regulation of the MOG-directed inflammatory response in EAE.

IL-10 could potentially be involved in downregulating all of the steps in the inflammatory feedback loop induced during EAE (See schematic page 166 for overview). Th1 cells entering the CNS produce cytokines such as IFNγ and TNFα that activate macrophages. These cytokines may also be involved in breaking down the blood-brain barrier (13). Activated migratory macrophages and DC, as well as resident microglia in turn produce chemokines to recruit further effector cells (198), and express MHC and costimulatory molecules to activate effector T cell
function within the CNS (176, 177). Activated DC may also migrate to the LN, to present CNS antigens and activate a fresh wave of Th1 cells.

IL-10 decreases expression of costimulatory molecules and peptide-MHC complexes on APC, and decreases IL-12 production (233, 318, 319). This may serve two functions during EAE: first, to reduce effector T cell functions in the CNS; second, to decrease the recruitment of fresh waves of T cells from the LN (by preventing activation of Th1-stimulating DC in the CNS and by downregulating effector T cell differentiation in the LN). IL-10 produced in the CNS may also act directly on the activated macrophages and T cells to turn off production of cytokines such as TNFα, IFNγ and IL-23. In this way, the inflammation will be quelled, and the stimuli involved in breaking the blood-brain barrier and recruiting new effector cells removed.

The results from this project suggest that a large proportion of the IL-10 found in the CNS is provided by CD4^+CD25^+ regulatory T cells. These cells accumulate in the CNS during recovery from EAE, so are in the prime location for downregulating inflammation. What is the source of these regulatory T cells? Their phenotype and the expression of FoxP3 within the CD4^+ compartment in the CNS suggest that at least some of these cells are derived from the "naturally-occurring" population of CD4^+CD25^+ cells constitutively present in normal mice (94). The impaired recovery observed when CD25^+ cells are depleted prior to EAE induction adds further weight to this proposal. If this were the case, the likely scenario is that CNS-specific CD4^+CD25^+ cells are expanded in draining LN in response to presentation of these antigens by DC migrating from the immunisation site or the inflamed CNS. Although proliferation of CD4^+CD25^+ cells has been observed in the inflamed gut epithelium (123), this was in a lymphopaenic model of colitis, and other non-lymphopaenic autoimmune models have found expansion to occur mainly in the draining LN (77, 122). After expansion in the LN (where they may also have some effects through interactions with DC and/or Th1 pathogenic cells), the regulatory cells will migrate to the CNS to downregulate inflammation there.
An alternative hypothesis for the source of IL-10-producing T cells in the CNS is that they are generated de novo from naïve T cells during the course of EAE, i.e. they are Tr1 cells. Since this process is likely to occur in the LN, where differentiating T cells are located, B cell production of IL-10 could feasibly be involved in this generation in two ways. First, B cell IL-10 may maintain local DC in an immature state (234), which in turn may induce the differentiation of IL-10 producing T cells (320, 321) specific for CNS antigens. The problem with this hypothesis is that the dendritic cells carrying antigen from the CNS are likely to already be in a mature state. Second, B cells presenting antigen and producing IL-10 could influence T cell differentiation directly.

Whether B cell IL-10 acts directly on T cells in EAE is at present unknown. B:T interactions form part of the normal course of an immune response (69). Indeed, these interactions are required for induction of the “regulatory” function of B cells, since chimaeras in which B cells alone are deficient in CD40 (and therefore unable to receive signals through CD40 ligation by CD40L on T cells) also fail to recover from EAE (59). As Ag-specific B cells expand and mature during an immune response, they become prime contenders to interact with and influence the developing T cell response. Thus, IL-10 may act directly on T cells later in the response. While B cells have been shown to be capable of activating naïve T cells in vitro (322), it is thought that in vivo B cells are more likely to interact with T cells that have first been activated through interactions with dendritic cells (69, 323). This is largely due to the separate locations of these cells in the LN or spleen: naïve T cells move into the T cell zone after arriving in the LN, while B cells (mostly) move towards follicles. Only after activation by antigenic stimuli will these cells move towards each other to interact (323). Nevertheless, T cell differentiation takes several rounds of stimulation and division before a polarised phenotype emerges (324), and T cells have been observed to interact with B cells at early stages after activation (323), so direct induction of Tr1s by B cells remains a possibility.
Another, perhaps more likely, hypothesis is that IL-10 from B cells will act to turn off the effector functions of previously activated T cells. It was thought for some time that only Th2 cells could enter B cell follicles, however Th1 cells have been shown to interact with B cells in follicles to aid the production of Th1-type antibody isotypes (325, 326). It is therefore likely that a proportion of T cells interact with B cells in the LN during their differentiation in the EAE model, before migrating to the CNS. However, since interaction with a B cell is not mandatory for an effector T cell before it leaves the LN, this mechanism of B cell regulation would be incomplete. Therefore, additional mechanisms of regulating effector cells in the CNS are likely to be required: since B cells do not enter the CNS in great numbers, IL-10 producing T cells could perform this role.

It was interesting to note that while depletion of CD25+ cells restored susceptibility to reinduction of EAE after recovery, these mice did not experience the impaired recovery observed in mice depleted of CD25+ cells prior to primary induction of EAE (Figures 5-11 and 5-6). This suggested that CD4+CD25+ cells are involved in recovery but are not acting alone, fitting with the observed requirement for B cells in recovery from EAE.
Schematic of regulatory circuits operating during recovery from EAE, showing the possible cellular targets of IL-10 produced by B cells and CD4⁺CD25⁺ (Tr) cells.
6.2. Questions remaining

This project has shed some light on the cellular sources of IL-10 during recovery from EAE, as well as defining a role for CD4⁺CD25⁺ cells in control of established disease in this model. From these results, some obvious new questions arise.

6.2.1. What is the mechanism of suppression by CD4⁺CD25⁺ cells in this model?

Although not yet proven, the mechanism of regulation by CD4⁺CD25⁺ cells in this model is suggested to be IL-10, since these cells were initially identified through their production of IL-10 in the CNS. IL-10 has been shown to be an effector mechanism for CD4⁺CD25⁺ cells in other models in which IL-10 deficiency also leads to more severe disease, such as colitis (105, 114, 115). Transfer of large numbers of naïve CD4⁺CD25⁺ cells was able to reduce EAE severity only if the transferred cells were able to produce IL-10 (50), suggesting that IL-10 plays a role at least in resistance to EAE. Transfer of CD4⁺CD25⁺ cells from the CNS of IL-10⁻/⁻ mice would be one way of determining whether IL-10 is indeed required for the function of CNS regulatory T cells.

Another immunoregulatory candidate for CD4⁺CD25⁺ function is TGFβ. An increase in TGFβ within the CNS during the recovery phase of EAE has been reported in two mouse models (229, 327), in one of these IL-10 was also investigated and found to increase along with TGFβ (229). Until recently, TGFβ was not considered to be a major effector mechanism for CD4⁺CD25⁺ cells, but it has now been shown to be important for regulation in mouse models of both diabetes and colitis. A cursory examination of surface-bound TGFβ (data not shown) found both CD4⁺CD25⁺ and CD4⁺CD25⁻ cells in the CNS to be TGFβ-negative, but production of soluble TGFβ was not investigated as IL-10 was the focus of this study.
6.2.2. What is the Ag-reactivity of the IL-10 producing B cells and CD4⁺CD25⁺ cells, and how are they expanded in vivo?

The antigen-reactivity of both B cells and CD4⁺CD25⁺ cells involved in the recovery from EAE remain unidentified. As discussed in the relevant chapters, the investigation of receptor specificity requires the production of tools to specifically label the BCR or TCR, such as fluorescently conjugated antigen, in the case of B cells, and tetramers or liposomes for T cells.

Knowing the target Ag(s) of regulatory cells may give some clues as to their origins and how they are expanded during EAE, an important question in terms of therapeutic applications. For example, if the CD4⁺CD25⁺ cells found in the CNS are expanded as a result of the immunisation with MOG(35-55), they will be reactive for this Ag, and it may also be possible to track the timing of their appearance and/or expansion in the LN. One complicating factor may be expansion of regulatory cell reactive against components of CFA. On the other hand, if CD4⁺CD25⁺ cells are expanded as a result of presentation of antigen by CNS-derived DC, the population may have reactivity for more than one CNS Ag. Expansion or induction of Tregs by DC migrating from the CNS versus the immunisation site may affect the timing and location of their appearance and/or expansion. Understanding the signals that lead to the accumulation of CD4⁺CD25⁺ cells in the CNS may point to methods for expanding these cells in the clinical setting of MS.

6.2.3. Is there a relationship between regulatory T cells and B cells

It has been shown that CD4⁺CD25⁺ cells may regulate B cells to prevent autoAb production (71, 72). There is little evidence available as to the effects of interactions with B cells on Tregs themselves. It has been observed that μMT mice have a decreased frequency of CD4⁺CD25⁺ T cells (312), and experiments in this project suggested that the CD4⁺CD25⁺ cells present in μMT mice may be defective compared to CD4⁺CD25⁺ cells from B cell-sufficient mice (Figure 5-4). It remains to be established whether B cells interact with CD4⁺CD25⁺ cells during recovery from EAE. This could be approached by investigating whether normal recovery is restored.
in µMT mice by transfer of CD4⁺CD25⁺ cells from wildtype mice, and conversely whether B cells from recovered mice are able to protect recipients that have been depleted of CD25⁺ cells prior to EAE induction.

6.3. Therapeutic potential of regulatory T cells.

The ultimate goal in autoimmunity is to restore a state of tolerance to self without affecting immune responses to foreign antigens. In healthy individuals, Tregs appear to play a major role in maintaining this desired balance, and disruption of their function may be involved in the development of autoimmunity. Therefore, regulatory T cells may be ideal candidates as tools for restoring the tolerant state. The results of this project suggest that Tregs are activated and expanded under inflammatory conditions as well as pathogenic cells. Other reports have shown directly that CD4⁺CD25⁺ cells may be expanded by immunisation with Ag in CFA or Ag-pulsed DC that have been activated by treatment with LPS. It is promising that Tregs can be expanded under these conditions, since patients with established disease present an inflammatory environment in which to attempt to restore regulation. However, approaches that are likely to lead to the activation of pathogenic cells along with Tregs are clearly not desirable. Several investigators have developed methods for inducing or expanding Tregs that (hopefully) circumvent this problem, and some of these will be discussed.

6.3.1. Ex vivo expansion of Tregs

The Bluestone and Steinman labs have recently described the in vitro expansion of regulatory T cells capable of both preventing and curing diabetes in mice (153, 154). In both studies, protection relied on the use of transgenic CD4⁺CD25⁺ cells with reactivity for islet antigens, while polyclonal NOD CD4⁺CD25⁺ cells were ineffective. This may reflect an inherent defect in regulatory T cells from NOD mice, or more likely a requirement for antigen-specificity in regulation. Thus, selective expansion of antigen-specific cells from the polyclonal population in humans is likely to be required if this is to become a useful therapy.
Hafler’s group have reported a reduced cloning efficiency (using a non-antigen-specific stimulus) of CD4^+CD25^+ cells from the peripheral blood of patients with MS (211). These cells also displayed reduced suppressive capacity compared to cells isolated from healthy controls. A similar reduction in numbers of peripheral CD4^+CD25^+ cells has been reported in patients with type-I diabetes (212). Expansion of a patient’s own regulatory cells may therefore turn out not to be an effective therapeutic tool.

In studies of human arthritis, CD4^+CD25^+ cells have been observed to accumulate in the inflamed joints (214, 215), and in this project CD4^+CD25^+ cells accumulated in the inflamed CNS. Therefore, the relevant antigen-reactive cells may be in sites not readily accessible for purification from humans, particularly those with MS.

The ex vivo expansion and reinfusion of patients’ own regulatory cells, while promising in concept, may ultimately prove too impractical (and expensive) for clinical applications. A more feasible approach may be to develop a regime capable of expanding pre-existing regulatory cells, or inducing them de novo, in multiple patients. Several potential methods for doing this have been developed in mice, and some have been attempted in clinical trials in humans.

### 6.3.2. Non-antigen-specific expansion of Tregs in vivo

Administration of agonistic anti-CD3 mAb has been shown to be an effective method for inducing tolerance in NOD mice with overt type I diabetes (328, 329). Anti-CD3 appears to have different effects on subpopulations of T cells in vivo: deleting Th1 cells but promoting Th2 cells (330). Chatenoud et al have recently demonstrated that remission from type I diabetes in NOD mice given anti-CD3 treatment corresponds with an enhanced frequency of CD4^+CD25^+ cells in vivo, particularly in the LN draining the pancreas (331, 332). In vivo neutralisation of TGFβ abrogates this
protective effect, and also prevents the increase in CD4^+CD25^+ cells (331), suggesting de novo generation of FoxP3^+ regulatory cells by TGFβ.

Humanised anti—CD3 mAb has been tested in small clinical trials involving patients with recently-diagnosed type-I diabetes (333) or allotransplantation of pancreatic islets (to treat type I diabetes) (334), with fairly impressive results. In the allotransplantation study, an increase in CD4^+CD25^+ cells was observed in patients with graft acceptance, and these cells were able to at least partially suppress responses to donor antigens in vitro (334).

Using a similar approach, Wood et al have induced tolerance to alloantigens by giving non-depleting anti-CD4 at the time of donor cell infusion in mouse models of allotransplantation (53, 335). Administration of anti-CD4 at the time of grafting appears to induce CD4^+CD25^+ regulatory T cells that are able to adoptively transfer specific tolerance to naïve recipients (53, 336).

The advantage of this type of approach is that the specific Ag involved in the autoimmune or rejection process do not have to be identified. These treatments do appear to target cells that are responding to Ag within a relatively short window of time, thereby conferring a degree of specificity and reducing the risks associated with the long-term general immunosuppressive therapies currently used.

6.3.3. Ag-specific Induction/Expansion of Tregs in vivo

Administration of antigen by the oral route has proved to be an effective mechanism for inducing tolerance against Ag encountered through systemic routes (56, 337). One of the mechanisms through which oral tolerance may act is via regulatory T cells. Indeed, two groups have demonstrated the induction (81) or expansion (82) of CD4^+CD25^+ regulatory cells by feeding Ag. In the EAE model, Weiner et al described that feeding mice with MBP induced CD4^+ regulatory T cells that could prevent EAE induction through secretion of TGFβ, so-called Th3 cells (248, 338). In
rats a similar phenomenon was observed, except the protective cells were CD8+ (339).

These promising results led to a clinical trial in which patients with MS were fed bovine myelin (340). While an increase in TGFβ production in response to MBP was observed in some of these patients, the therapy was not found to be beneficial clinically. It may be that regulatory cells activated via the oral route are able to act in lymph nodes to prevent activation of pathogenic cells, but are unable to home to the CNS to quell inflammation there. Therefore the ability of orally-induced Th3 cells to mediate remission in MS remains debatable.

It has recently been demonstrated that injection of MBP-coated DC that have been pre-treated with TGFβ can protect against EAE through the generation of CD8+ regulatory T cells in mice (57). Indeed, therapeutic administration of DC is attractive in many fields of immunology due to the ability to target T cells in an antigen-specific manner, and also the potential to manipulate the type of response those DC will induce. Immature DC have been shown to induce tolerance (320), and while this has largely been attributed to the lack of costimulatory molecules on these cells, induction or expansion of regulatory T cells may also play a part (341).

DC manipulation requires purification and culture of individual patients’ DC. A more simple approach is to give peptide directly. Wraith and colleagues have shown that intranasal administration of peptide may induce Ag-specific Tr1 cells. Unlike Weiner et al, who found TGFβ produced in response to orally administered antigen (56), intranasal peptide administration resulted in IL-10 producing Tr1 cells able to transfer tolerance (63). Administration of low doses of peptide via the intravenous route (81), or continuous infusion of low doses of peptide by a subcutaneously-placed osmotic pump (83), have also been shown to induce Ag-specific CD4+CD25+ cells in vivo.
Administration of Ag can therefore induce regulatory T cells in vivo, in a route- and dose-dependent manner. However, one drawback of these approaches is that the relevant Ag are to some extent still unclear in many autoimmune diseases, and may differ between patients. The ability of regulatory T cells to mediate bystander suppression may compensate if at least one antigen is known. These studies have been performed in laboratory mice that, unlike humans, are not subject to concomitant challenge by infectious agents, which may provide "danger" signals to the immune system, leading to active immunity rather than tolerance. Also, it must be considered that pathogenic effector or memory T cells, which require less costimulation than naïve cells, may be reactivated by this protocol. However, currently used general immunosuppressive treatments also carry risks, and specifically stimulating the body's own regulatory mechanisms still seems a desirable alternative for therapy of autoimmunity and allergy.

6.4. Conclusions

Diverse mechanisms exist to prevent or control immune responses against self tissues. When these break down autoimmune disease results. Therapy of autoimmune disease, as well as allergy and transplantation, aims to restore or establish tolerance under conditions of inflammation. While tolerance protocols that are effective in mouse studies may turn out to be beneficial in humans, many offer an approach akin to shutting the stable door after the horse has bolted, in that they are effective on naïve cells, but may not affect or may even activate effector cells.

Four studies have recently shown that CD4⁺CD25⁺ cells can restore tolerance after disease has been established, i.e. they can act on effector cells in an inflammatory environment (121, 123, 153, 154). This project has additionally shown that regulatory T cells may act to mediate spontaneous resolution of autoimmune inflammation without a requirement for infusion of high numbers of CD4⁺CD25⁺ cells or the requirement for the use of cells from TCR transgenic mice. The importance of IL-10 production by B cells in models of inflammation, including
EAE, must not be forgotten. Determining the most effective methods of enhancing physiological regulatory mechanisms, under conditions similar to those present in patients with active disease, may lead to the establishment of long-term tolerance to specific antigens without the current requirement for general immunosuppression.
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### Table A: Summary of cloning attempt

Table A shows source and number of clones scored positive at different timepoints. B shows results of testing of clones for levels of IFNγ, IL-10 and proliferative responses to MOG(35-55).
<table>
<thead>
<tr>
<th>Source of cells</th>
<th>No. of cells/ well</th>
<th>no. of initial wells</th>
<th>+ve clones 1 week</th>
<th>+ve clones 6 wks</th>
<th>+ve clones 9 wks</th>
</tr>
</thead>
<tbody>
<tr>
<td>LN CD4+ CD25+</td>
<td>50-100</td>
<td>235</td>
<td>46</td>
<td>0.13</td>
<td>13</td>
</tr>
</tbody>
</table>

Table A-2: Summary of cloning attempt 2 Purified CD4+CD25+ cells initially cultured with $5 \times 10^5$ APC, 10mM MOG(35-55), 50U/ml IL-2, 200pg/ml IL-10 and 100ng/ml IL-15, and positive wells restimulated weekly. After the first stimulation the concentration of MOG(35-55) was decreased to 4mM. After the first two rounds of stimulation IL-10 was no longer included. After 9 weeks the positive wells still did not appear to be expanding significantly. The concentration of IL-2 was increased to 500U/ml in an attempt to boost expansion, but this resulted in giant cell formation and death.
Figure A-1: Titration of anti-CD3 for suppression assays
2x10^4 MACS-sorted CD4+CD25- cells were cultured with CD4+CD25+ cells at the ratios indicated in graph key. 5x10^4 irradiated splenocytes were added as APC. Cultures were stimulated with the indicated concentrations of anti-CD3 for 72 hours, with tritiated thymidine added 6 hours before harvesting.
Figure A-2: Testing different APC numbers in suppression assay
2x10^4 MACS-sorted CD4^+CD25^+ cells were cultured with the indicated number of CD4^+CD25^+ cells and APC per well. Two sources of irradiated APC were used: whole splenocytes or splenocytes that had been depleted of T cells using Thy1-microbeads (Miltenyi Biotec). Cultures were stimulated with 0.5µg/ml anti-CD3 for 72 hours, with tritiated thymidine added for the last 6 hours of culture.