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T cells in experimental autoimmune encephalomyelitis

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M.Sc., B.Sc., P.Grad.Dip.

A thesis submitted to

Trinity College Dublin

For the degree
of
Doctor of Philosophy

Supervisor: Prof. Kingston H.G. Mills

Immune Regulation Research Group
School of Biochemistry and Immunology
& Institute of Neuroscience

Trinity College Dublin

2010
Declaration of Authorship

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Stephen Lalor

Signature

Stephen Lalor
Abstract

Multiple sclerosis (MS) is an inflammatory, demyelinating disease of the central nervous system (CNS) that is both clinically and pathologically heterogeneous. Studies in experimental autoimmune encephalomyelitis (EAE), an animal model for MS, have demonstrated that T cells specific for self antigens mediate pathology in these diseases. Th1 cells were thought to be the main effector T cells responsible for the autoimmune inflammation. However, recent studies have highlighted an important pathogenic role for CD4+ T cells that secrete interleukin (IL)-17, termed Th17 cells. IL-17 induces IL-1, tumour necrosis factor (TNF)-α and IL-6 expression in inflamed tissues, and IL-1, IL-6, IL-21 and IL-23 are thought to drive Th17 cell development in the presence of TGF-β. However, CD8+, γδ T cells and natural killer (NK) T cells, neutrophils and microglia have also been found to secrete IL-17.

This study examined the time course and distribution of IL-17-producing T cells involved in the pathogenesis of EAE. Significant infiltration of CD4+ T cells and γδ T cells into the brain and spinal cord of mice was observed immediately prior to the onset of clinical signs of EAE. A high frequency of γδ T cells expressed IL-17, but not IFN-γ, in the acute phase of disease. These cells were found to be CD27- and predominantly of the Vγ4 subset. IL-17 production by CD4+ Th17 cells was also significantly elevated at the onset of clinical signs and in the acute phase of disease. These findings correlated with increased expression of IL-17, IL-23, IL-1β, IL-6 and TGF-β mRNA in the cerebral cortex, cerebellum and spinal cord. Conversely, IL-17 expression, as well as that of IL-1β, IL-6 and IL-23, was significantly upregulated in the spleens of mice 7 days after induction of EAE, and decreased thereafter. This coincided with a significant increase of Th17 cells in the spleen and lymph nodes at this stage. IL-17 expression by NK cells and CD8+ T cells did not change significantly over the course of disease.

It is demonstrated here that γδ T cells constitutively express the IL-23 receptor (IL-23R) and the key Th17-associated transcription factor retinoic acid receptor-related orphan receptor (ROR)γt, which was enhanced by stimulation with IL-23. Furthermore, stimulation of γδ T cells with IL-1β and IL-23, without T cell antigen-receptor (TCR) engagement promoted IL-17A, IL-17F, IL-21, IL-22, TNF-α and IFN-γ mRNA expression. Administration of IL-1β and IL-23 in vivo also resulted in elevated IL-17 gene expression and increased frequency of IL-17+ γδ T cells in draining lymph nodes.

IL-1 is critically required early in the differentiation of Th17 cells and IL-1-deficient mice are resistant to EAE. It is demonstrated here that pharmacological inhibition of caspase-1, essential for processing of IL-1β to its active form and secretion from the cell, significantly attenuates EAE. This was associated with decreased antigen-specific IL-17 production, reduced infiltration of Th17 and Th1 cells into the CNS and reduced accumulation of macrophages and neutrophils in the chronic phase of disease.

These findings are consistent with an essential role for IL-1β and IL-23 in the development and effector function of Th17 cells and the pathogenesis of EAE. Moreover, this study demonstrates that γδ T cells are an important innate source of IL-17 that may be pathogenic in the neuroinflammation associated with MS and EAE. As a small, discrete population uniquely defined by restricted TCR usage and tissue specific localization, γδ T cells may represent an attractive therapeutic target over the pan-T cell therapies currently in use for MS.
Acknowledgements

First and foremost, I would like to thank Kingston for his direction, support and constructive criticism throughout my Ph.D. and particularly in the preparation of this thesis. Kingston has a lot of faith in all of us and our abilities in the lab, and that was really encouraging when experiments didn’t work or results were not what we expected. I was fortunate to undertake my Ph.D. in a lab where funding for consumables or otherwise was never an issue, and that comes from Kingston’s extremely hard work ethic and fantastic ability to get grants (I’m still waiting to sample those irradiated strawberries!). Despite this, Kingston’s door has always been open and he was always available with advice, encouragement and enthusiasm for my project. I would also like to thank Tom Connor and Marina Lynch who share a great enthusiasm in the area of neuroimmunology and who were also always there with advice and encouragement throughout my time in Trinity College.

A big thank you to all the members of the Immune Regulation Research Group for their ideas, technical assistance and guidance throughout much of these studies; but mostly for making my time in the lab one full of memories of great times. Thanks, in particular, to Áine for keeping it neuro! and just being a great friend. Thanks also to Barry for all his help making sense of endless dotplots. Sween and Sharpie – to Team Transfer!! You’re up next, best of luck with it. Sweeney especially, your fantastic attitude was brilliant in the reading room – you’re already being missed down there. And Fiona – I’m looking to collecting the bet!! Thanks, also to Jean for regular chats about all things IL-17, and more recently to Aisling for being so helpful with my new foray into the world of inflammasomes. Thanks to Karen - it was nice to have someone to share the late evenings and frustration with figures whilst putting this thesis together. Vivienne, Maureen and Oonagh were all brilliant steering the ship (and cracking the whip!). Thanks for everything. And to Catherine for her deftness with all matters
monetary. Caroline, thank you for all your help and hard work on the Immunity paper, it’s a nice one to have in the bag already! And an especially big thank you to Corinna and Sarah; you really helped me get up and going when I started in the lab and, together with Aine, were always there to help with big experiments and just generally adding a bit of fun around the lab! But to everybody, Padraig, Neil, Helen, Fionnuala, Pam, Deirdre, Brian, Kev, Fen, Keith, PJR, Aileen, Pat, Lisa, Michelle, Ania, Anna, Lara, Paddy, Anne, Ed, Edel and all the Lavelle lab, as well as all my friends in TCIN – it really has been a great few years.

Special thanks to Ruthie. You have been so patient these last couple years, never mind the last couple weeks, and it has been fantastic to have someone special like that. We’ve had a lot of fun just going for pints or going away, and it was always wonderful to have that to look forward to after the tough days in the lab. To all my family and friends, Mam and Dad, Joss, Katie and Buddy in particular, you have put up with my long hours and probably a mopey attitude when I got home late. But you were always supportive and interested in my work, and that was really encouraging. Thanks so much for everything.

Finally, I would like to thank the Health Research Board for the funding to carry out this research.
Publications


Murphy, A.C.*, Lalor, S.J.*, Lynch, M.A. and Mills, K.H.G. (2010). Infiltration of Th1 and Th17 cells and activation of microglia in the CNS during the course of experimental autoimmune encephalomyelitis. *Brain Behav Immun.* doi:10.1016/j.bbi.2010.01.014 *These authors contributed equally to this work


<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>αβ</td>
<td>Alpha beta</td>
</tr>
<tr>
<td>AKT</td>
<td>Serine/threonine kinase</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>Allophycocyan</td>
<td></td>
</tr>
<tr>
<td>ASC</td>
<td>Apoptosis-associated speck-like protein containing a CARD</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone marrow-derived dendritic cells</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase-activating recruitment domain</td>
</tr>
<tr>
<td>CCL</td>
<td>Chemokine (C-C motif) ligand</td>
</tr>
<tr>
<td>CCR</td>
<td>Chemokine (C-C motif) receptor</td>
</tr>
<tr>
<td>CXCL</td>
<td>Chemokine (C-X-C motif) ligand</td>
</tr>
<tr>
<td>CXCR</td>
<td>Chemokine (C-X-C motif) receptor</td>
</tr>
<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>CFA</td>
<td>Complete Freund’s adjuvant</td>
</tr>
<tr>
<td>CIA</td>
<td>Collagen-induced arthritis</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine-guanine dinucleotide</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>CSIF</td>
<td>Cytokine synthesis inhibitory factor</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>DAMP</td>
<td>Damage associated molecular pattern</td>
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<td>Abbreviation</td>
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<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ds</td>
<td>Double stranded</td>
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<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>EBAO</td>
<td>Ethidium bromide acridine orange</td>
</tr>
<tr>
<td>ECSIT</td>
<td>Evolutionarily conserved signaling intermediate in Toll pathways</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
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<tr>
<td>FACS</td>
<td>Fluorescence associated cell sorting</td>
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<tr>
<td>FCS</td>
<td>Foetal calf serum</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>γδ</td>
<td>Gamma delta</td>
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<tr>
<td>GDNF</td>
<td>Glial cell line-derived neurotrophic factor</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-macrophage colony stimulating factor</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's balanced salt solution</td>
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<tr>
<td>HLA</td>
<td>Human leukocyte antigen</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IGF</td>
<td>Insulin-like growth factor</td>
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<tr>
<td>I-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B-cells inhibitor</td>
</tr>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>ILN</td>
<td>Inguinal lymph nodes</td>
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<td>IL-1F</td>
<td>Interleukin 1 family</td>
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<td>IL-1Ra</td>
<td>IL-1 receptor antagonist</td>
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<td>IRAK</td>
<td>IL-1 Receptor Associated Kinase</td>
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<td>IL-1RI</td>
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<td>IL-1RII</td>
<td>Type II IL-1 receptor</td>
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<td>IL-6R</td>
<td>IL-6 receptor alpha subunit</td>
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<td>IL-23R</td>
<td>Interleukin 23 receptor</td>
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<td>Ion</td>
<td>Ionomycin</td>
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<td>i.p.</td>
<td>Intraperitoneally</td>
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<td>JAK</td>
<td>Janus kinase</td>
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<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
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<tr>
<td>KO</td>
<td>Knock out</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>MAP</td>
<td>Mitogen-activated protein</td>
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<td>MBP</td>
<td>Myelin basic protein</td>
</tr>
<tr>
<td>MCP</td>
<td>Macrophage chemoattractant protein</td>
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<td>MDP</td>
<td>Muramyl dipeptide</td>
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<td>MHC</td>
<td>Major histocompatibility complex</td>
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<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
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<tr>
<td>mg</td>
<td>Milligram</td>
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<tr>
<td>ml</td>
<td>Millilitre</td>
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<td>Full Form</td>
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<tr>
<td>MMP</td>
<td>Matrixmetalloproteinases</td>
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<tr>
<td>MOG</td>
<td>Myelin oligodendrocyte glycoprotein</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
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<tr>
<td>Mtb</td>
<td><em>Mycobacterium tuberculosis</em></td>
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<tr>
<td>ng</td>
<td>Nanogram</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
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<td>NLR</td>
<td>NOD-like receptor</td>
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<td>NLRC4</td>
<td>NLR family, CARD domain containing 4</td>
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<td>NOD</td>
<td>Nucleotide-binding oligomerization domain</td>
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<td>OA</td>
<td>Osteoarthritis</td>
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<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>PE</td>
<td>Phycoerythrin</td>
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<tr>
<td>PI3K</td>
<td>Phosphoinositide-3-kinase</td>
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<tr>
<td>PLP</td>
<td>Proteolipid protein</td>
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<td>PMA</td>
<td>Phorbol mystic acid</td>
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<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>PP</td>
<td>Primary progressive</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>PRRs</td>
<td>Pattern recognition receptors</td>
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<td>PT</td>
<td>Pertussis toxin</td>
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<td>RA</td>
<td>Rheumatoid arthritis</td>
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<tr>
<td>rh</td>
<td>Recombinant human</td>
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<tr>
<td>RORγt</td>
<td>Retinoic acid receptor-related orphan receptor γt</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>RR</td>
<td>Relapsing-remitting</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse transcription-polymerase chain reaction</td>
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<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
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<td>SP</td>
<td>Secondary progressive</td>
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<tr>
<td>SOCS</td>
<td>Suppressor of cytokine signaling</td>
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<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
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<td>TAB</td>
<td>TGF-β-activated protein kinase 1-binding protein</td>
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<td>TAK</td>
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<td>T cell receptor</td>
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<td>TGF-β</td>
<td>Transforming growth factor-β</td>
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<td>Toll/IL-1 receptor</td>
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<td>Toll-like receptor</td>
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<td>TNF</td>
<td>Tumour necrosis factor</td>
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<td>TNF receptor associated factor</td>
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<td>Treg</td>
<td>Regulatory T cell</td>
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<td>Tyk</td>
<td>Tyrosine kinase</td>
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<td>Full Name</td>
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<td>μg</td>
<td>Microgram</td>
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<tr>
<td>μl</td>
<td>Microlitre</td>
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<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
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<tr>
<td>VLA</td>
<td>Very late antigen-4</td>
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<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
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<tr>
<td>YVAD-cmk</td>
<td>Tyr-Val-Ala-Asp-Chloromethyl Ketone</td>
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The experimental method is nothing but bringing observation and experiment into operation in order to get access to scientific truth. Some use the results of observation and experiment to build theories that they no longer put to test. They could be called *a priorists*. Instead, one’s inferences are to be tested by new experiments. This is the true experimental method *a posteriori* method. However, this is not yet sufficient. Even when attempting to verify one’s inference by an experiment or an observation, it is necessary to remain the slave of the observation, as well as of the experiment. One must not be overcome by one’s inductive idea that is nothing but a hypothesis. I can say that I follow such a precept. Thus, the verification of my inferring hypothesis, whatever its likelihood, does not blind me. I hold conditionally to it. Therefore, I am trying as much to invalidate as to verify my hypothesis. In short, I do research with an open mind. This is the reason why I so often found results I was not looking for while investigating other things I could not find. The truth must be the goal of our studies. Being satisfied by plausibility or likelihood is the true pitfall.’

Claude Bernard (1877)
General Introduction

Chapter 1
1.1 The Immune System

The very foundation of immunity is the ability of an organism to discriminate between self and non-self. In this regard, the immune system functions to clear ‘foreign’ pathogens such as bacteria and viruses from the body. The immune system consists of a number of organs, including the bone marrow, thymus, spleen and lymphatic system, and several different cell types. All cells of the immune system, tissue cells and white blood cells or leukocytes, develop from pluripotent stem cells in the bone marrow. These haemopoietic stem cells also give rise to red blood cells or erythrocytes. The development of leukocytes is through two main pathways of differentiation (Fig. 1.1). The lymphoid lineage gives rise to T- and B-lymphocytes and natural killer (NK) cells, while the myeloid pathway produces mononuclear and polymorphonuclear (PMN) leucocytes, as well as platelets and mast cells. Derived from megakaryocytes, platelets are involved in blood clotting and inflammation, while mast cells are similar to basophils but are found in tissue.

The immune system is broadly comprised of two components – the innate and the adaptive immune systems. The innate immune system is the body’s first line of defense, initiating an immediate, generalized, but short-lived response against viral, bacterial, fungal or parasitic intruders. Unlike the innate immune system, the adaptive immune system is characterized by its specificity and memory. The specificity of the adaptive system originates from the development of a diverse repertoire of T and B cell receptors that recognize a specific peptide sequence or “antigenic epitope”. Mature lymphoid cells can also be long lived and may survive for many years as memory cells.
Chapter I

1.1.1 The Innate Immune System

The healthy individual is protected from potentially harmful microorganisms in the environment by a number of mechanisms present from birth, which do not require prior exposure to any particular pathogen. These innate defense mechanisms are non-specific in that they are effective against a wide range of potentially infectious agents, and fall into two broad categories: constitutive and inducible immunity.

1.1.1.1 Constitutive Innate Immunity

The intact skin and mucous membranes of the body afford a high degree of protection against pathogens. The sebaceous secretions and sweat of the skin contain bactericidal and fungicidal fatty acids and these constitute an effective protective mechanism against many potential pathogens. The mucus covering the respiratory tract acts as a trapping mechanism for inhaled particles. Cilia sweep the secretions, containing the foreign material, toward the oropharynx so that it is swallowed; acidic secretions in the stomach then destroy most of the microorganisms present. Nasal secretions and saliva contain mucopolysaccharides capable of blocking entry of some viruses, and tears and the mucous secretions of the respiratory, alimentary and genitourinary tracts contain lysozyme that is particularly active against some Gram-positive bacteria. The washing action of tears and the flushing of urine are also effective in stopping invasion by microorganisms. Commensal organisms in the gut and bacteria normally present on the skin provide a further layer of constitutive innate immunity. This normal bacterial flora produce various antimicrobial substances, such as bacteriocines and acids, while at the same time compete with potential pathogens for essential nutrients (Brock, 1998).
Figure 1.1  Cells of the immune system

Both the lymphoid and myeloid lineages derive from pluripotent stem cells present in the bone marrow. A common lymphoid progenitor cell gives rise to NK, T- and B-cells. NK cells and B cells develop in the bone marrow; B cells becoming antibody-producing plasma cells when fully differentiated. Lymphoid progenitors that mature in the thymus differentiate into either CD4⁺ or CD8⁺ T cells. Myeloid progenitors are capable of producing the granulocytes (basophils/mast cells, eosinophils, and neutrophils), megakaryocytes that give rise to platelets, and monocytes that differentiate into macrophages when they enter the tissue, or dendritic cells. (Figure adapted from The Biology Project, a University of Arizona online interactive resource (http://www.biology.arizona.edu/immunology)).
1.1.1.2 Inducible Innate Immunity

The inducible arm of the innate immune system relies on either innate cells or soluble protein effectors, and is triggered upon pathogen recognition by a set of pattern recognition receptors (PRRs; Janeway & Medzhitov, 2002). The principle functions of PRRs include opsonization, activation of complement, phagocytosis, activation of proinflammatory signaling pathways, and induction of apoptosis (Janeway & Medzhitov, 2002). PRRs are structurally and functionally heterogeneous proteins and can be categorized according to their function (Medzhitov, 2001). The best known PRRs are the Toll-like receptor (TLR) family and the intracellular nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) proteins. NLRs are expressed in the cytosol, where they detect intracellular pathogens, such as viruses and bacteria, and their products, that have gained access to intracellular compartments. The TLRs are a large family of PRRs that detect pathogens either at the cell surface or at lysosome / endosome membranes (Akira et al., 2006).

PRRs recognize conserved microbial components called pathogen associated molecular patterns (PAMPs; Janeway & Medzhitov, 2002). Some of the better known examples include lipopolysaccharide (LPS) from Gram-negative bacteria, lipoteichoic acid (LTA) from Gram-positive bacteria, cytosine-guanine dinucleotide (CpG) motifs, lipoproteins and double stranded (ds) RNA, produced by viruses during their infection cycle. These structures are only expressed by particular classes of microorganisms allowing the innate immune system to distinguish between self and non-self molecules. Expression of specific PAMPs is often shared by large groups of microorganisms allowing a limited number of germline-encoded PRRs to recognize a immense variety of potential pathogens (Medzhitov, 2001). Recognition of PAMPs by PRRs activates conserved host defense signaling pathways that control the expression of a variety of immune response genes (Medzhitov, 2001).
1.1.1.3 Cells of the Innate Immune System

The inflammatory innate immune response is primarily mediated by myeloid phagocytic cells, such as PMN, macrophages and dendritic cells (DC), that either kill the invading microbe or secrete proteins called cytokines, which mediate the influx of defensive cells to the site of infection. NK cells of the lymphoid lineage, however, also contribute to innate defense during certain viral infections. Viruses can only replicate within host cells, thus it is important to eliminate such cells before progeny virus is released. NK cells, which account for up to 15% of blood lymphocytes, recognize changes on virus infected cells and destroy them via an extracellular killing mechanism. On binding to infected cells, NK cells produce membrane-disrupting molecules that lead to the destruction of the target cell. This innate defense mechanism occurs without prior exposure to the infectious agent. Using a similar mechanism, NK cells are also thought to play a role in host defense against tumors, recognizing changes in the cell membrane of transformed cells (Lanier, 2005). The cytokines interleukin (IL)-12 and tumor necrosis factor (TNF)-α act in synergy to stimulate the release of large quantities of another cytokine, interferon (IFN)-γ, which plays a critical role in controlling many infections before T cells have been activated. A small population of T cells, particularly CD8⁺ T cells, also express NK cell markers, and these are referred to as NKT cells (Biron & Brossay, 2001).

PMN, also known as granulocytes, comprise predominantly of neutrophils, but also eosinophils and basophils. All PMN are short-lived cell types. Neutrophils are the most abundant type of phagocyte, accounting for up to 50-60% of all circulating leukocytes. Neutrophils engulf pathogens, and internalize them into phagosomes for destruction. Phagosomes fuse to membrane-bound granules called lysosomes, which contain antimicrobial substances that are released into the phagosome, destroying the pathogen. Eosinophils are present in
the blood of normal individuals at a very low frequency (<1%), but their numbers increase in patients with parasitic infections and allergies (Brock, 1998). They are not efficient phagocytic cells but their granules release molecules that are highly toxic to parasites. They also release prostaglandins, leukotrienes and cytokines, which amplify the proinflammatory response. Basophils are the least common of the granulocytes, accounting for 0.1 – 0.3% of circulating leukocytes, and have a similar role to eosinophils. They release histamine upon activation resulting in vasodilation, thereby allowing recruitment of other immune cells to the site of infection. Mast cells also contain granules rich in histamine are similar to basophils, but are found within tissue.

Circulating monocytes give rise to a variety of tissue-resident macrophages throughout the body, as well as to specialized cells such as DC. Monocytes originate in the bone marrow from a common myeloid progenitor that is shared with PMN, and are then released into the circulation, where they constitute 5 to 10% of peripheral-blood leukocytes in humans. They may circulate for several days before entering tissues and replenishing the tissue macrophage populations (Gordon & Taylor, 2005). Macrophages are primarily phagocytic cells that engulf pathogens and destroy them in intracellular vesicles. They can also be stimulated to express major histocompatibility complex (MHC) class II and costimulatory molecules, enabling them to function as antigen presenting cells (APC; Hume, 2008). In this regard, macrophages can link the innate and adaptive immune responses.

DC, however, are the principal cell type bridging the innate and adaptive immune systems. As so-called professional APC, DC are very efficient at internalizing antigen, either by phagocytosis or by receptor-mediated endocytosis, and then displaying a fragment of the antigen, bound to an MHC class II
molecule, on their membrane (Guermonprez et al., 2002). DC can be divided into numerous subsets based on phenotype, function and location. By expressing a high concentration of TLRs, DC serve as sentinels against invading pathogens (Cutler et al., 2001). Besides acting as immunological sensors through the expression of PRRs, DC can also sense pathogens indirectly by detecting inflammatory mediators produced by various cells, including macrophages, NK cells and endothelial cells (Pulendran & Ahmed, 2006). These inflammation-associated tissue factors include cytokines, produced in response to microbes, chemokines, eicosanoids, heat shock proteins (HSPs) and extracellular matrix components.

Immature DC internalize pathogens/exogenous antigens and process them for MHC class II-mediated presentation. In their immature state, they express low levels of MHC class II and costimulatory molecules, and cannot prime T cell responses. Upon activation, however, DC migrate to the lymph nodes and undergo a maturation process, shutting off their antigen capturing and processing capacity. Mature DC express high levels of MHC and costimulatory molecules, including CD80, CD86, CD40 and CD83, rendering them capable of priming naïve T cells (Fig. 1.2) (Pulendran, 2004). DC also secrete cytokines which help determine the phenotypic lineage of effector T cells. T cells are initially activated as Th0 cells, and their differentiation into the various Th cell subsets is influenced by the cytokines in the surrounding environment (Fig. 1.3). Mature DC, therefore, are primed by pathogens to activate pathogen-specific T cells with signals that determine effector function.
Figure 1.2 DC maturation and antigen presentation

Upon exposure to antigens, immature DC mature into APC expressing MHC peptide and co-stimulatory receptors on their surface following ligation of PRRs (A). Mature DC then provide three signals to naïve T cells that mediate activation and polarization to the different Th cell subsets (B). The peptide-MHC complex engages the TCR, co-stimulatory molecules such as CD80 and CD86 trigger CD28 signaling in the T cell, while soluble cytokines released by the DC provide the polarizing signal required for differentiation to specific Th subsets. The nature of the third signal depends on activation of particular PRRs by PAMPs or inflammatory tissue factors and often requires feedback stimulation by CD40 ligand (CD40L) expressed on T cells after activation via the TCR and co-stimulatory molecules. Adhesion molecules such as DC-SIGN and ICAM-3 maintain stability of the synapse during antigen presentation. (Figure adapted from Sarkar and Fox, Frontiers in Bioscience 10, 656-665, 2005).
1.1.2 The Adaptive Immune System

Microorganisms that overcome or circumvent the non-specific innate immune mechanisms, or are administered deliberately by active vaccination, are met by the host’s second line of defense, the adaptive or acquired immune system. Initiation of this form of immunity requires presentation of antigens of the invading pathogen by APC of the innate immune system to lymphoid cells of the adaptive immune system. The lymphocytes that respond are precommitted, because of their surface receptors, to respond to a particular epitope of the antigen. This response takes two forms, humoral and cell-mediated immunity, that usually operate in parallel. B cells respond to APC-presented antigen by differentiating into antibody-producing plasma cells (humoral immunity), while T cells are responsible for cell-mediated immunity (Brock, 1998). Lymphocytes constitute about 20% of all leukocytes present in the adult circulation. These mononuclear cells are heterogeneous in terms of size and morphology; T- and B-cells are typically small and agranular, whilst plasma cells are larger and contain cytoplasmic granules. T cells and B cells are distinguished by their site of differentiation, the thymus and the bone marrow, respectively, as well as the mechanism of their effector response.

1.1.2.1 B cells

B cells constitutively produce immunoglobulins which are inserted into the cell surface membrane where they act as antigen-specific B cell receptors (BCR). The majority of human B cells in peripheral blood express two immunoglobulin (Ig) isotypes, IgM and IgD, on their surface. Less than 10% of B cells in the circulation express IgG, IgA or IgE, although these are present in larger numbers in specific locations of the body (Delves & Roitt, 2001). The majority of B cells express MHC class II antigens, which are important for cognate interactions with T cells.
Chapter 1

Following activation, many B cells mature into terminally differentiated plasma cells. Plasma cells are infrequent in the blood, and are normally restricted to the secondary lymphoid organs and tissues, as well as bone marrow (Delves & Roitt, 2001). Antibodies produced by a single plasma cell are of one specificity and Ig class. Antibodies can coat a pathogen making them easier to phagocytose, a process known as opsonisation. Furthermore, they can activate the complement system which leads to the destruction of bacterial cells. Most plasma cells have a short lifespan, surviving for a few days before dying by apoptosis.

1.1.2.2 T cells

T cells are derived from lymphoid precursors, which are produced in the bone marrow and differentiate in the thymus, where they acquire the ability to distinguish self from non-self. T cells are the effector cells of the adaptive immune response, recognizing antigen via a clonally distributed TCR heterodimer. The expression of TCR molecules is acquired intrathymically during T cell maturation. The TCR expressed on 95% of T cells is a heterodimer consisting of an α-chain and a β-chain at the variable domains of the antibody arms, which together form the TCR complex. Less than 5% of T cells express a TCR consisting of the γ- and δ-chains. Following recognition of MHC-bound antigen through the TCR, they respond to endogenous and exogenous antigens in a cell-mediated manner.

Gamma delta (γδ) T cells are unconventional in that their recognition is carried out directly, in contrast to the MHC-dependent antigen presentation process employed by αβ T cells. The lymph nodes and T cell areas of the spleen are the only known structures in which a wide variety of antigens are presented to cognate lymphocytes, resulting in their differentiation and clonal expansion.
Because γδ T cells are found in relatively low numbers in these tissues suggests that these cells do not usually rely on professional APC for antigen recognition (Hayday, 2000). As such, a ‘first line of defense’ hypothesis has been adopted, which proposes that γδ T cells respond to unique ‘stress antigens’ that are markers of cell infection or transformation, rather than a diversity of microbial antigens (Janeway et al., 1988; Hayday, 2009). γδ T cells are thought to respond to non-peptide antigens, phosphoantigens, alkylamines, heat-shock proteins, and non-classical MHC class I molecules, and have been shown to proliferate in response to pathogenic infection by *Mycobacterium tuberculosis*, *Plasmodium sp.*, *Toxoplasma gondii*, *Yersinia enterocolitica*, Epstein-Barr virus and early HIV infection (Guerrero et al., 2003).

γδ T cells, like their αβ counterparts, exist as a number of different subtypes, distinguishable by their use of the variable regions of both the γ and the δ genes. In addition, they express unique combinations of adhesion molecules which determine differences in migration and tissue localization (Poggi et al., 2007). Murine γδ T cells are distinguished by their Vγ usage, with Vγ1 and Vγ4 the two main peripheral γδ T cell subsets in the mouse. Vγ1 γδ T cells are typically tissue derived, found in the spleen, lymph nodes and intestine. They are auto-reactive to Hsp60, and produce IL-17 during lung infection (Romani et al., 2008). Vγ4 are the circulatory γδ T cells, found in the blood, spleen and lymph nodes. Among the remaining five subtypes, Vγ5 γδ T cells are thymus-dependent and predominantly found in the skin, while Vγ7 γδ T cells are the predominant subtype in the intestine and can develop independent of the thymus.

Although relatively rare in the circulation (2-4%), γδ T cells comprise the majority of intraepithelial lymphocytes, where they may play a role in protecting the mucosal surfaces of the body. The different tissue-specific subsets of γδ T
cells are thought to represent an innate-like defense recognizing ligands induced by stress, inflammation or infection, while also providing other tissue-specific functions, including the regulation of wound healing (Hayday & Tigelaar, 2003). In humans, however, specific \( \gamma\delta \) T cell subsets are not as prominent or as restricted in their diversity, highlighting differences between human and murine \( \gamma\delta \) T cell populations. In many respects \( \gamma\delta \) T cells are thought to function largely like their \( \alpha\beta \) counterparts. In addition, however, they have recently been demonstrated to efficiently process and display peptide antigen to \( \alpha\beta \) T cells, displaying features of professional APC (Brandes et al., 2005). \( \gamma\delta \) T cells, therefore, have characteristics of both the adaptive and innate immune systems, and perhaps represent a conserved, primitive form of immunity (Modlin & Sieling, 2005).

\( \gamma\delta \) T cells have recently come to be acknowledged as a major source of IL-17. Like conventional IL-17-producing \( \alpha\beta \) T cells, IL-17-producing \( \gamma\delta \) T cells require the transcription factor ROR\( \gamma \)t for their development (Ivanov et al., 2006). In bacterial infections, \( \gamma\delta \) T cells can rapidly produce IL-17 (Lockhart et al., 2006; Shibata et al., 2007), and in some models have been demonstrated to be the main source of IL-17 (Umemura et al., 2007). Interestingly, naïve \( \gamma\delta \) T cells have also been demonstrated to produce IL-17 upon stimulation (Stark et al., 2005; Romani et al., 2008), an ability established by their lack of CD27 expression during thymic development (Ribot et al., 2009). Thus, it seems possible that certain, perhaps tissue-specific, subsets of \( \gamma\delta \) T cells are already differentiated in the periphery, and are capable of rapidly producing IL-17 upon activation.

\( \alpha\beta \) T cells are subdivided into two distinct, non-overlapping populations; T cells expressing the CD4 co-receptor on the cell membrane have a central function in the generation, maintenance and regulation of both the humoral and
the cellular arm of the immune response. As such, they are defined as 'helper' T (Th) cells, which are further subdivided based on their cytokine expression profile and associated functions. Conversely, differentiated T cells expressing the CD8 receptor are defined as 'cytotoxic' as they develop an armory of cytolytic proteins that enable them to kill pathogen-infected cells by activating an apoptotic program. CD4⁺ T cells recognize their specific antigen in association with MHC class II molecules, whereas CD8⁺ T cells recognize antigen in association with MHC class I molecules. Thus, the presence of either the CD4 or CD8 co-receptor on the surface of the T cell restricts the type of cell with which it can interact.

Classically, effector CD4⁺ T cells have been divided into two lineages based on their cytokine profile (Mosmann et al., 1986). This Th1 / Th2 dichotomy subdivides Th cells into those specialized for cell-mediated defense against intracellular pathogens, particularly viruses but also some bacteria (Th1 cells), and those that mediate humoral immune responses against large extracellular pathogens such as helminths (Th2 cells; Fig. 1.3). IL-12 and IL-18, proinflammatory cytokines produced by subsets of DC and macrophages, are the main polarizing factors in the differentiation of naïve T cells into Th1 cells (Okamura et al., 1995; Trinchieri, 2003); however, IL-27 (Lucas et al., 2003), type I interferons (IFNs; Kadowaki et al., 2000) and cell surface-expressed intracellular adhesion molecule (ICAM)-1 (Salomon & Bluestone, 1995) may also be involved. The anti-inflammatory cytokine IL-4 promotes the induction of Th2 cells from naïve T cells, augmented by the DC-derived factors chemokine (C-C motif) ligand 2 (CCL2) and OX40 ligand (Ohshima et al., 1998). Th1 cells are dependent on IFNγ to mediate their responses, whereas Th2 cells secrete IL-4, IL-5 and IL-13, and induce B cell growth and differentiation, and in turn, Ig production. The discovery of various Th1- (T-bet and Stat-4) and Th2-related transcription factors (GATA-3 and Stat-6) further added to the validity of the Th1 / Th2 paradigm. Moreover, in many instances Th2 cytokines are antagonistic to
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Th1 cell development and function, and Th1 cytokines inhibit Th2 responses. Many autoimmune diseases have long been thought to depend on a Th1 response to autoantigens (O’Garra *et al.*, 1997). In contrast allergy and asthma result from an imbalance in favour of a Th2 response (Cohn *et al.*, 2004). For over two decades, this dogma has been a cornerstone of our understanding of immune responses.

Recently, a T cell population that produces IL-17, but not IFN-γ or IL-4 and therefore distinct from Th1 and Th2 cells, has been described (Aggarwal *et al.*, 2003; Langrish *et al.*, 2005). IL-6 and transforming growth factor (TGF)-β were initially shown to induce the differentiation of naïve murine T cells into Th17 cells (Bettelli *et al.*, 2006; Mangan *et al.*, 2006; Veldhoen *et al.*, 2006). IL-1 signaling has since been found to be critically required for early development of Th17 cells (Sutton *et al.*, 2006; Chung *et al.*, 2009). IL-23 is necessary for the full differentiation of activated T cells into effector Th17 cells (McGeachy *et al.*, 2009). Additionally, IL-21 was found to be a key cytokine in the differentiation of murine and human CD4⁺ Th17 cells, where it appears to act in an autocrine loop involving IL-6 and IL-23 (Korn *et al.*, 2007; Nurieva *et al.*, 2007; Zhou *et al.*, 2007). Expansion of memory Th17 cells is promoted by IL-1 and IL-23 (Langrish *et al.*, 2005; Higgins *et al.*, 2006; Sutton *et al.*, 2006). The nuclear receptor retinoic acid receptor-related orphan receptor (ROR)γt is the key transcription factor required for the development of Th17 cells (Ivanov *et al.*, 2006). Differentiation of Th17 cells is inhibited by IL-12, IFN-γ and IL-4 (Cua *et al.*, 2003; Harrington *et al.*, 2005). In addition to IL-17A, Th17 cells have been shown to produce a variety of cytokines, including IL-17F, IL-21 and IL-22 (Fig. 1.3).
IL-17, or IL-17A, is the founding member of the IL-17 family of cytokines, which comprises 6 members in total, designated IL-17A-F. IL-17F shares the greatest homology (55%) with IL-17, and both are produced by Th17 cells. Conversely, IL-17B-D are produced by non-T cell sources. IL-25 (IL-17E) functions to promote differentiation toward Th2 cell fate in an IL-4-dependent manner (Fort et al., 2001). IL-17A and F may exist as homodimers or as IL-17A-IL-17F heterodimers; however, IL-17A homodimers are the most efficient at inducing chemokine expression by epithelial cells (Liang et al., 2007). IL-17 induces IL-1, TNF-α and IL-6 expression, as well as a number of leukocyte-specific chemokines, in inflamed tissues, and Th17 cells have been shown to be the chief pathogenic cell type in animal models of autoimmunity, including experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA) (Langrish et al., 2005; Murphy et al., 2003).

A population of T cells with regulatory or suppressive activity, termed regulatory T cells (Treg), have also been described, which can control the various effector T cell and other inflammatory responses during infection (Mills, 2004). These have been divided into several subsets with distinct phenotypes and mechanisms of action. Natural (n)Treg cells are generated in the thymus and represent 5-10% of the total peripheral CD4⁺ T cell population. These CD4⁺CD25⁺ cells express the transcription factor Forkhead box P3 (Foxp3), which is essential for directing regulatory function, and control autoimmunity by mediating immunological tolerance to self antigens (Sakaguchi et al., 1995). They are profoundly immunosuppressive and block CD4⁺ and CD8⁺ T cell proliferation. Conversely, adaptive or inducible Tr1, Th3 and various subsets of CD8⁺ Treg cells are derived in the periphery from uncommitted naïve T cells stimulated by antigen under the influence of the immunosuppressive cytokines IL-10 and TGF-β (Groux et al., 1997). The suppressor activity of these Treg cells is cell-cell contact independent and is mediated primarily through the actions of the
anti-inflammatory cytokines IL-10 and TGF-β. Tr1 cells secrete high levels of IL-10, with low levels of IL-5 and TGF-β, and inhibit antigen-specific T cell responses (Groux et al., 1997). Th3 cells preferentially secrete TGF-β together with varying amounts of IL-4 and IL-10. Mainly mucosal in origin, Th3 cells were originally identified in mice during experiments on the induction of oral tolerance to myelin basic protein (MBP) in EAE, where they mediated suppression in a TGF-β-dependent manner (Weiner, 2001). However, since thymically derived nTreg cells are specific for self antigens, they are thought to play a dominant role in preventing autoimmunity, whereas inducible Treg cells are more likely to be generated in response to foreign antigens during infection. Various autoimmune disorders have been described to result from alterations in the function of these regulatory T cells (Viglietta et al., 2004).
Figure 1.3 The differentiation and regulation of CD4 T cell subsets

Naïve T cells primed by APC such as DC can differentiate into Tr1/Th3, Th1, Th2 or Th17 cells depending on the cytokine environment. Priming in the presence of IL-4 promotes the differentiation of Th2 cells, which mediate their effects via the anti-inflammatory cytokines IL-4, IL-5 and IL-13. Th1 cells differentiate in response to IL-12, and produce IFN-γ and IL-2. Combinations IL-6, IL-1 and IL-23 promote the differentiation of Th17 cells, which are negatively regulated by Th1 or Th2 cells. IL-10 and TGF-β are involved in the differentiation of Tr1 and Th3 cells, which also secrete IL-10 and TGF-β and can suppress effector cell responses, primarily by suppressing APC function.
1.2 The Nervous System

The primary function of the nervous system is to allow the body to sense and react to changes in the environment, both internally and externally. The vertebrate nervous system is divided into the central nervous system (CNS) and the peripheral nervous system (PNS). The CNS consists of the cerebrum, the cerebellum, the brainstem, the spinal cord and the optic and olfactory nerves. The cranial nerves and their roots, the spinal nerves and their roots, the dorsal root ganglia, and the peripheral nerves comprise the PNS. The two systems intersect where the cranial nerves enter and leave the brainstem, and where the dorsal roots enter and the ventral roots exit the spinal cord.

1.2.1 The Peripheral Nervous System

The PNS is mainly composed of axons and the Schwann cells that myelinate them; however, neuronal bodies are also present in the dorsal root ganglion. This system connects the CNS to the tissues and organs of the body. In this way, the PNS is responsible for conveying input and output signals to and from the CNS.

1.2.2 The Central Nervous System

The CNS integrates and controls the entire nervous system, receiving information about changes in the internal and external environments, interpreting and assimilating this information, and then sending out signals for the execution of activities. The CNS is made up of multiple specialized populations of neurons and glia, which, together with their vascularization, are enclosed in a protective sheath called the meninges. The meninges, composed of three connective tissue membranes - the dura mater, the arachnoid and the pia mater - are located between
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the brain and cranial bones and between the spinal cord and the vertebral column, and serves to support and protect the CNS.

1.2.2.1 Cells of the CNS

With the exception of microglia, all cells of the CNS are derived from neuroepithelial stem cells (Holland, 2001). Neurons consist of a cell body or soma, the metabolic centre of the neuron, and of numerous protoplasmic processes called dendrites and axons. Dendrites are short and convey impulses towards the cell body. Axons convey impulses away from the cell body and can vary in length from microns to meters. Axons are usually insulated with a sheath of myelin which increases the conduction velocity of the nerve impulse along the axon; the thicker the myelin sheath, the faster the conduction velocity. Neurons signal to muscle cells and gland cells, and from neuron to neuron, via synapses formed between the axonal terminals and the muscle or gland cell, or dendrites or the soma of post-synaptic neurons.

In addition to neurons, there are four other main cell types in the CNS. Microglial cells, the only CNS-resident cells derived from a myeloid lineage, are phagocytes that engulf the debris resulting from injury, infections or diseases in the CNS. Together with astrocytes, they form a tissue-specific innate immune system for the CNS, acting as both sentinel cells and the first line of defense by responding to non-specific "danger" signals (Carson & Sutcliffe, 1999). Astrocytes, are large star-like cells with numerous processes and, comprising approximately 70% of all cells, are the most numerous cell-type in the CNS. End-feet at the tips of these processes surround capillaries and, with a minor input from microglial cell processes, form the perivascular glia limitans. Tight junctions between brain vascular endothelial cells and between the epithelial cells lining the
Figure 1.4 Neuroepithelial stem cell origin of CNS resident cells

With the exception of microglia, which are derived from a myeloid lineage, all cells of the CNS are derived from neuroepithelial stem cells. Neurons and glia-restricted precursors are both derived from the same multipotent stem cells. The glial-restricted precursors give rise to both astrocyte-restricted precursors and O2A progenitors, which develop into both oligodendrocytes and astrocytes. Type 1 and type 2 astrocytes differ in morphology and marker expression. Glial-restricted precursor cells also give rise to radial glial cells during development, from which ependymal cells ultimately derive (not shown). (Figure adapted from Holland, EC. Nature Reviews Genetics 2, 120-129, 2001).
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choroid plexus, where cerebro-spinal fluid (CSF) is made, function with the glia limitans to form the blood-brain barrier and blood-CSF barrier, respectively, limiting the entry of blood- or CSF-borne proteins and cells into the CNS. In addition, astrocytic end-feet processes also form a protective covering just inside the meninges called the external limiting membrane or glial membrane. Astrocytes play numerous other roles, including production of neurotrophic factors, necessary for neuronal survival, and removal of certain neurotransmitters from synaptic clefts. Oligodendrocytes are small glial cells responsible for the formation and maintenance of CNS myelin (a function performed by Schwann cells in the PNS). The myelin sheath is formed by oligodendrocyte processes wrapping around the axon to form a tight spiral, with each oligodendrocyte enveloping up to 40 or 50 axons. It has recently been shown that oligodendrocytes also produce certain neurotrophic factors, including insulin-like growth factor (IGF)-1 and glial cell line-derived neurotrophic factor (GDNF), which may promote the growth of damaged CNS axons (Wilkins et al., 2003). Finally, ependymal cells line the fluid-filled cavities, or ventricles, of the brain and the central canal of the spinal cord, and bear cilia at the surface that promote the circulation of the cerebrospinal fluid (CSF).
1.2.3 Neuroimmunology

Neuroimmunology is the study of the interaction between the nervous system and immune system, and how they influence one another in health and disease. For years, the two systems have been regarded as largely independent, functionally and biochemically distinct. The general view was that the nervous system was more hard-wired, laid down during development and unchanging thereafter. The immune system, on the other hand, was understood to be more reactive; ever growing and evolving in response to a myriad insults, and learning from past encounters in order to mount a swifter, more specific response upon re-infection with a pathogen. Moreover, the idea developed that the CNS assumed a special ‘immune privileged’ status, required to protect its delicate network of complex nerve connections from potential damage by immune cells in their rush to eliminate a potential threat.

It has been known for a century or so that neurons communicate with one another via synapses. Recently, it has become apparent that immune cells also interact through synapse-like junctions, and share many common features with nerve cell synapses, including some molecules. In the immune system, MHC molecules on the surface of APC take a peptide fragment of antigen from an invading pathogen and present it to T cells in order that they can recognise the foreign body and eliminate it. A number of insightful experiments have recently shown that MHC class I molecules are essential in the developing nervous system for neurons to determine which other cells to connect to as the brain “wires up” (Huh et al., 2000). Expression of the TCR-β locus in neurons of the murine CNS during development has also been demonstrated (Syken & Shatz, 2003). Conversely, neurotrophins, a family of growth factors that induce the survival, development and function of neurons, have been shown to be involved in the regulation of MHC molecule expression (Collawn & Benveniste, 1999). Toll, another highly conserved molecule, long known for its role in the development of
the early nervous system in the fruit fly Drosophila *melangaster*, has recently been recognized as critical to that species' resistance to fungal infection in adult life (Lemaitre *et al.*, 1996). Moreover, Toll homologues, TLRs, have since been identified across a whole series of species, including mice and humans, and have become one of the most intensely studied families of receptors in both host response to infection and autoimmune disease research. Finally, semaphorins, a family of secreted or transmembrane proteins that regulate cell motility and attachment in axon guidance in the developing nervous system, have been demonstrated to be abundantly present on certain immune cells, where different members are thought to regulate proliferation, activation and effector function (Kikutani and Kumanogoh, 2003; Czopik *et al.*, 2006).

It is clear, therefore, that these two pillars of vertebrate biology do not operate in isolation. Not only is it now accepted that these two systems have the ability to interact with one another, but also that they share many of the same molecules. With this in mind, we must no longer view disease from the perspective of one systems influence on the passive other, but rather how the two systems interact with and influence one another in health and disease.

1.2.4 Peripheral Immune Cells in the CNS

Classically, the brain has been thought of as being devoid of cells of the immune system, largely due to some early studies that showed that tissue grafts transplanted there survive longer than similar grafts in other sites (Barker &Billingham, 1977). This 'immune-privileged' nature of the brain was ascribed to the existence of the blood-brain barrier, but also the lack of lymphatic drainage, a lack of constitutive expression of MHC molecules, and the possible presence of certain nervous system-restricted chemical molecules that might retard
lymphocyte traffic. However, numerous recent studies have shown that immune responses proceed in the nervous system in a manner relatively similar to that in other organs; namely, that activated T cells preferentially migrate from the blood into tissues. Whilst the blood-brain barrier may limit the access of circulating antibodies to the nervous system, it does not appear to limit T cell access, as activated T cells of any specificity, but not resting ones, can readily cross to the CNS (Wekerle et al., 1987). Activated and memory T cells express adhesion molecules, chemokines and integrins that allow them to cross the blood-brain and blood-CSF barriers (Ransohoff et al., 2003). Immune surveillance of the healthy brain is critical for the protection of the CNS against infection and manifestations of cancer. Indeed, it has recently been demonstrated that blocking immune surveillance by targeting adhesion molecules with natalizumab (Tysabri), a monoclonal antibody therapy for the treatment of multiple sclerosis (MS) which prevents T cell migration across the blood-brain barrier but may also block APC-T cell interaction, thus compromising T cell-mediated immunity, can lead to the expansion of chronic, latent viral infections of the CNS, resulting in the sometimes fatal condition progressive multifocal leukoencephalopathy (PML; Langer-Gould et al., 2005).

Low numbers of T cells are consistently demonstrable in normal human and rat brains (Booss et al., 1983; Wekerle et al, 1987), indicating that the CNS is continuously patrolled by activated T cells. However, the initiation of neuroinflammation in CNS autoimmunity requires reactivation of myelin-specific T cells by APC presenting self antigen (Ransohoff et al., 2003). T cell reactivation triggers the release of cytokines and chemokines by many cell types, resulting in the recruitment of other inflammatory cells, and, in turn, disruption of the blood-brain barrier and the mass influx of non-specific leukocytes from the periphery. The nervous system is a critical yet delicate organ, with its intricate network of specialized cells and subcellular components. As such, its status as a
special immune-privileged site is indispensable for damage limitation during inflammation. This privilege, however, is less a complete barrier against immune system components entering the CNS but more strong local regulation of inflammatory mechanisms by CNS-resident cells and their microenvironment.

1.3 Multiple Sclerosis

MS is an inflammatory disease of the CNS that is caused by demyelination of axons, either directly or indirectly, by cells of the immune system and is characterised by inflammatory infiltrates composed of T cells, B cells and macrophages and focal demyelinating plaques within the CNS (Stromnes & Goverman, 2006). It is both clinically and pathologically heterogeneous, perhaps due to some complex genetic trait that translates into different immune abnormalities and/or increased vulnerability of CNS tissue to inflammatory insult or reduced ability to repair damage (Sospedra & Martin, 2005). Furthermore, there is a wide range of potential target antigens in the CNS, even within a single specialised structure such as the myelin sheath. The segmental and topographical organisation of the nervous system may also add to the diversity of clinical presentations (Pender and McCoombe, 1995). MS leads to substantial disability through deficits of sensation and of motor, autonomic, and neurocognitive function.

There are three main forms of MS. Relapsing-remitting (RR)-MS is the most frequent, affecting 85–90% of patients, and is characterised by clinical exacerbations interspersed by periods of inactivity (Keegan & Noseworthy, 2002). Most RR-MS patients later develop secondary progressive (SP)-MS, a stage of chronic disease in which patients see no let-up of clinical signs. This stage of
disease is resistant to treatment, possibly due to neurodegenerative mechanisms within the disease plaques. About 10% of patients present with insidious disease onset and steady progression, termed primary progressive (PP)-MS (Keegan & Noseworthy, 2002). This heterogeneity is mirrored in morphological alterations of the brain as found by magnetic resonance imaging (MRI) or histopathological evaluation, as well as in clinical presentation; for example, which CNS areas are primarily affected and whether a patient responds to treatment.

The age of onset of MS is highly variable, but usually peaks between 20 and 40 years of age. As such, it is the most common CNS disorder of young people. MS affects roughly 2.5 million people worldwide (McKeown et al., 2003). There is a preponderance of females to males of at least 2-3:1. The most common clinical symptoms are: monocular visual loss due to optic neuritis; weakness of the lower limbs, with or without upper limb weakness; sensory loss or paraesthesia of the limbs or trunk; sensory or cerebellar ataxia; cranial nerve symptoms and signs including facial sensory disturbance, diplopia (double vision), oscillopsia (swinging vision) and nystagmus (rapid, rhythmic and repetitious involuntary eye movements), due to brain stem involvement; bladder and bowel disturbance; and memory and cognitive impairment (Pender and McCoombe, 1995). Clinical evidence has shown involvement of the same or different regions of the CNS in different attacks (Pender and McCoombe, 1995).

The etiology of MS is complex and largely unknown. It is most probable that MS has a number of causal factors that differ between individuals, with both genetic and the environmental factors involved. The concordance rate for MS in monozygotic twins was found to be 25.9%, much higher than that in dizygotic twins (2.3%) or non-twin siblings (1.9%) (Ebers et al., 1996). While multiple genes appear to be involved, genetic susceptibility seems greatest for the MHC
class II region histocompatibility leukocyte antigen (HLA) genes and T cell receptor (TCR) genes (Keegan & Noseworthy, 2002). However, the relatively low concordance rate in identical twins suggests that other, possibly environmental, factors may play a role. Diet, infections, and differences in exposure to sunlight have all been implicated in the etiology of MS. Data on the epidemiology of MS is quite variable, however, MS is prevalent in less than 5/100,000 people in equatorial regions, up to 20/100,000 people in Southern USA and Southern Europe, but more than 100/100,000 in Northern Europe, Northern USA and Canada (WHO and MSIF, 2008). Interestingly, Japan, on similar latitude, has a relatively lower prevalence (Compston, 1997). Moreover, the susceptibility to MS is higher in people who migrate from low risk geographical areas to high risk areas, and those that relocate to low risk areas before the age of 15 lose their increased susceptibility (Kurtzke, 2000). The significantly greater prevalence of MS in females also indicates that hormones may play some part.

The pathological hallmark of MS, whether determined histopathologically or by MRI, is the inflammatory plaque (Frohman et al., 2006). It is thought that this originates from a breach of the blood-brain barrier in a person who is genetically predisposed to the disease. Some form of systemic infection may result in the activation of peripheral lymphocytes as well as the up-regulation of adhesion molecules on the endothelium of the brain and spinal cord. Activated lymphocytes home to the CNS, attach to the endothelium and traverse the vessel walls. If lymphocytes programmed to recognize myelin antigen exist within the cell infiltrate, they may be reactivated by antigenic myelin fragments presented in an MHC-restricted pattern by endogenous APC, including microglia and perivascular macrophages, resulting in the release of pro-inflammatory cytokines, opening up of the blood-brain barrier and further leukocyte chemotaxis. This is followed by a second, larger wave of inflammatory cell recruitment, and leakage of antibody and plasma proteins; ultimately triggering a cascade of events.
resulting in the formation of an acute inflammatory, demyelinating lesion (Frohman et al., 2006). These typically develop in the white matter where they target the myelin sheath and the myelinating cell, the oligodendrocyte. MS lesions can occur anywhere in the CNS but the most common sites of involvement are the optic nerves, spinal cord and the periventricular regions of the cerebral hemispheres (Pender and McCoombe, 1995). Grey matter lesions are also known to occur (Frohman et al., 2006).

Whatever the pathological events leading to lesion formation, the key morphological feature of MS is primary demyelination of nerve axons leading to signal conduction block or conduction slowing at the site of demyelination. If conduction block occurs simultaneously in a significant proportion of fibres within a given pathway, neurological symptoms and signs will occur (McDonald & Sears, 1970). During clinical recovery, it is thought that there is restoration of CNS conduction due to glial ensheathment and remyelination. In contrast, axonal loss is irreversible and is most likely an important cause of neurological dysfunction in chronic MS (Pender and McCoombe, 1995).

Currently available therapies for MS are primarily aimed at reducing the number of relapses and slowing progression of disability. The standard immunomodulatory therapies include glatiramer acetate and IFN-β, although the mechanism of action of both drugs is largely unknown. Administration of glatiramer acetate (formerly copolymer-1; Copaxone) is thought to promote development of Th2-polarized CD4+ T cells, which may dampen inflammation within the CNS (Weber et al., 2007). Furthermore, it was recently reported that the deficiency in CD4+CD25+Foxp3+ Treg cells in multiple sclerosis is restored following treatment with glatiramer acetate (Hong et al., 2005). IFN-β (Avonex, Rebif, Betaseron) is thought to reduce antigen presentation and modulate co-
stimulatory molecules on APC, inhibit proliferation of Th1 cells and block the proinflammatory properties of IFN-γ and IL-12. IFN-β also inhibits differentiation of Th17 cells and leads to increased production of the regulatory cytokines IL-10, IL-27 and IL-4; again, generally shifting the cytokine profile from pro- to anti-inflammatory (Vosoughi et al., 2010). Both of these drugs are well tolerated by patients, however they are only modestly effective first line therapies and about one third of RR-MS patients develop recurrent relapses and an increase in sustained disability, while others can develop neutralizing antibodies to IFN-β. Patients who suboptimally respond to first line therapies are considered for treatment with natalizumab (Tysabri). A humanized monoclonal antibody that targets the α4-chain of α4β1 integrin (VLA-4), natalizumab inhibits leukocytes binding to vascular cell adhesion molecule (VCAM)-1 and fibronectin, thus reducing leukocyte traffic across the blood-brain barrier and, possibly, APC-T cell interactions (Rice et al., 2005). Natalizumab is more efficacious than any of the first line therapies for MS. Although well tolerated in most patients, around 1 in every 2200 patients treated with natalizumab have developed the sometimes fatal viral brain infection PML (Martin, 2010). Mitoxanthrone is another drug approved for the treatment of worsening RR-MS and both PP- and SP-MS. It works by suppressing the proliferation of T cells and B cells, and macrophages, and impairing antigen presentation and decreasing proinflammatory cytokine secretion. Further, Mitoxantrone enhances T cell suppressor function, inhibits B cell function and antibody production and limits macrophage-mediated myelin degradation (Fox, 2004). However, use of this drug is severely restricted because it can be toxic to the heart and has been linked to secondary leukemias in almost 3% of patients (Pascual et al., 2009; Martin, 2010).
1.3.1 Experimental Autoimmune Encephalomyelitis

EAE is a demyelinating disease of the CNS that shares clinical and pathological features with MS and is used as a model for the human disease. EAE is induced in susceptible strains of mice, rats or non-human primates by immunization with whole spinal cord, myelin proteins, or even peptides corresponding to the major encephalitogenic regions of these proteins, all emulsified in complete Freund's adjuvant (CFA; Stromnes & Goverman, 2006a). SJL mice express the proteolipid protein (PLP)-specific TCR at high frequency and are thus highly susceptible to a relapsing-remitting form of disease upon immunization with either PLP protein or the peptides PLP\textsubscript{139-151} and PLP\textsubscript{178-191}. Immunization of SJL mice with myelin basic protein (MBP) or the peptide MBP\textsubscript{84-104} also induces a relapsing form of disease, but requires the administration of pertussis toxin (PT) at the time of immunization and again 48 h later (Zamvil et al., 1985). Although the precise effect of PT in EAE is unknown, it is thought to facilitate immune cell entry to the CNS, as well as promoting proliferation and cytokine production by T cells and breaking T cell tolerance (Waldner et al., 2004). Myelin oligodendrocyte glycoprotein (MOG) is expressed at relatively low levels on the surface of myelin sheaths but, together with the peptide MOG\textsubscript{35-55}, is highly encephalitogenic in C57BL/6 mice. Again, administration of PT is required for disease induction with MOG\textsubscript{35-55} in C57BL/6 mice, which predominantly develop a chronic-progressive course of disease (Schreiner et al., 2009). The majority of transgenic mouse strains are developed on the C57BL/6 genetic background. Other mouse stains, including B10.PL and PL/J mice, develop an acute monophasic disease course (Schreiner et al., 2009). EAE can also be induced by adoptive transfer of \textit{in vitro} activated myelin-specific CD4\textsuperscript{+} T cells, isolated from primed mice, into naïve hosts (Stromnes & Goverman, 2006b). In addition, a number of TCR transgenic mice that spontaneously develop EAE with variable incidence have been developed. These include the 2D2 C57BL/6 (H-2\textsuperscript{b})/MOG\textsubscript{35-55}, 5B6 SJL (H-2\textsuperscript{b})/PLP\textsubscript{139-151} and 19G B10.PL (H-}
2^{10})/MBPAC1-11 TCR transgenic strains (Bettelli et al., 2003; Waldner et al., 2000; Lafaille et al., 1994).

The various EAE models have proven very useful in dissecting specific aspects of the pathogenesis of MS in rodents and have been invaluable for the development of new therapies, including glatiramer acetate (Teitelbaum et al., 1971) and natalizumab (Yednock et al., 1992). It is accepted that the artificial induction of disease in such models may by-pass important pathogenic mechanisms operating in human disease. This is highlighted by the fact that only very few therapeutics that were successful in pre-clinical trials in EAE have shown similar efficacy in MS patients (Friese et al., 2006). Moreover, certain therapies have had opposite outcomes in these two diseases. For example, administration of IFN-γ or anti-TNF-α antibodies were protective in EAE, but exacerbated MS. This is reflected in the limited number of treatments currently available for MS sufferers.

1.3.2 Effector T cells in MS and EAE

Patients with MS and healthy controls appear to have similar numbers of T cells in the peripheral blood that react with one or more antigens located in the myelin of the CNS (Frohman et al., 2006). However, myelin-reactive T cells from patients with MS exhibit a memory or activated phenotype, whereas these same antigen-specific cells in a healthy person appear to have a naïve phenotype (Lovett-Racke et al., 1998). The cytokine producing phenotype of auto-reactive T cells determines the ability of these cells to cause inflammation in the CNS. Myelin-reactive T cells from patients with MS were found to produce cytokines more consistent with a Th1-mediated response, whereas myelin-reactive T cells from healthy persons were more likely to produce cytokines with a Th2-type
profile (Crawford et al., 2004). IL-12 is required for differentiation of Th1 cells, and it was found that IL-12p40-defective (IL-12p40\(^{-/-}\)) mice were resistant to EAE (Segal et al., 1998). Taken together, it was considered that MS and EAE are CD4\(^+\) Th1-mediated autoimmune diseases.

This theory was first challenged by the observation that other T cell populations including CD4\(^+\) Th2 cells (Lafaille et al., 1997) and CD8\(^+\) myelin-specific T cells (Huseby et al., 2001) could also mediate forms of EAE, depending on the model system. Furthermore, IFN-\(\gamma\)^{+/−} or STAT1\(^{-/-}\) mice lacking Th1 cells, were found to develop more severe EAE (Ferber et al., 1996; Bettelli et al., 2004). More recently, it has been demonstrated that an IL-17 producing T cell subset, distinct from Th1 and Th2, is the critical cell type mediating the pathogenesis of EAE (Langrish et al., 2005). It was found that the perceived central role for IL-12 in autoimmune inflammation, by inducing differentiation of a Th1-type T cell and its production of IFN-\(\gamma\), had been misinterpreted (Cua et al., 2003). The cytokine IL-23 had been discovered as the pairing of the IL-23p19 subunit with the IL-12p40 subunit, also shared with IL-12 (Oppmann et al., 2000). Sedgwick and colleagues demonstrated that only IL-23p19\(^{-/-}\) mice and IL-12p40\(^{-/-}\) mice, but not IL-12p35\(^{-/-}\) (restricted to IL-12) were resistant to EAE (Cua et al., 2003). This provided conclusive evidence that IL-23, and not IL-12, is essential for the development of CNS autoimmune inflammation. In addition to a possible direct proinflammatory role, IL-23 was shown to promote a distinct population of memory CD4\(^+\) T cells characterized by the production of IL-17, referred to as Th17 cells (Aggarwal et al., 2003). Cua and colleagues subsequently showed that both IL-12- and IL-23-expanded PLP-specific Th1 and Th17 cells, respectively, could migrate to the CNS upon adoptive transfer into naïve mice, but that only the IL-23-driven Th17 cells brought about disease (Langrish et al., 2005). Th17 cells have now been defined as a distinct subset of CD4\(^+\) T cells that promote inflammation and are pathogenic in many autoimmune disorders. In addition, the
orphan nuclear receptor RORγt was discovered to be the critical transcription factor required for development of Th17 cells (Ivanov et al., 2006a). Despite deriving their name from IL-17, Th17 cells produce a range of cytokines including IL-17A, IL-17F, IL-6, IL-21, IL-22 and TNF-α.

It was initially reported that TGF-β and IL-6 were required for differentiation of murine Th17 cells, whereas IL-23 was required for their expansion (Mangan et al., 2006). This was based on findings that naïve T cells do not express the IL-23R and do not differentiate into Th17 cells in response to IL-23 in vitro (Langrish et al., 2005; van Beelen et al., 2007). Conversely, it was reported that IL-23 could expand a population of Th17 cells in vitro (Langrish et al., 2005; Aggarwal et al., 2003). It now appears, however, that TGF-β functions to suppress Th1 and Th2 cells, the products of which can inhibit the differentiation of Th17 cells (Das et al., 2009). Das and colleagues demonstrated that TGF-β blocks expression of the transcription factors signal transducer and activator of transcription (STAT) 4 and GATA-3, thus preventing Th1 and Th2 cell differentiation. In contrast, TGF-β had no effect on the expression of RORγt. Furthermore, in mice deficient in both in STAT-6 and T-bet, which are unable to generate Th1 and Th2 cells, IL-6 alone was sufficient to induce differentiation of Th17 cells, whereas TGF-beta had no effect (Das et al., 2009). Thus, the role of TGF-β in Th17 differentiation appears to be indirect by inhibiting IFN-γ and IL-4 and, in their absence, TGF-β is dispensable for Th17 cell development.

IL-6 is a pleiotropic cytokine produced by various types of lymphoid and non-lymphoid cells in response to a variety of signals, such as bacterial pathogens, IL-1β, TGB-β and TNFα, which up-regulate Il6 expression via a large number of transcription factors that includes NF-κB and c-Jun (Naka et al., 2002). IL-6
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signals through the IL-6 receptor composed of two different subunits, the IL-6 receptor alpha subunit (IL-6R) which mediates ligand specificity and the IL-6 signal transducer gp130, a receptor subunit shared with other cytokines of the IL-6 family (Hirano, 1998). Cells that express gp130, but not IL-6R, can be activated by IL-6 and the soluble IL-6R which is produced by shedding from the cell surface by the metalloproteinases ADAM17 and ADAM10 (Matthews et al., 2003). Binding of IL-6 to its receptor initiates cellular events including activation of Janus kinase (JAK) 1 / STAT3 and Extracellular signal-regulated kinase 1 and 2 (ERK1/2) signaling pathways (Naka et al., 2002). Activated JAK1 phosphorylates STAT3, which dimerizes and is translocated to the nucleus to activate transcription of genes containing STAT3 response elements. Up-regulation of Suppressor of cytokine signaling (SOCS)3 gene transcription by STAT3 leads to the termination of IL-6 cytokine signaling (Zhang et al., 2006).

IL-6−/− mice are resistant to EAE while gp130−/− mice, defective in IL-6 signaling, do not develop a Th17 response but instead have a peripheral repertoire that is dominated by Foxp3+ Treg cells, and are thus protected from EAE (Eugster et al., 1998; Korn et al., 2008). The role of IL-6 in driving Th17 differentiation however, is not clear. It has been demonstrated that IL-6 negates the suppressive effect of Foxp3 on RORγt function and inhibits the reciprocal generation of Foxp3+ Treg cells induced by TGF-β (Zhou and Littmann, 2009; Bettelli et al., 2006). IL-21 is an autocrine growth factor induced by IL-6 and IL-23, and IL-21 itself, that has a crucial role in driving the Th17 response (Nurieva et al., 2007; Zhou et al., 2007; Korn et al., 2007). IL-21 signaling also upregulates RORγt expression (Ivanov et al., 2006a; Zhou et al., 2007). IL-6, IL-21, and IL-23 share the ability to activate STAT3, which was shown to be critical for Th17 cell differentiation in mouse and man (Wei et al., 2007; Milner et al., 2008; Chen et al., 2006). STAT3 directly regulates the IL-17 and IL-21 genes but also regulates IL-23R expression (Wei et al., 2007; Chen et al., 2006). Furthermore, IL-1, IL-6
and IL-23 acting via STAT3 induce RORγt (Ivanov et al., 2006a). Thus, as naïve T cells do not express IL-23R on their surface, it is thought that IL-6, by inducing IL-21, upregulates IL-23R in a STAT3-dependent manner upon ligation of the TCR and thus promotes Th17 cell differentiation in the presence of IL-23 (Zhou et al., 2007).

IL-1 signaling is also critical for the early development of Th17 cells. The inflammatory activities of IL-1 are partially derived by transcriptionally inducing expression of a number of inflammatory molecules, amongst which include the cytokines IL-6, TGFβ and TNFα. IL-1α and IL-1β are encoded by two different genes and synthesized as 31 kDa proteins (pro-IL-1α and pro-IL-1β). IL-1α and IL-1β bind to and signal through the IL-1 type I receptor (IL-1RI). The activity of IL-1α and IL-1β is regulated by a decoy non-signaling type II receptor and by IL-1 receptor antagonist (IL-1Ra), a specific inhibitor of IL-1α and IL-1β which competitively binds IL-1RI with high affinity. IL-1RI is similar in structure to the TLR family, which recognise conserved PAMPs. IL-1RI and TLR both possess an internal Toll/IL-1 receptor (TIR) domain, which mediate the internal signalling for the receptor (Akira et al., 2001; Medzhitov, 2001). On binding of IL-1 to IL-1RI, the accessory protein IL-1RαcP is recruited to form a high affinity heterodimeric receptor complex, leading to activation of IL-1 Receptor Associated Kinase (IRA)-1 and IRAK-2, and initiating the IL-1 signalling cascade (Braddock and Quinn, 2004). IRAK-1 activates and recruits TNF receptor associated factor (TRAF)6 to the IL-1 receptor complex. TRAF6 activates two pathways, one leading to NF-κB activation and another leading to c-Jun activation. The TRAF associated protein ECSIT (evolutionarily conserved signaling intermediate in Toll pathways) leads to c-Jun activation through the mitogen-activated protein (MAP) kinase / c-Jun N-terminal kinase (JNK) signaling system (Kopp et al., 1999). TRAF6 also signals through the TGF-β-activated protein kinase 1-binding protein (TAB)1/ TGF-β-activated kinase
(TAK)1 kinases to trigger the degradation of I-κB, and activation of NF-κB (Akira et al., 2001).

IL-1R1^- mice have defective Th17 responses and are resistant to the induction of EAE (Sutton et al., 2006). Adoptive transfer of encephalitogenic T cells into IL-1R1^- mice, however, could induce disease. Consistent with these findings, it was demonstrated that IL-1 synergizes with IL-6 and IL-23 to induce expression of the transcription factors interferon regulatory factor (IRF) 4 and RORγt, thereby driving Th17 polarization (Chung et al., 2009).

IL-23-deficient mice are resistant to EAE and IL-23 is thought to be the critical cytokine for autoimmune inflammation of the brain (Cua et al., 2003). The IL-23 receptor is composed of the IL-12Rβ1 chain and a unique IL-23 receptor subunit, IL-23R. IL-23R constitutively associates with JAK2 and in a ligand-dependent manner with STAT3, with limited Th1-associated STAT4 phosphorylation. IL-12Rβ1 interacts directly with Tyrosine kinase 2 (Tyk2) (Parham et al., 2002). IL-23 induced activation of STAT3 leads to up-regulation of RORγt expression as well as direct binding of phosphorylated STAT3 to Il17a and Il17f promoters (Yang et al., 2007; Chen et al., 2006). IL-23 induced JAK2 activation also triggers the Phosphoinositide-3-kinase (PI3K)/RAC-alpha serine/threonine kinase (AKT) pathway, which leads to phosphorylation of STAT3, and the NF-κB pathway, both of which are required for IL-17 production. SOCS3 may inhibit JAK2 activity, thereby decreasing IL-23 induced Il17a and Il17f expression (Chen et al., 2006).

Although not yet fully resolved, it seems that IL-23 may not be required for lineage commitment, but is essential for the terminal differentiation and full
pathogenic activity of Th17 cells. Studies in an EAE model, involving adoptive transfer of T cells into IL-23−/− mice, showed that IL-23 played a critical role in the development but not in the effector function of encephalitogenic T cells (Thakker et al., 2007). In an experimental autoimmune uveitis model, neutralization of IL-23 reduced IL-17 production and attenuated autoimmununty when administered immediately before and after induction of disease, but not at the effector stage of the disease (Luger et al., 2008). These findings are consistent with a report that IL-23R is required for full differentiation to pathogenic Th17 cells as well as the generation of large numbers of effector Th17 cells in vivo (McGeachy et al., 2009). Cua and colleagues demonstrated that the early activation of Th17 cells is normal in the absence of IL-23R, with the expansion of antigen-specific T cells and the production of IL-17. However, these cells failed to progress in their differentiation to become true effector Th17 cells and could not enter the blood; consequently, fewer Th17 cells infiltrated the CNS and these failed to successfully initiate inflammation (McGeachy et al., 2009).

The function of IL-23 may also extend beyond the activation of Th17 cells, as it has recently been reported that IL-23 is required for the homing of MOG-specific T cells to the CNS during EAE (Gyulveszi et al., 2009). The importance of CNS homing in the development of EAE was confirmed by the fact that deficiency in CCR6, a chemokine receptor expressed on Th17 cells, conferred resistance to EAE, although CCR6−/− mice still developed peripheral Th17 responses. Susceptibility was restored by transfer of wild-type cells, after which effector cells were recruited independently of CCR6 (Reboldi et al., 2009).

Although early studies on Th17 cells dismissed a role for Th1 cells, recent reports suggest that both cell types may play distinct roles in pathology. It has been reported that adoptive transfer of highly purified Th1 cells could induce
EAE, whereas Th17 cells, devoid of IFN-γ producing cells, could not induce disease (O’Connor et al., 2008). It was suggested that only Th1 cells could initially access the CNS, and that this facilitated subsequent recruitment of Th17 cells. Russell and colleagues, however, demonstrated that an encephalitogenic Th1 cell line induces recruitment of host IL-17-producing T cells to the CNS during the initiation of EAE and that these cells contribute to the incidence and severity of disease (Lees et al., 2008). Evidence that recruited host Th17 cells contributed to disease was provided by the demonstration that adoptive transfer of Th1 cell lines derived from wild-type mice into IL-17-deficient mice resulted in reduced EAE clinical outcomes (Lees et al., 2008). Interestingly, although Th1 or Th17 cells induce clinically similar EAE, the pathological features appear to be different, with Th1 cells promoting expression of monocyte attracting chemokines and macrophage rich infiltrates into the spinal cord, whereas Th17 cells activate neutrophil-attracting chemokines and promote neutrophil recruitment and activation, particularly in the brain (Kroenke et al., 2008; Stromnes et al., 2008). Thus, both Th1 and Th17 cells may play complementary roles in the pathogenesis of EAE.

MS has traditionally been viewed as a CD4+ T cell mediated autoimmune disease, however, the frequency of CD8+ T cells is greater than that of CD4+ T cells in inflamed plaques, and CD8+ T cells show oligoclonal expansion in plaques, CSF and blood, all suggestive of a pathogenic role in MS (Friese & Fugger, 2009). Studies involving cell transfer have suggested that CD8+ T cells are pathogenic in EAE (Huseby et al., 2001). Furthermore, IL-17 production by both human and murine CD8+ T cells has been demonstrated (Shin et al., 1999; Happel et al., 2003). However there is also evidence of a regulatory role for CD8+ T cells in EAE, with increased disease severity in mice deficient in or depleted of CD8+ T cells (Weiss et al., 2007).
While much effort has focused on IL-17-producing CD4^+ Th17 cells, it is becoming clear that there are other sources of IL-17. Human memory CD8^+ T cells have been shown to produce IL-17 in vitro following stimulation with PMA and ionomycin (Shin et al., 1999). Murine CD8^+ T cells too, were found to produce IL-17 in response to IL-1 and IL-23 or conditioned medium from DC activated with Klebsiella pneumoniae (Sutton et al., 2006; Happel et al., 2003). Neutrophils, in addition to T cells, have been shown to produce IL-17 in response to IL-15 (Ferretti et al., 2003). And, IL-17 was secreted by microglia in vitro in response to IL-23 or IL-1β (Kawanokuchi et al., 2008).

Unconventional T cells such as γδ T cells, NKT cells and lymphoid tissue inducer (LTi)-like cells also secrete innate IL-17. Murine IL-17 was originally cloned from NKT cells (Kennedy et al., 1996). Recent evidence has demonstrated that NKT cells constitutively express the IL-23R and RORγt and secrete IL-17 after stimulation with αCD3 and IL-23 or the synthetic ligand α-galactosylceramide (Michel et al., 2007; Rachitskaya et al., 2008). It has also been reported that LTi-like cells constitutively express RORγt and IL-23R and rapidly produce IL-17 and IL-22 in response to IL-23 stimulation (Ivanov et al., 2006b; Takatori et al., 2009).

γδ T cells are another major source of IL-17. Like αβ Th17 cells, IL-17-producing γδ T cells also require RORγt for their development (Ivanov et al., 2006a). In bacterial infections, γδ T cells can rapidly produce IL-17 (Lockhart et al., 2006; Shibata et al., 2007), and in some models have been demonstrated to be the main source of IL-17 (Umemura et al., 2007). Naïve γδ T cells have been demonstrated to produce IL-17 in response to IL-23, a response similar to memory Th17 cells (Lockhart et al., 2006; Shibata et al., 2007). Interestingly, it has been shown that IL-17 production by γδ T cells is independent of IL-6, an essential
cytokine for driving the development of Th17 cells (Lochner et al., 2008). There is evidence that IL-17^γδ T cells may have a pathogenic role in CIA (Roark et al., 2007). Therefore, it is possible that γδ T cell-derived IL-17 may contribute to CNS pathology in EAE and MS.

It has been considered for some time that γδ T cells may play a role in both MS and EAE. Clonally expanded γδ T cells were found in acute MS brain lesions and in the cerebrospinal fluid of MS patients with recent disease onset, suggesting that γδ T cells contribute to neuroinflammation (Wucherpfennig et al., 1992; Shimonkevitz et al., 1993). In the EAE model, deletion of these cells immediately prior to disease onset or during the chronic phase of disease significantly reduced clinical and pathological expression of EAE (Rajan et al., 1996). Disruption of the TCR δ chain gene led to reduced severity of disease in one study, although not in another (Spahn et al., 1999; Clarke & Lingenheld, 1998). It is thought that γδ T cells may participate in the development of disease by regulating leukocyte transfer across the blood-brain barrier and acting as a source of proinflammatory molecules. γδ T cells have been detected in the CNS prior to the onset of disease in EAE and were found to secrete IFN-γ and TNF-α (Smith & Barnum, 2008). Moreover, Brosnan and colleagues showed a marked decrease in IFN-γ expression at all stages of disease in γδ depleted mice, as well as reduced expression of IL-1, IL-6 and TNF-α at disease onset, but returning to levels comparable to controls in the chronic phase of disease (Rajan et al., 1998). Thus, γδ T cells may play a role in facilitating the activation and migration of myelin-reactive T cells into the CNS by upregulating expression of appropriate proinflammatory cytokines and chemokines. Such effects may not require large numbers of cells and could be facilitated by the numbers of γδ T cells routinely found in the spinal cord and brain of mice with EAE (Rajan et al., 1998).
1.4 Aims

CD4⁺ T cells are considered to be the major source of IL-17, particularly in autoimmune diseases. However, recent evidence has suggested that other lymphocyte populations can produce IL-17. The hypothesis underlying this thesis was that cells other than conventional αβ CD4⁺ Th17 cells might also produce IL-17 during CNS autoimmunity and contribute to the neuroinflammation associated with EAE. Hence, IL-17-expressing T cell populations identified during the pathogenesis of EAE were characterized. In order to determine the potential roles of the various Th17 cell-associated cytokines, receptors and transcription factors, the kinetics and distribution of their gene expression and *ex vivo* cytokine responses were analyzed in the pre-clinical, acute and chronic phases of disease. With indications of where and when molecules of importance to Th17 cell differentiation and effector function were expressed, the cells that may be involved in IL-17-mediated disease pathology were investigated, in particular those accumulating in the CNS but also in the periphery during the different stages of development of disease. The precise functions of IL-1, IL-6, IL-21 and IL-23 in driving Th17 cell development and IL-17 secretion are not yet clear, therefore this study investigated how different combinations of these cytokines promoted IL-17 production by T cells that were found to have a role in CNS inflammation in EAE. Finally, having established which cytokines were critical in inducing IL-17-mediated neuroinflammation, the aim was to target these as a therapeutic approach to EAE and potentially MS.
Methods

Chapter 2
2.1 Animals

Specific pathogen-free C57BL/6 mice were purchased from Harlan UK Ltd., (Bicester, Oxon, UK) and were maintained according to the guidelines and regulations of the Irish Department of Health and Children. Experiments were performed under licence from the Department of Health and Children and with the approval of the University of Dublin BioResources Ethics Committee. All mice used were female and 6-12 weeks old at the initiation of experiments.

2.2 EAE

EAE was induced in C57BL/6 mice by s.c. injection at one site in the centre of the lower back of 100 μg of myelin oligodendrocyte glycoprotein (MOG)35-55 (GenScript) emulsified 1:1 in 100 μl complete Freund's adjuvant (CFA; Chondrex Inc.), containing 4 mg/ml H37 Ra Mycobacterium tuberculosis (Mtbc). Control mice were immunized with PBS in CFA or PBS alone. Mice were also injected i.p. with 500 ng pertussis toxin (PT; Kaketsuken, Japan) on days 0 and 2. Animals were monitored daily for signs of clinical disease. Disease severity was graded as follows: grade 0 – normal; grade 1 – flaccid tail; grade 2 – wobbly gait; grade 3 – hind limb weakness; grade 4 – hind limb paralysis; grade 5 – tetraparalysis/death.

2.3 Treatment of Mice with a Caspase-1 Inhibitor

Ac-YVAD-cmk (Merck-Calbiochem), an inhibitor of the caspases 1, 4 and 5, was reconstituted in 500 μl dimethyl sulfoxide (DMSO) and diluted to 5 mg/ml stock concentration with sterile PBS. In the EAE model, mice were administered 25 μg YVAD-cmk or an equal
concentration of DMSO (as a control) s.c. with the MOG/CFA on day 0 and again on days 1 and 2, on days 0, 1 and 2 and every second day from day 5 to day 21, or every second day from day 5 to day 21.

2.4 Direct Immunization with Adjuvants

C57BL/6 mice were immunized by s.c. injection into the footpad (25 μl/footpad) with PBS, Mtb (50 μg/footpad), Ac-YVAD-CMK (25 μg/footpad), or Mtb and Ac-YVAD-CMK together. Mice were sacrificed and popliteal lymph nodes collected after 4 and 24 h, and snap frozen in liquid N₂, until analysis of cytokine expression.

2.5 Cell Culture

Cells were cultured in an incubator at 37°C with an atmosphere of 95% humidity and 5% CO₂.

2.6 Cell Counting

2.6.1 Haemocytometer

Cell counting was performed by diluting 10 μl cells (1/10 dilution for CNS tissue and lymph nodes, and 1/20 dilution for spleens) in Ethidium Bromide-Acridine Orange (EBAO). A 10 μl volume of the cell suspension was then loaded onto a disposable haemocytometer (Hycor Biomedical, UK). The number of viable cells (green) and dead cells (orange) were then counted using a fluorescent microscope. The number of cells per ml was calculated according to the
manufacturer's protocol by multiplying the cell number in 9 small squares, within 1 large square, by $10^4$, by the dilution factor:

$$\text{Number of cells/ml} = \text{cell number in 9 small squares} \times 10^4 \times \text{dilution factor}$$

### 2.6.2 FACS

To determine the absolute number of cells infiltrating a particular organ or tissue in each animal, a 50 μl sample of digested tissue was removed to a FACS tube both before and after Percoll separation. These cells were washed, incubated with 1 μg/ml anti-CD16/CD32 (Fcγ Block; BD Pharmingen) for 15 min at room temperature and then stained with anti-CD45 and anti-CD3 for 15 min in the dark. Cells were washed in FACS buffer and resuspended in 400 μl FACS buffer for analysis. 20 μl (for CNS and lymphoid tissue) or 50 μl (for spleen tissue) CytoCount Beads (DakoCytomation) were added to each tube just prior to FACS analysis and the contents vortex mixed. CytoCount beads are manufactured at a concentration of 1100 beads/μl. Thus, the absolute number of CD45$^+$ and CD3$^+$ cells can be determined by multiplying:

$$\frac{\text{# cell population acquired}}{\text{# beads acquired}} \times \frac{\text{CytoCount}}{\text{volume dilution}} = \frac{\text{total cell #/sample}}{\text{concentration}}$$

(tissue)
2.7 Generation of Murine Bone Marrow-derived Dendritic Cells

Bone marrow-derived dendritic cells (BMDC) were generated from C57BL/6 mice, using a method similar to that described by Lutz et al. (1999). Mice were euthanized and their femurs and tibiae removed and dissected from the surrounding muscle tissue. The bone marrow was flushed out with complete Roswell Park Memorial Institute-1640 medium (cRPMI; appendix) using a 27 G needle attached to a 20 ml syringe. The cell aggregates were dissociated using a 19 G needle attached to a 20 ml syringe. The cell suspension was pelleted by centrifugation (300 x g for 5 min at 4°C) and cells were resuspended in 2 ml of ammonium chloride lysis solution (warmed to 37°C; appendix) for 2 min, to lyse red blood cells. The cells were washed in cRPMI medium, then pelleted by centrifugation (300 x g for 5 min at 4°C) and resuspended in 10 ml cRPMI medium. Cell viability was assessed by EBAO staining (section 2.6.1). Immature BMDC were cultured at 1 x 10^6/ml in cRPMI medium containing 20 ng/ml of GM-CSF in the form of supernatant from a GM-CSF expressing J558 cell line. After 3 d incubation, 20 ml of fresh cRPMI medium containing 20 ng/ml GM-CSF was added to each culture flask. 3 d later, the flasks were gently removed from the incubator and the cell culture supernatant was carefully decanted to eliminate contaminating cells (e.g. granulocytes) from the culture. 25 ml of sterile PBS (BioSera), warmed to 37°C, was added to each flask and the flasks were gently agitated before the PBS suspension was transferred to 50 ml tubes containing 10 ml cRPMI medium. 20 ml of sterile EDTA (0.02%; Sigma), warmed to 37°C, was added to each culture flask before returning the flask to the incubator for 10 min. Meanwhile, the cells removed in the PBS step were pelleted by centrifugation (300 x g for 5 min at 4°C) and resuspended in cRPMI medium. Culture flasks were removed from the incubator and the EDTA solution repeat-pipetted over the layer of remaining cells, before being added to fresh cRPMI medium warmed to 37°C, and centrifuged at 300 x g for 5 min. The cell pellet
was resuspended in cRPMI medium and pooled with the pellet obtained in the PBS step. Cells were then re-cultured at $1 \times 10^6$ cells/ml in cRPMI medium supplemented with 20 ng/ml GM-CSF. After a further 2/3 d of incubation (d 8/9), 20 ml cRPMI medium containing 20 ng/ml GM-CSF was added to each flask of cells. After a further 2 d in culture, the loosely adherent cells were harvested by gentle repeat pipetting of the culture medium. Viability of BMDC was assessed and the cells were cultured at required concentrations (e.g. $1 \times 10^6$/ml) in tissue culture plates. DC were left overnight in medium containing 10 ng/ml GM-CSF before stimulation.

2.7.1 In Vitro Stimulation of BMDC

Precise conditions for stimulation of DC in vitro are outlined in each figure legend. Briefly, for studies examining the effect of Mtb or LPS and adenosine triphosphate (ATP) on cytokine production, BMDC ($1 \times 10^6$ cells/ml) were cultured in the presence of Mtb (10 and 50 µg/ml; Difco) or LPS (100 ng/ml; Alexis Biochemicals) +/- ATP (5mM; Sigma) in 96 well tissue culture plates and supernatants removed at various time points and analyzed for cytokine concentrations by ELISA. For co-culture experiments with T cells, BMDC ($1 \times 10^6$/ml) were cultured with Mtb (10 and 50 µg/ml) +/- the caspase-1 inhibitor Ac-YVAD-CMK (2 - 50 µM), or cRPMI medium alone for 24 h in 6-well tissue culture plates (2 ml/well), washed in cRPMI medium, counted and added to purified T cells. For analysis of protein by Western blot, BMDC ($1 \times 10^6$/ml) were cultured in 24-well tissue culture plates (1 ml/well) with LPS (100 ng/ml) +/- ATP (5mM), Mtb (10 and 50 µg/ml), or medium only. Supernatants were harvested at appropriate time points and frozen at -20°C. When required, BMDC were pre-treated with the caspase-1 inhibitor for 1 h prior to incubation with other stimuli.
2.8 Mononuclear Cell Isolation from CNS Tissue

Mice were anaesthetized with 40 µl pentobarbital sodium (Euthetal), perfused intracardially with 20 ml ice-cold PBS and brain, spinal cord, inguinal lymph node and spleen tissue isolated to Hanks Balanced Salt Solution containing 3% FCS (HBSS/FCS). Tissue was dissociated through a 70 µm cell strainer (Nunc), washed through with HBSS/FCS, centrifuged at 170 x g for 10 min at 18°C, and enzymatically digested in Collagenase D (1 mg/ml) and DNase I (10 µg/ml) for 1 h at 37°C, with gentle agitation. Cells were washed in HBSS/FCS and resuspended in 9 ml 1.088 g/ml Percoll (appendix). This was underlayed with 5 ml 1.122 g/ml Percoll, and overlayed with 9 ml 1.072 g/ml Percoll; 9 ml 1.030 g/ml Percoll, and; 9 ml of 1X PBS. Percoll gradients were centrifuged at 1250 x g for 45 min at 18°C. MNCs were removed from the 1.088 : 1.072 and 1.072 : 1.030 g/ml interfaces and washed twice in HBSS/FCS.

2.9 *Ex Vivo* Restimulation and FACS Analysis

Isolated cells were restimulated for 6 h or overnight at 37°C / 5% CO2 with PMA (10 ng/ml) and ionomycin (1 µg/ml), and cytokine secretion blocked with brefeldin A (5 µg/ml). Cells were then washed in FACS buffer (appendix), non-specific binding blocked by incubation with 1 µg/ml anti-CD16/CD32 (Fcy Block; BD Pharmingen) for 15 min and stained for surface markers with anti-CD45, anti-CD3, anti-CD4, anti-CD8, anti-NK, anti-F4/80, anti-Ly-6G, anti-γδ TCR, anti-CD27, anti-Vγ4, anti-Vγ5, anti-Vδ6.3/2, anti-CD11b or anti-CD11c. Cells were fixed and permeabilized with Caltag Fix & Perm Kit, and stained for intracellular cytokines with anti-IFNγ and anti-IL-17. Flow cytometric analysis was performed using a CyAn ADP flow cytometer (DakoCytomation). The flow cytometer was calibrated using the compensation function in the Summit software in conjunction with BD Comp Beads (BD Biosciences). Results were analysed...
using Summit (DakoCytomation) or FloJo (Stanford University) software, with gating determined using the fluorescence-minus-one (FMO) technique, in which a tube of cells contains antibodies conjugated to fluorochromes for all molecules being examined except the one of interest.

2.10 Stimulation of Spleen and Lymph Node Cells

*Ex Vivo*

Single cell suspensions were prepared by homogenizing spleen, lymph node, brain or spinal cord tissue through a 70 μm cell strainer (Nunc). Cells were centrifuged at 300 x g for 5 min and counted using EBAO (2.6.1). Cells were resuspended at 2 x 10^6/ml and 100 μl of the cell suspension was added to wells of a 96-well ‘U-bottomed’ tissue culture plate. 100 μl of 2 μg/ml, 10 μg/ml or 50 μg/ml MOG, 10 ng/ml PMA (Sigma) and 1 μg/ml αCD3 (Pharmerin), or medium alone as positive and negative controls, respectively, was added to triplicate wells. In some instances, CD3^+^, CD4^+^ or γδ^+^ T cells were purified using MACS beads (Miltenyi Biotec), according to the manufacturer’s instructions. CD3^+^ and CD4^+^ T cells were isolated by negative selection, via depletion of all non-CD3^+^ or CD4^+^ T cells. γδ^+^ T cells were isolated from CD3-purified cells by positive selection of the γδ TCR. Isolated cells were tested for cell purity by FACS staining (2.9) and cultured with recombinant cytokines. T cells were also cultured in the presence of BMDC (at a ratio of 4:1, T cells to DC) that had been stimulated for 24 h with Mtb (10 – 50 μg/ml) +/- the caspase-1 inhibitor Ac-YVAD-CMK (2 – 50 μM). Cells were cultured for 72 h (48 h for γδ T cell cultures) after which supernatants were removed for analysis of cytokine production by ELISA, and cells resuspended in TRIzol solution (Invitrogen) for analysis of mRNA expression.
2.10.1 Preparation of Antigen Presenting Cells

Single cell suspensions prepared from murine spleens were suspended in ice-cold PBS in a 15 ml tube. Cells were irradiated at a dose of 30 Gy in a Gammacell 3000 Elan irradiator (Nordian), to prevent subsequent cell proliferation. Cells were counted in EBKO (2.6.1) and used at a ratio of 20:1 (Splenocytes to T cells) as APC.

2.11 Western Blot Analysis

2.11.1 Preparation of Protein from Supernatants

BMDC were stimulated as described in 2.7.1 and supernatants (500 µl) were collected in sterile eppendorfs from each well. 500 µl methanol and 125 µl chloroform were added, samples vortexed and centrifuged for 5 min at 13,000 x g at room temperature. The upper phase was removed and another 500 µl methanol added and samples again centrifuged for 5 min at 13,000 x g. The supernatant was removed and the pellet dried by heating to 50°C for 5 min. 60 µl of 1 X loading buffer was added to each eppendorf, mixed well and boiled at 95°C for 5 min, after which samples were briefly centrifuged.

2.11.2 Gel Preparation

A 15% separating gel (appendix) was prepared and pastuer-pipetted between two glass plates, overlaid with dH2O and allowed to set. The H2O was poured off and a 5% stacking gel (appendix) carefully layered on top of the separating gel and a comb immediately inserted between the plates. The comb was removed after 30 min when the gels had set and the gels were either used straight away or wrapped in tissue paper and thoroughly soaked in dH2O, wrapped
in clingfilm and stored O/N at 4°C. The plates were placed in the gel rig before the addition of running buffer. 20 μl of sample was loaded into the required lanes, and 6 μl of a molecular weight marker (Precision Plus Protein Standards, Bio-Rad) added to lane 1. The gels were run at 120 V until the dye front was coming near the end of the separating gel.

2.11.3 Western Blotting

Directly following SDS-PAGE, protein bands were transferred onto polyvinylidene fluoride (PVDF) membrane using a wet transfer system. PVDF was activated by immersion in MeOH before rinsing in transfer buffer. Gels were gently removed from between the glass plates and kept moist in transfer buffer. Gels were placed on moist PVDF between layers of moist filter paper, and sandwiched in the cassette between two moist scotchbrite pads, making sure there were no trapped air bubbles. The cassette was placed in the transfer apparatus which was filled with transfer buffer. Transfer was achieved by applying a constant current of 110 mA for 1.5 h. Membranes were blocked in 5% alkali soluble casein (Novagen) in 1 X PBS-tween for 2 h on a rocker at room temperature, before incubation with 5 ml rat anti-mouse IL-1β or anti- mouse caspase-1 primary antibody (R&D Systems; 1:500 in 5% casein) in a 50 ml tube on a roller for 1 h at room temperature or O/N at 4°C. Membranes were briefly washed in PBS-tween for 3 X 5 min on a rocker and incubated with polyclonal rabbit anti-rat immunoglobulins/HRP (Dako; 1:1000 dilution in 5% casein) in a 50 ml tube on a roller for 1 h at room temperature. Membranes were again washed as above in PBS-tween and blots developed by chemiluminescence (ECL Western Blotting Detection Reagents, GE Healthcare) according to the manufacturer’s instructions.
Chapter 2

2.12 Measurement of Cytokine Concentration by Enzyme Linked Immunosorbent Assay (ELISA)

2.12.1 IL-23p19 ELISA

The concentration of IL-23p19 in supernatants from stimulated cells, which had been stored at -20°C until analysis, was measured using commercially available ELISA kits (eBioscience). High binding certified 96-well microtitre plates (Greiner Bio-one) were coated overnight at 4°C with 50 μl/well of purified anti-mouse IL-23 (2 μg/ml) capture antibody in eBioscience coating buffer. Plates were washed 3 times in ELISA wash buffer (PBS / 0.05% Tween 20) and non-specific binding sites blocked by addition of 200 μl/well of blocking buffer (eBioscience assay diluent) for 2 h at room temperature. Plates were then washed five times before the addition of 50 μl per well of sample supernatant or an eight point two-fold serial dilution from the top working standard recombinant protein for IL-23p19 (4000 pg/ml in eBioscience assay diluent). Plates were incubated overnight at 4°C. Plates were washed five times and incubated for 1 h at room temperature with 50 μl/well biotinylated anti-mouse IL-23p19 detection antibody (2 μg/ml) diluted in eBioscience assay diluent. Plates were again washed three times and incubated with 50 μl/well HRP-conjugated strepavidin (eBioscience, 1:250 in eBioscience assay diluent) for 30 min at room temperature in the dark. Plates were washed seven times, soaking the wells for 1 min between washes, and 50 μl TMB substrate solution (eBioscience) added to each well. The enzyme reaction was quenched by addition of 25 μl stop solution (1M H₂SO₄, appendix) per well when the standard curve for each cytokine had developed sufficiently, and absorbance was read at 450 nm on a Versamax Tunable Microplate Reader (Molecular Devices). Cytokine concentration in each unknown sample was determined by reference to the standard curve, prepared using recombinant mouse IL-23p19 of known concentration, and following subtraction of the blank absorbance reading from each unknown sample.
2.12.2 IL-1β, IL-10, IL-17 and TNF-α ELISA

Concentrations of the cytokines IL-1β, IL-10, IL-17 and TNFα were measured using commercially available ELISA kits (R&D Systems). High binding certified 96-well microtitre plates (Greiner Bio-one) were coated overnight at 4°C with 50 µl/well of rat anti-mouse IL-1β (4 µg/ml), IL-10, IL-17 or TNF-α (all 2 µg/ml) capture antibody in PBS. Plates were washed 3 times in ELISA wash buffer (PBS / 0.05% Tween 20) and non-specific binding sites blocked by incubation with 200 µl blocking buffer (1% BSA and 5% sucrose in PBS) per well for 2 h at room temperature. Plates were again washed 3 times before the addition of 50 µl per well of sample supernatant or an eight point two-fold serial dilution from the top working standard recombinant protein for each cytokine (IL-1β and IL-17 - 1000 pg/ml; IL-10 and TNFα - 2000 pg/ml, all diluted in 1% BSA in PBS). Plates were incubated overnight at 4°C. Plates were washed three times and incubated for 2 h at room temperature with 50 µl/well biotinylated goat anti-mouse IL-1β (600 ng/ml), IL-10 (400 ng/ml), IL-17 (200 ng/ml) or TNF-α (300 ng/ml) detection antibodies diluted in 1% BSA. Plates were again washed three times and incubated with 50 µl/well horseradish peroxidase (HRP)-conjugated strepavidin (R&D, 1:200 in 1% BSA) for 30 min at room temperature in the dark. Plates were washed three times and 50 µl substrate solution (appendix) added to each well. The enzyme reaction was quenched by addition of 25 µl stop solution (1M H₂SO₄, appendix) per well when the standard curve for each cytokine had developed sufficiently, and absorbance was read at 492 nm on a Versamax Tunable Microplate Reader (Molecular Devices). Cytokine concentration in each unknown sample was determined by reference to the standard curve, prepared using recombinant mouse IL-1β, IL-10, IL-17 and TNF-α of known concentration, and following subtraction of the blank absorbance reading from each unknown sample.
2.12.3 IL-4, IL-18 and IFN-γ ELISA

Concentrations of the cytokines IL-18 (Medical and Biological Laboratories Co.), IL-4 and IFN-γ (BD Pharamingen) were measured using commercially available paired antibodies. High binding certified 96-well microtitre plates (Greiner Bio-one) were coated overnight at 4°C with 50 µl/well of purified anti-mouse IL-4, IL-18 or IFN-γ (all 1 µg/ml) capture antibody in PBS. Plates were washed 3 times in ELISA wash buffer (PBS / 0.05% Tween 20) and non-specific binding sites blocked by incubation with 200 µl/well 5% w/v non-fat dried milk (Marvel) in PBS for 2 h at room temperature. Plates were again washed 3 times before the addition of 50 µl per well of sample supernatant or an eight point two-fold serial dilution from the top working standard recombinant protein for each cytokine (IL-4 - 2500 pg/ml, IL-18 - 1000 pg/ml and IFN-γ - 10 ng/ml; all diluted in PBS). Plates were incubated overnight at 4°C. Plates were washed three times and incubated for 1 h at room temperature with 50 µl/well biotinylated rat anti-mouse IL-4, IL-18 or IFN-γ detection antibodies (all 1 µg/ml) diluted in PBS. Plates were again washed three times and incubated with 50 µl/well HRP-conjugated strepavidin (BD Pharamingen, 1:1000 in PBS) for 20 min at room temperature in the dark. Plates were washed three times and 50 µl substrate solution (appendix) added to each well. The enzyme reaction was quenched by addition of 25 µl stop solution (1M H₂SO₄, appendix) per well when the standard curve for each cytokine had developed sufficiently, and absorbance was read at 492 nm on a Versamax Tunable Microplate Reader (Molecular Devices). Cytokine concentration in each unknown sample was determined by reference to the standard curve, prepared using recombinant mouse IL-4, IL-18 and IFN-γ of known concentration, and following subtraction of the blank absorbance reading from each unknown sample.
2.13 Real Time Quantitative RT-PCR

Posterior cortex and cerebellar regions of the brain, whole spinal cord, spleen and inguinal lymph nodes were isolated to RNAlater (Ambion) or cultured cells resuspended in TRIzol, and both stored at -20°C until analysis. Total ribonucleic acid (RNA) was extracted from cells or tissue using the TRIzol/chloroform method. Briefly, fixed tissue was transferred to 1 ml TRIzol reagent in sterile RNase-free tubes and homogenized using a Polytron tissue homogenizer (Kinematica). Cells were transferred to sterile RNase-free tubes and disrupted by repeat pipetting in TRIzol. 200 μl chloroform was added to each tube, vortex mixed, stood at room temperature for 3 min and then centrifuged at 12,000 x g for 15 min at 4°C. The resulting upper aqueous phase was removed to a sterile RNase-free tube, and 500 μl isopropanol added, mixed vigorously and stood at room temperature for 15 min, before centrifugation at 12,000 x g for 15 min at 4°C. Supernatants were decanted and the pellet washed twice (7,600 x g for 3 min) in 1 ml 75% ethanol in nuclease-free H2O. Finally, remaining ethanol was removed with a pipette and the tubes inverted and allowed to air-dry for 10 min. RNA was resuspended in 1 μl nuclease-free H2O / mg original tissue sample or 20 μl for cells, incubated on ice for 10 min, heated to 65°C for 10 min and then stored on ice for 20 min. RNA concentration was determined spectrophotometrically using an Eppendorf BioPhotometer 6131 (Eppendorf) by diluting 2 μl RNA in 98 μl nuclease-free H2O and reading the absorbance at 260 nm and determining the 260/280 nm ratio. 300 ng - 1 μg of each RNA sample was reversed transcribed into cDNA using the Applied Biosystems High Capacity cDNA Reverse Transcription kit, according to the manufacturer’s protocol, and the cDNA reverse transcription product diluted 1:8 with nuclease-free H2O. Transcripts were quantified by real time quantitative PCR on an ABI 7500 Fast Real Time PCR System with Applied Biosystems predesigned Taqman Gene Expression Assays (Table A.4, appendix) and reagents according to the manufacturer’s instructions. For each sample, mRNA concentration was
normalised to the amount of 18S ribosomal RNA (rRNA) and is expressed as fold difference compared to naïve control mice.

2.14 Confocal Microscopy

Stably transfected ASC-YFP\(^+\) macrophages were a kind gift from Dr. Eicke Latz, University of Massachusetts, USA. ASC-YFP cells, cultured in cDMEM and passaged twice weekly, were set up at $5 \times 10^5$ per ml the day before use on 35 mm glass bottom culture dishes. Cells were pre-treated for 3 h with LPS (100 ng/ml) where indicated and stimulated with ATP (5 mM) or Mtb (50 µg/ml) for 1 h. Imaging was performed on an Olympus FluoViewTM FV1000 laser scanning confocal microscope equipped with a temperature and CO\(_2\) controlled chamber. A UPlanSAPO 60X/1.35 NA oil objective was used and the images were acquired with the accompanying software.

2.15 Statistics

Statistical analyses were performed using GraphPad Prism (v4.03) statistical analysis software. Group differences were analyzed by unpaired, two-tailed Students \(t\) test or two-way ANOVA with multiple comparisons followed by Bonferroni post-test comparisons for three or more groups. A Wilcoxon signed-rank test was used to test for column differences relative to controls in PCR analysis and a standard unpaired, two-tailed \(t\) test used to determine significance where appropriate. Differences between groups for clinical scores in EAE were analyzed by two-way ANOVA with repeated measures. P-values of 0.05 or less were considered significant.
Temporo-spatial expression of inflammatory cytokines over the course of EAE

Chapter 3
3.1 Introduction

Cytokines, together with chemokines and adhesion molecules, are thought to be some of the key mediators of histological damage and repair, and clinical relapse and remission in MS and EAE. It is thought that signaling between the CNS and the immune system leads to inflammation at the blood-brain barrier where endothelial cells and astrocytes, activated by T cell cytokines, express adhesion molecules and produce chemokines, which facilitate the directed passage of T cells, and subsequently macrophages and plasma cells, into the CNS (Merrill and Benveniste, 1996). In turn, parenchymal astrocytes and microglia are recruited to the inflammatory process, either directly by cell-cell contact, or indirectly, in response to the release of pro-inflammatory cytokines by infiltrating inflammatory cells. These glial cells secrete IL-1, IL-6, IL-17, IL-23, TNF-α, TGF-β and GM-CSF (Constantinescu et al., 2005; Li et al., 2007; Kawanokuchi et al., 2008; Luo et al., 2007; Zaheer et al., 2007).

IL-17 promotes the production of other cytokines and chemokines from a variety of cell types and act as a chemoattractant for monocytes and neutrophils (Hofstetter et al., 2007). IL-17 has been shown to induce IL-1, IL-6 and TNF-α in inflamed tissues, as well as a variety of chemokines and costimulatory molecules, such as ICAM-1 (Albanesi et al., 1999).

The differentiation of naïve T cells into IL-17 expressing memory/effector T cells is dependent on IL-23 (Aggarwal et al., 2003; Langrish et al., 2005), and independent of the Th1-driving cytokine, IL-12. Moreover, mice deficient in IL-23 or the IL-23 receptor (IL-23R) are resistant to EAE (Cua et al., 2003; McGeachy et al., 2009). A significant increase in IL-23 mRNA has also been
demonstrated in patients with active RR-MS; an effect that is decreased by treatment with IFN-β, possibly in an IL-10-dependent manner (Krakauer et al., 2008). Differentiation of naïve precursors to Th17 cells is directed by the nuclear receptor RORγt (Ivanov et al., 2006a). IL-6−/− mice or mice deficient in the IL-6 family receptor subunit, gp130, and which are defective in IL-6 signaling, are also resistant to EAE (Eugster et al., 1998; Korn et al., 2008), and while there is some debate regarding the precise role of IL-6 in the induction of Th17 differentiation, it is known that IL-17 activates cells of the local inflammatory environment to produce IL-6 (Shimada et al., 2002).

The development of Th17 cells from naïve precursor cells is inhibited by IFN-γ, as is the function of CD4 memory/effector cell lineages that secrete IL-17 (Park et al., 2005). IFN-γ−/− mice are highly susceptible to EAE (Ferber et al., 1996). Indeed, treatment with anti-IFN-γ antibodies has been shown to exacerbate EAE (Billiau et al., 1988). Despite this, it is understood that there is a requirement for IFN-γ, or at least some unidentified Th1-type cytokine, in some capacity for the development of EAE. Therefore, the widespread use of IFN-γ as a marker for Th1 cells may be misleading.

IL-10, formerly cytokine synthesis inhibitory factor (CSIF), is known to suppress pro-inflammatory cytokine production, possibly by inhibition of the induction of a set of NFκB-regulated pro-inflammatory genes, and therefore represents one of the most important immune-regulating cytokines (Conti et al., 2003). Moreover, IL-10-deficient mice are more susceptible and develop a more severe EAE compared with wild type mice and mice transgenic for IL-10 are completely resistant to disease (Bettelli et al., 1998).
Many cytokines also have important regulatory roles in both the inflammatory and repair processes. Certain cytokines in the CNS might function early on to amplify the disease process and later to attenuate it (Merrill & Benveniste, 1996). For example, IL-6 increases blood brain barrier permeability, induces fever and gliosis, upregulates pro-inflammatory cytokine and antibody production (Campbell et al., 1993), and yet is thought to have a role in the survival, migration and differentiation of oligodendrocyte precursors, required for the repair of damaged myelin (Barres et al., 1993). TGF-β is thought to be required for the generation of effector Th17 cells (Bettelli et al., 2006). Glial-derived TGF-β is also critical for the initiation of EAE in a Th17-independent manner. This cytokine is chemotactic for monocytes, neutrophils and T cells, and also activates microglia and astrocytes, thereby inducing leukocyte infiltration and gliosis (Luo et al., 2007). Yet TGF-β is a potent inhibitor of activated innate immune cells (Merrill & Zimmermann, 1991; Krieglstein et al., 1995). TGF-β also induces differentiation of oligodendrocyte precursors into myelin-producing cells in vitro, and may therefore be doubly beneficial in the repair of lesions in MS and EAE, where it is produced locally in or near the lesion site (Issazadeh et al., 1995; De Groot et al., 1999; Merrill & Benveniste, 1996). This apparent discrepancy of cytokine function is most likely explained by the timing of the appearance of the cytokines in relation to the stage of inflammation and the location in which they are produced in the CNS, as well as by the state of activation of the putative target cell (Merrill & Benveniste, 1996). The recent debate regarding the inflammatory or protective role of IFN-γ in the pathogenesis of EAE may in the end conclude that it too is governed by the temporal and spatial relationship between it and its receptor-bearing target cell in the CNS and the immune system.
3.1.1 Aims and rationale for the study

It is thought that IL-17-producing T cells are activated in the periphery early after induction of EAE, and subsequently migrate to the CNS, where they stimulate local production of pro-inflammatory cytokines by resident glial cells. The present study was undertaken to determine the kinetics of gene expression for a number of the molecules associated with the differentiation and production of IL-17 by T cells, both in the periphery and the CNS.

The dynamics of mRNA expression for inflammatory molecules associated with Th17 cell development, effector function and regulation were investigated at time-points reflecting the preclinical, acute and chronic phases of EAE, as well as controls and mice that had just been immunized with MOG in CFA and pertussis toxin. Therefore, at 12 hours, and 7, 14, and 21 days post immunization, gene expression was measured for the pro-inflammatory cytokines IL-17, TNF-α and IFN-γ, the transcription factor required for the differentiation of IL-17-producing T cells, RORγt, some of the cytokines thought to have a role in driving this differentiation including IL-1β, IL-23p19, IL-6 and TGF-β, as well as that of the other main regulatory cytokine in addition to TGF-β, IL-10. The concentration of mRNA expression at each time point was compared to the level of expression of that cytokine in control mice (sacrificed at the same time points), following normalization to the endogenous control, 18S rRNA. Tissues examined include the inguinal lymph nodes and spleen in the periphery, and the cerebral cortex, cerebellum and spinal cord in the CNS. In addition, antigen-specific IL-17 and IFN-γ production by spleen and lymph node cells was measured at each time point.
Following internalization of pathogens or exogenous antigen, immature DCs migrate to the draining lymph nodes and undergo a maturation process, rendering them capable of priming naïve T cells (Pulendran, 2004). DCs can also secrete cytokines that help determine the phenotypic lineage of effector T cells. The induction of EAE involves the injection of myelin peptides emulsified in CFA, s.c. in the back of susceptible mice. Mice are also injected i.p. with pertussis toxin on the day of immunization and again two days later, in order to help initiate a systemic immune response and possibly open up the blood-brain barrier to allow infiltration of peripheral immune cells. Because of the sites of injection, the local draining lymph nodes are the inguinal lymph nodes (ILN), and so these lymph nodes were examined in all studies below.

As well as having haematologic and vascular reservoir functions, the spleen is also an important lymphoid organ. The structure of the spleen enables it to remove older erythrocytes from the circulation by phagocytosis, recycling iron at the same time, and play an important role in the removal of blood-borne microorganisms and cellular debris (Mebius & Kraal, 2005). It functions as part of both the innate and adaptive immune systems. The spleen provides nonspecific defenses, by producing opsonins, resulting in the capture and destruction of pathogens. Blood-borne DCs, having captured bacteria in the circulation and transported them to the spleen, there mediate the initial differentiation and survival of B cells to become antibody-producing plasmablasts. On entry of APCs to the white pulp, T cells become activated, which can then help the B cells (Mebius & Kraal, 2005). As such, the spleen is an important immune-related organ and the relative expression of different cytokines here will be important in the differentiation and activation of specific T cell subsets during the development of disease.
Whilst understanding that the brain works as a whole by inter-relating its component parts, it is possible to divide the brain into individual regions discernable by anatomy and function. The two hemispheres of the cerebral cortex and the corpus callosum which connects them form a large grey matter area at the highest level of the brain, and lesions are commonly found here and in the subcortical white matter in MS patients (Calabrese et al., 2007; Bö et al., 2006). The cerebellum is located at the lower back of the brain, just above the brain stem, and is involved in the coordination of voluntary motor movement, balance and equilibrium, and muscle tone. It is immature at birth, but develops through childhood and adolescence, reaching its full structural growth by the fifteenth to the twentieth year of life (Leiner et al., 1993). The cerebellum contains more neurons than all the rest of the brain combined, and is a more rapidly acting mechanism than any other part of the brain. The cerebellum is connected to the cerebral cortex by approximately 40 million nerve fibres, allowing it to process a torrent of information from sensory and motor areas of the cerebral cortex, as well as cognitive and language areas, and even areas involved in emotional functions (Leiner et al., 1993). In addition, the choroid plexus of the fourth ventricle is situated just in front of the cerebellum. The CCR6-CCL20 axis in the choroid plexus has recently been identified as an important regulator of lymphocyte entry into the uninflamed CNS (Reboldi et al., 2009). These two important parts of the brain were both examined for their expression of mRNA for the inflammatory cytokines described above.

The spinal cord is the structure through which the brain communicates with all parts of the body below the head. In vertebrates, the spinal cord runs length-wise inside the vertebral column, or spine, and connects with the spinal nerves. Impulses for the general sensations such as touch and pain that arise in the limbs, trunk and neck must pass through the spinal cord to reach the brain, where they are perceived. Commands for voluntary movements in the limbs,
trunk and neck originate in the brain and must pass through the spinal cord to reach the spinal nerves that innervate the appropriate muscles. Thus, damage to the spinal cord may result in the loss of general sensations and the paralysis of voluntary movements in parts of the body supplied by the spinal nerves (Young et al., 2008). Anatomically, the spinal cord is the closest part of the CNS to the site of immunization in EAE, and could be an early access point for T cells following activation in the periphery. Analysis of molecular changes in the CSF, recovered by the spinal tap procedure, is also one of the most important tools used for molecular diagnosis of MS. Thus, whole spinal cord was also analyzed for changes in expression of inflammatory cytokine mRNA.
Chapter 3

3.2 Results

3.2.1 Clinical course of EAE

EAE was induced in C57BL/6 by s.c. injection of 100 μg of MOG35-55 emulsified in CFA, containing 4 mg/ml H37 Ra *M. tuberculosis*. Control mice were immunized with PBS in CFA or PBS alone. All mice were injected i.p. with 500 ng pertussis toxin (PT) on days 0 and 2. Animals were monitored daily for signs of clinical disease. The incidence of disease in mice immunized with MOG and CFA was 100%. Clinical disease presented from about day 11 post immunization, signs beginning with loss of tail tone (stage 1), and progressing through varying degrees of hind limb weakness, leading ultimately to hind limb paralysis (stage 4; Fig. 3.1). None of the control mice developed any clinical signs. Mice were sacrificed 12 h, and 7, 14, and 21 days post-immunization and the spleen, ILN, cerebral cortex, cerebellum and spinal cords removed for analysis of inflammatory molecule mRNA and protein expression.

3.2.2 IL-17A mRNA expression is upregulated early in peripheral immune organs, but not until after the onset of clinical signs in the CNS

IL-17 has recently emerged as a critical cytokine in T cell-mediated autoimmune inflammation, and can induce the production of IL-1, IL-6 and TNF-α in inflamed tissues. Compared to control mice that were administered PBS and PT only, IL-17 mRNA expression was significantly (P = 0.011) increased in the spleen, 7 days after induction of EAE (Fig. 3.2). IL-17 expression remained significantly (P < 0.05) higher than in control mice at day 14, albeit at a much lower expression than at day 7, and returned to control levels by day 21. Similarly, in the ILN, IL-17 mRNA expression increased 130-fold over controls 7 days post immunization, and returned to near control levels thereafter. In all three
areas of the CNS examined, an increase in IL-17 mRNA expression was not observed until day 14 post-immunization. In the cortex and cerebellum this increase persisted to day 21, while in the spinal cord, IL-17 mRNA expression increased 35 – 70-fold on day 14 to day 21. The relative increase in IL-17 expression in the CNS was more marked in the cerebellum and spinal cord, than in the cortex. The lack of significance despite substantial upregulation of IL-17 mRNA in the ILN and CNS of mice with EAE is due to an inability to detect IL-17 transcripts in these tissues in some naïve mice, reducing statistical power in the unpaired Student’s t test.

3.2.3 MOG-specific IL-17 production by spleen and lymph node cells from mice with EAE

Having demonstrated a substantial increase in the expression of IL-17 mRNA in the spleen and ILN, early in the development of EAE, we then examined MOG-specific IL-17 protein production by spleen and ILN cells. IL-17 secretion by MOG-specific T cells from the spleens of mice with EAE was detected as early as day 7 post immunization (Fig. 3.3A). At the higher concentration of MOG (25 μg/ml), IL-17 production by spleen cells was significantly (P < 0.001) greater than that from control mice. Although slightly lower on day 14 (P < 0.01), the concentration of IL-17 in the supernatants peaked at 600 pg/ml 21 days post-immunization (P < 0.01). IL-17 secretion was also induced in a concentration-dependant manner in response to MOG-restimulation by lymph node cells from mice on days 7, 14 and 21 post-induction of EAE (Fig. 3.3B).
3.2.4 Elevated expression of RORγt in the lymph nodes, but only moderate increases in the CNS

RORγt, coded for by the Rorc gene, is the transcription factor required for the differentiation of IL-17-producing T cells. The level of expression of RORγt mRNA did not change in the spleen over the course of disease (Fig. 3.4). In the ILN, there was an increase in the expression of RORγt mRNA as early as 12 h post-immunization, increasing through day 7 and rising to a 27-fold difference over controls by day 14. RORγt mRNA expression in the ILN declined after day 14. The inability to detect RORγt mRNA in the ILN of some naïve mice, likely affected the lack of significance of elevated RORγt in the ILN of mice with EAE. In all three areas of the CNS examined, RORγt mRNA expression fluctuated around the same level as controls. Surprisingly, however, there was a significant increase in expression in the spinal cord and cerebellum (both P < 0.001) 12 h after immunization. RORγt mRNA was also significantly elevated in the cerebellum on day 14. In the spinal cord, RORγt mRNA decreased again after the 12 h increase, and continued to decline until by day 21 it was actually significantly (P < 0.01) decreased compared to control levels.

3.2.5 IFN-γ mRNA expression is upregulated early in peripheral immune organs, but not until after the onset of clinical signs in the CNS

IFN-γ is the prototypical Th1-type cytokine. mRNA transcripts for IFN-γ were significantly (P < 0.01) increased in the spleen 7 days after induction of EAE, returning to the concentration of control mice by day 14, and continuing to decline significantly (P < 0.01) below this level by day 21 (Fig. 3.5). In the ILN, IFN-γ mRNA expression was 21-fold higher on day 7 in mice with EAE than in controls, and this increased further by day 14. While this level of expression began to decrease thereafter, it remained significantly (P < 0.01) higher on day 21 compared to control mice. IFN-γ mRNA expression in the spinal cord showed a
substantial increase on days 14 and, significantly (P < 0.01) so on day 21. IFN-γ expression was also significantly (P < 0.05) increased on day 14 in the cerebellum, and increased nearly 3-fold further again by day 21 (P < 0.05). IFN-γ mRNA expression in the cerebral cortex showed a similar expression pattern to that in the cerebellum, if somewhat less dramatic in terms of fold increases over control mice.

3.2.6 MOG-specific IFN-γ production by spleen and lymph node cells from mice with EAE

IFN-γ mRNA was upregulated early in the development of disease in the periphery, but not until later in the CNS. Whilst a small amount of MOG-specific IFN-γ was detectable in the supernatants of MOG-restimulated spleen and lymph node cells isolated from mice with EAE on day 7, a significant increase in MOG-specific IFN-γ production was not seen until 21 days post-immunization in both tissues (Fig. 3.6). Spleen cells isolated 21 days post-immunization, produced almost 14 ng/ml IFN-γ in response to MOG-restimulation in vitro; this was significantly (P < 0.01) more than the 450 pg/ml secreted by cells from control mice (Figure 3.6A). There was a similar but less pronounced increase in production of IFN-γ by cells from the ILN (Fig. 3.6B), again peaking on day 21 at almost 6 ng/ml (P < 0.05) in response to restimulation with 25 µg/ml MOG.

3.2.7 TNF-α mRNA expression was significantly elevated in all areas of the CNS after the onset of clinical signs of EAE

TNF-α is an very important pro-inflammatory cytokine known to be involved in the pathogenesis of a number of autoimmune disorders, and its production is induced in inflamed tissues by IL-17. TNF-α mRNA expression was only moderately upregulated in the spleen of mice with EAE 7 days post
induction compared to controls, and did not show any change in expression in the ILN until day 21, at which stage there was a significant (P < 0.05) increase in expression (Fig. 3.7). In contrast, TNF-α mRNA expression was significantly increased in the cortex and spinal cord (both P < 0.05) of mice 7 days after induction of EAE, when compared with control mice. TNF-α expression was further elevated by day 14 in the cortex and spinal cord (both P < 0.01), but also in the cerebellum (P < 0.01). By day 21, the fold increase in TNF-α mRNA expression compared with control mice was highly significant in the cortex, cerebellum and spinal cord (all P < 0.001).

3.2.8 IL-1β mRNA expression is significantly upregulated early both in the periphery and the CNS

IL-1β is an innate cytokine produced by numerous cells of both the immune system and CNS, and is thought to have a role both in the induction upstream and effector functions downstream of IL-17 signaling. IL-1β mRNA expression was modestly, but significantly (P < 0.001), upregulated as early as 12 h post immunization in the spleen (Fig. 3.8). Expression peaked at day 7 (P < 0.001), and remained significantly elevated over control levels 14 days (P < 0.001) after induction of EAE. In the ILN, IL-1β mRNA expression was upregulated on day 7 of EAE, and was elevated 100-fold over control levels by day 14, declining thereafter, but remaining higher than controls even after 21 days. The largest increase in IL-1β mRNA expression was observed in the cerebellum. 7 days after immunization, mRNA expression was significantly (P < 0.001) higher than in control mice, and continued to increase, up to 156-fold higher (P < 0.01) than that seen in the cerebellum of control mice, before beginning declining, but still very significantly (P < 0.001) elevated on day 21. IL-1β mRNA expression was considerably increased in the cortex 7 and 14 days post induction of EAE, and decreased by day 21, but concentrations still remained 11-fold higher than those seen in control mice. Finally, whilst the fold changes in
IL-1β mRNA expression in the spinal cord were less pronounced than elsewhere in the CNS, it was significantly elevated over controls at all time-points examined. At 12 h post immunization, IL-1β mRNA expression decreased (P < 0.05), before significantly (P < 0.01) increasing over control levels by day 7, and further increasing (P < 0.01) by day 14. IL-1β mRNA expression remained significantly (P < 0.001) higher than that seen in control mice, 21 days after induction of EAE.

3.2.9 IL-23p19 mRNA is upregulated early in the periphery, but exhibits little change in expression in the CNS over the course of EAE

IL-23p19 is produced by numerous cell types, mostly phagocytic APC, both in the CNS and the periphery, and is thought to have a role in either the differentiation of IL-17-producing T cells or the induction of IL-17 production by innate and memory T cells, or both. IL-23p19 mRNA expression increased roughly 16-fold in both the spleen (P < 0.05) and ILN 7 days post induction of EAE, and whilst the expression in the spleen quickly returned to the level of control mice, expression in the ILN more than doubled again by day 14, before returning to control levels (Fig. 3.9). In the cortex, there was a modest yet significant (P < 0.01) increase in IL-23p19 mRNA expression 14 days after immunization. There was also a significant (P < 0.01) increase in IL-23p19 expression in the cerebellum at this stage. However, in the spinal cord, the only real changes were a significant decrease in expression 7 and 21 days (both P < 0.05) post-immunization.

3.2.10 IL-6 mRNA is upregulated early in the periphery during the development of EAE and after the onset of clinical signs in the CNS

IL-6 production is induced in inflamed tissues in response to IL-17, and is also thought to play a role in the differentiation of Th17 cells. IL-6 mRNA
expression in the spleen increased significantly \( (P < 0.001) \) 7 days after induction of EAE, and remained significantly elevated \( (P < 0.01) \) over control mice by day 14, before declining thereafter (Fig. 3.10). In the ILN, IL-6 expression was also substantially upregulated on day 7 of EAE, and increased to over 100-fold compared to controls, by day 14, before returning to normal levels. At the same time, IL-6 mRNA expression in all parts of the CNS measured was decreased early in the development of disease. In the cerebellum, expression decreased significantly \( (P < 0.001) \) just 12 h after immunization, and in the cerebellum \( (P < 0.001) \), cortex \( (P < 0.001) \) and spinal cord \( (P < 0.05) \), IL-6 mRNA expression was significantly down-regulated on day 7. On day 14, after the onset of clinical signs, there was a modest increase in IL-6 expression in the spinal cord and cortex, compared to that in control mice. In the cerebellum, however, the expression of IL-6 mRNA was almost 200-fold greater \( (P < 0.01) \) compared with that in the cerebellum of control mice.

3.2.11 TGF-β is modestly upregulated in the CNS late in the development of EAE

TGF-β is an important regulatory cytokine produced by nTreg cells and inducible Th3 cells. It has been shown to be important in the amelioration of signs in EAE, but paradoxically is also thought to play a role in the differentiation of naïve precursors into Th17 cells. TGF-β mRNA expression in the spleen fluctuated around the level of control mice, showing no significant change over the course of EAE (Fig. 3.11). In the ILN, there was a modest 2.5-fold increase over control levels 12 h and 7 days after immunization, and then showed an over 8-fold increase over control mice on day 14, before decreasing slightly by day 21. In the cortex and cerebellum (both \( P < 0.05 \)), there was a small but significant decrease in TGF-β mRNA expression 7 days after immunization. By day 14, however, there was a significant increase in TGF-β mRNA expression in both tissues (cortex, \( P < 0.05 \); cerebellum, \( P < 0.01 \)), compared with controls, returning
to normal levels by day 21. The production of TGF-β mRNA expression in the spinal cord demonstrated a different profile, with no change in expression up to and after the onset of clinical signs, but increasing significantly ($P < 0.05$) over controls by day 21.

### 3.2.12 IL-10 mRNA expression is upregulated late in EAE in both the ILN and the CNS

IL-10 is an important regulatory cytokine produced primarily by inducible Tr1 cells, and thought to inhibit pro-inflammatory cytokine production and have a role in the regulation of EAE. There was no significant change in IL-10 mRNA expression in the spleen throughout the course of EAE (Fig. 3.12). In the ILN, there was a slight increase in IL-10 mRNA expression on day 14, and a smaller but significant ($P < 0.01$) increase over controls on day 21. In the cortex, IL-10 mRNA was significantly ($P < 0.05$) upregulated 14 days post induction of EAE, and further increased on day 21 ($P < 0.01$). There was also a large (approximately 30-fold) increase in IL-10 mRNA expression over control levels in the cerebellum on days 14 and 21. In the spinal cord there was a significant decrease in expression of IL-10 mRNA at 12 h ($P < 0.05$) and 7 days ($P < 0.01$) post-immunization, followed by a return to control levels by day 14.
3.3 Discussion

IL-17 has recently emerged as a critical cytokine in organ specific T cell-mediated autoimmune inflammation. Multiple cell types, including CD4$^+$ and CD8$^+$ T cells, γδ T cells, NKT cells and neutrophils have been shown to produce IL-17, which mediates inflammation by stimulating the production of IL-1, IL-6, TNF-α and other inflammatory cytokines and chemokines in inflamed tissues and promoting the recruitment of neutrophils and macrophages.

This study found a large increase in the expression of IL-17 mRNA in the CNS after the onset of clinical signs of EAE. In the cortex and cerebellum this increased expression persisted through to day 21 post immunization, whilst it almost doubled again in the spinal cord. It is known that microglia can secrete IL-17 in vitro in response to IL-1β and IL-23 (Kawanokuchi et al., 2008), but the most potent producers of IL-17 are T cells. As such, the elevated IL-17 seen in the CNS of mice with clinical signs of EAE is most likely the product of autoinflammatory T cells that have infiltrated from the periphery. In support of this theory, we found significant upregulation of IL-17 mRNA in the spleen and ILN of mice before the onset of clinical signs of EAE, which waned as disease progressed.

MOG-specific IL-17 production was also observed in cells isolated from the spleen and lymph nodes of mice with EAE as early as 7 days post-immunization. This IL-17 production occurred in a concentration and antigen-specific manner and was also evident in cells isolated on days 14 and 21, but not in cells from control mice and suggests that the spleen and lymph nodes are important sites for the differentiation of IL-17-producing T cells early in the development of EAE.
Taken together, it is likely that IL-17-producing T cells are generated in lymphoid organs early after immunization, from which they enter the blood and circulate until they are reactivated upon contact with their cognate antigen, in this case myelin components produced by oligodendrocytes in the CNS. Activated T cells can easily penetrate the blood-brain barrier and enter the CNS, regardless of their antigen specificity (Wekerle et al., 1986). However, only T cells that recognize CNS antigen persist. Their presence likely initiates infiltration by other leukocytes, such as neutrophils, NK cells and unconventional T cells, which might amplify the production of IL-17, and recruit endogenous glia to the inflammatory process.

RORγt has been identified as one of the critical transcription factors involved in the generation of IL-17-producing T cells (Ivanov et al., 2006a). Encoded by the Rorc locus, RORγt is expressed only by haematopoietic cells, and participates in the formation of lymph nodes and Peyer’s patches (Dzhagalov et al., 2004). IL-17-producing T cells are absent from the intestinal lamina propria in RORγt deficient mice, and transduction of naïve T cells with a RORγt-encoding retrovirus induces IL-17 production (Ivanov et al., 2006a). This study found an incremental increase in RORγt mRNA expression over the course of development of disease in the ILN, peaking 14 days after immunization and then declining again on day 21. There was no change in the expression of RORγt in the spleen, and no real deviation from control levels in any region of the CNS examined, throughout the course of EAE. Whilst, elevated expression was limited to the lymph nodes, it did appear that RORγt was highly constitutively expressed throughout the CNS and the spleen at all stages of disease, and in control animals, as determined by the low cycle threshold at which exponential amplification of the Rorc transcripts occured. Thus, either the majority of IL-17-producing T cell differentiation occurs in the lymph nodes, or basal levels of RORγt expression in cells of naïve mice is sufficient to mediate the generation of IL-17-producing
effector T cells. In support of the latter possibility, it is currently thought that Foxp3 and RORγt are both expressed by TCR-activated CD4⁺ T cells, but that in the presence of TGFβ, Foxp3 binds to RORγt, antagonizing its function. In the presence of IL-6 or IL-21, however, RORγt is released, which then induces IL-23 receptor expression on T cells, conferring responsiveness to IL-23 and promoting Th17 cell differentiation (Bettelli et al., 2006; Zhou and Littman, 2009).

The differentiation of naïve T cells into the different effector or regulatory populations is controlled by a number of factors, including the cytokine milieu at the site of antigen presentation. It was initially reported that IL-23 was the key cytokine involved in the differentiation of IL-17-producing T cells (Langrish et al., 2005). Subsequently it was shown that naïve T cells do not express the IL-23R and cannot differentiate into IL-17-producing T cells in response to IL-23 alone. However, work from our laboratory has demonstrated a role for IL-23 in synergy with IL-1α or IL-1β in promoting IL-17 production by T cells (Sutton et al., 2006). T cells from IL-1 receptor type I-deficient (IL-1RI⁻) mice failed to induce IL-17 production in response to IL-23 and the induction of antigen-specific Th17 cells was abrogated in IL-1RI⁻ mice (Sutton et al., 2006). Furthermore, it was recently demonstrated that IL-23 is absolutely required for the development of encephalogenic T cells and that, although not required for lineage commitment, IL-23 is essential for the terminal differentiation and full pathogenic activity of Th17 cells (Thakker et al., 2007; McGeachy et al., 2009). Both IL-1 and IL-23, therefore, play key roles in the differentiation of IL-17-producing T cells.

The present study found a significant increase in the expression of IL-23p19 mRNA in the spleen and ILN 7 days after the induction of EAE, and that expression was further elevated in the ILN at least until day 14. While the precise mechanism by which IL-23 contributes to inflammation is still unknown, IL-23 is
known to induce the proliferation and secretion of IL-17 from memory CD4^ T cells (Aggarwal et al., 2003), and we have shown that IL-23 can induce IL-17 production in γδ T cells in the absence of TCR engagement (Sutton et al., 2009). Examining mRNA expression 7 days after the induction of EAE may have precluded observation of the initial responses of DCs following maturation, however, the increased expression of IL-23p19 mRNA in the periphery before the onset of clinical signs, demonstrates a potential role for IL-23 in the differentiation or activation of IL-17-producing T cells, and as such coincides well with the upregulation of IL-17 mRNA in these organs at this time point. Furthermore, a growing body of evidence implicates IL-23 in the acquisition of CNS homing properties by autoimmune effector T cells. It has been demonstrated that T cells defective in IL-23 signaling expanded to a similar extent as WT antigen-specific CD4^ T cells, but failed to accumulate in the CNS at the peak of EAE (Gyulveszi et al., 2009). This study supports findings by Prat and colleagues (2007) who demonstrated that IL-23-stimulated peripheral blood CD4^ T cells are particularly efficient at penetrating an in vitro model of the blood-brain barrier (Kebir et al., 2007).

IL-23 is produced rapidly by numerous cell types, mostly phagocytic APC, in both the CNS and the periphery after exposure to pathogen-derived molecules (McKenzie et al., 2006). It is probable that after activation by TLR ligands DC, as well as monocytes and macrophages, secrete IL-23 which in turn amplifies local expression of pro-inflammatory cytokines, including IL-1β, TNF-α, IL-17, IL-12 and IFN-γ (Langrish et al., 2004; Belladonna et al., 2002). In this way, IL-23 may drive inflammation by triggering a pro-inflammatory cytokine cascade. This study found little change in IL-23 expression in the CNS over the course of disease. However, the time of sampling may have missed the peak of expression, and it is possible that IL-23 is produced by microglia and astrocytes in response to
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signals from infiltrating T cells, and may play a role in the re-activation of T cells coming into contact with their cognate antigen.

IL-1β is an innate cytokine produced by numerous cells of both the immune system and CNS, and is thought to have a role both upstream and downstream of IL-17 signaling. Numerous groups have demonstrated a role for IL-1β in the activation of resting naïve and memory antigen-specific T cells (Badovinac et al., 1998; Nakae et al., 2003a,b). Immunization of IL-1R1−/− mice with MOG and CFA failed to induce MOG-specific Th17 cells (Sutton et al., 2006). In addition to its role in the differentiation of IL-17-producing T cells, IL-1β plays a significant role in leukocyte recruitment to the CNS (Gibson et al., 2004). IL-1β may also contribute to blood-brain barrier permeability in MS (Argaw et al., 2006). Thus, IL-1β is capable of over-riding the intrinsic resistance of the CNS to leukocyte infiltration, resulting in acute cellular recruitment to the brain parenchyma (Shaftel et al., 2007). IL-1β expression is induced in many cells in inflamed tissues in response to IL-17 (Jovanovic et al., 1998), where it promotes the proliferation and activation of local cells, such as microglia and astrocytes in the CNS, and upregulates an array of genes, including those for chemokines, adhesion molecules, and cytokines such as IL-6 and TNF-α (Simi et al., 2007). IL-1β, therefore, is a key cytokine in the pathogenesis of EAE, and, furthermore, its concentration in the CSF of MS patients has been shown to correlate with disease activity (Hauser et al., 1990).

This study found an immediate and significant increase in IL-1β mRNA expression in the spleen, 12 h after immunization with MOG and CFA and the administration of pertussis toxin. IL-1β was further upregulated in the spleen and ILN before the onset of clinical signs and into the acute phase of disease. The increased expression of IL-1β in the lymphoid organs of mice with EAE before
and around the same time as IL-17 expression was elevated is consistent with a role for IL-1 in the development of IL-17-producing T cells early in the pathogenesis of EAE. The sustained expression of IL-1β in these tissues after the expression of IL-17 has diminished might reflect the elevated expression of IL-1β by monocytes and macrophages in response to IL-17 signaling, forming an autocrine loop and resulting in its chronic expression.

IL-1β mRNA expression was significantly upregulated in all areas of the CNS when examined 7 days after induction of EAE. Expression was further elevated through days 14 to 21. IL-1β is rapidly produced by microglia and astrocytes initially and subsequently by neurons and vascular endothelial cells in response to infection or damage in the CNS. Infiltrating leukocytes are thought to contribute to this neuroinflammatory response. It has been shown that there is an initial modest influx of T cells into the brain of mice with EAE before a second, larger wave of infiltration (Wekerle et al., 1986). It is possible that T cells activated in the periphery early after immunization with MOG and CFA migrate across the blood-brain barrier to the CNS, where they act upon resident glia, inducing production of a range of pro-inflammatory cytokines, including IL-1β. In addition, it was recently reported that auto-antigenic CD4⁺, CD8⁺ and particularly IL-17⁺ γδ T cells all secrete IL-1β, and that increased IL-1β expression induces re-localization of the lymphoid chemokine CXCL12 at the blood-brain barrier, permitting leukocyte infiltration of the CNS parenchyma (McCandless et al., 2009). This pathologic pattern of CXCL12 expression at the blood-brain barrier was also found to correlate with disease severity in MS (McCandless et al., 2008).

Elevated IL-1β expression has been demonstrated to cause leakage of the blood-brain barrier, and play a significant role in leukocyte recruitment to the
CNS (Argaw et al., 2006; Shaftel et al., 2007). Astrogliosis and neuroinflammation have repeatedly been described as preceding clinical signs of autoimmunity (Luo et al., 2007; Ponomarev et al., 2005), and it is possible that the infiltration of a small number of activated antigen-specific T cells early during the development of disease leads to increased expression of IL-1β, both by infiltrating T cells and resident glia, disruption of the blood-brain barrier and the subsequent influx of a host of antigen-specific and non-specific T cells, macrophages, NK cells and neutrophils. This second, larger wave of infiltrating immune cells may also induce IL-1β production by CNS resident cells, perhaps accounting for the substantial upregulation of IL-1β mRNA found after the onset of clinical signs of EAE. This in turn might synergize with the elevated levels of IL-23 at this time-point to bring about the production of IL-17 by infiltrating T cells and microglial cells, both of which have been demonstrated to secrete IL-17 in response to IL-1β and IL-23 (Sutton et al., 2006; Kawanokuchi et al., 2008). In support of this, increased expression of IL-17 mRNA was observed from 14 to 21 days after induction of EAE.

IL-1 enhances production of IL-6 and TNF-α (Simi et al., 2007), two cytokines also induced in response to IL-17 signaling (Bettelli et al., 2007). While it is known that IL-23 drives IL-17 production by memory T cells and IL-23-deficient mice lack IL-17-producing T cells in the CNS, and are resistant to EAE, it is also thought that IL-6 in combination with TGF-β is required for the differentiation of naïve T cell precursors to Th17 cells (Bettelli et al., 2006). IL-6^{-/-} mice are resistant to EAE and mice lacking the gp130 receptor subunit, and therefore defective in IL-6 signaling, do not generate a Th17 response (Eugster et al., 1998; Korn et al., 2008). Naïve T cells do not express IL-23R on their surface, and it is thought that IL-6, possibly by inducing IL-21, upregulates IL-23R upon ligation of the TCR and thus promotes Th17 cell differentiation in the presence of IL-23 (Zhou et al., 2007). Furthermore, it has been demonstrated that
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IL-6 negates the suppressive effect of Foxp3 on RORγt function and inhibits the generation of Foxp3⁺ T reg cells induced by TGFβ (Zhou and Littmann, 2009; Bettelli et al., 2006). IL-6 expression in the CNS is induced in response to IL-1 signaling (Simi et al., 2007), and IL-17 has also been demonstrated to activate cells of the local inflammatory environment to produce IL-6 (Shimada et al., 2002).

Analogous to the findings with IL-1β, and perhaps in response to its signaling, this study revealed a rapid increase in IL-6 expression in the periphery after induction of EAE. IL-6 mRNA was substantially upregulated in the spleen and ILN around the onset of clinical signs of EAE. Examination of the expression of IL-6 mRNA in total ILN tissue does not permit discrimination between the possible role of IL-6 in inducing the differentiation of Th17 cells or whether it is being induced in response to or produced by IL-17-secreting T cells. Unlike IL-1β, however, IL-6 expression in the lymph nodes returns to control levels 21 days after induction of EAE, perhaps supporting the theory that it functions primarily in the differentiation of naïve precursors to Th17 cells in the peripheral lymphoid tissues.

Conversely, in the CNS IL-6 mRNA expression remained homeostatic until after the onset of clinical signs of EAE. The present study found a large increase in IL-6 mRNA expression 14 days post induction of EAE, likely induced in resident cells of the CNS by IL-17 and IL-1β signaling, expression of which were already shown to be elevated in the CNS at this stage. Th17 cells also produce IL-6 (Langrish et al., 2005), and this is likely to contribute to the elevated concentration of IL-6 in the CNS following inflammatory cell infiltration from the periphery. Indeed, the greatest increase in IL-6 expression was observed in the cerebellum, and it was recently demonstrated that the cerebellum, as well as the
brain stem, is particularly susceptible to blood-brain barrier breakdown and cellular infiltration (Smorodchenko et al., 2007; Silwedel and Forster, 2006; Muller et al., 2005). Finally, it is interesting to note that there was a significant decrease in IL-6 expression 7 days post immunization in all areas of the CNS examined. This coincided with the peak of IL-6 expression in the periphery, and is at odds with the early upregulation of IL-1β at this stage of disease in the CNS. The functional significance of this is uncertain.

When combined with inflammatory cytokines such as IL-6 or IL-21, TGF-β is a strong inducer of Th17 cell differentiation in vitro and promotes expression of the critical transcription factor for the differentiation of Th17 cells, RORγt (Bettelli et al., 2006; Mangan et al., 2006; Korn et al., 2007). Indeed, blocking TGF-β locally at the time of immunization abrogates induction of EAE (Veldhoen et al., 2006). Conversely, TGF-β is known to drive the reciprocal conversion to CD25+ Treg cells, by inducing expression of the lineage-specific transcription factor Foxp3 (Bettelli et al., 2006). Until recently, TGF-β was considered solely an anti-inflammatory cytokine, whose upregulated expression has been demonstrated during the remission phase of EAE (Chen et al., 1998), and whose production by Treg cells was found to mediate oral tolerance in this disease (Chen et al., 1994). Furthermore, administration of soluble TGF-β1 can ameliorate EAE (Racke et al., 1991). Thus, TGF-β appears to function early to amplify the disease process and later to attenuate it.

In the present study, TGF-β mRNA expression in the peripheral lymphoid organs did not change significantly over the course of EAE. Despite a modest increase in the ILN 14 days post induction, it appears the requirement for TGF-β in the initial differentiation of Th17 cells is met by the presence of normal levels of this cytokine in the lymphoid organs. Indeed, it has been reported that TGF-β
does not directly promote Th17 cell differentiation but rather acts indirectly by blocking expression of the transcription factors STAT 4 and GATA-3, thus preventing Th1 and Th2 cell differentiation (Das et al., 2009). Moreover, IL-6 alone was sufficient to induce differentiation of naïve precursors to Th17 cells in Stat-6<sup>-/-</sup> T bet<sup>-/-</sup> mice, which are unable to generate Th1 and Th2 cells (Das et al., 2009). It is possible that the increased expression of TGF-β on day 14 in the ILN derives from inducible antigen-specific Th3 regulatory T cells, which have been shown to be induced in response to myelin protein in EAE (Weiner, 2001).

Wyss-Coray and colleagues described the requirement for early expression of TGF-β1 by glial cells in the CNS in order to create a permissive environment for the initiation of autoimmune inflammation (Luo et al., 2007). The present study, however, found no increase in TGF-β1 signaling before the onset of clinical signs of EAE, and even observed a slight decrease in TGF-β expression in the cortex and cerebellum 7 days after immunization. It is possible that background levels of TGF-β may suffice to bring about the chemotactic signaling required for macrophage infiltration and gliosis associated with lesion development. Alternatively, other molecules may be involved in this aspect of disease progression and TGF-β may be solely required for the differentiation of naïve T cell precursors into Th17 cells in the periphery. The reduced expression of IL-6 at this stage of disease might result from the reduced availability of TGF-β in the CNS, as IL-6 was also found to be induced in response to TGF-β signaling (Jones, 2005).

A small but significant increase in TGF-β mRNA expression was detected in the acute and chronic stages of EAE. While TGF-β is initially produced by astrocytes and microglia predominantly (Luo et al., 2007), it is subsequently upregulated in neurons (Liu et al., 2006) and produced by infiltrating T cells
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(Korn et al., 2007). Activation and proliferation of Foxp3 \(^+\) Treg cells has been demonstrated in the inflamed CNS (O'Connor et al., 2007), as has the accumulation of peripherally expanded Tregs, despite failing to control autoimmune inflammation (Korn et al., 2007). However, Liu and colleagues (2006) described the generation of neuron-dependent Treg cells, via neuronal secretion of TGF-\(\beta\), and the conversion of effector T cells to Treg cells that can suppress encephalitogenic T cells and ameliorate the signs of EAE (Liu et al., 2006). Thus, the later upregulation of TGF-\(\beta\) in the spinal cord and cerebellum may result from neurons and activated Treg cells, suggestive of a role in regulating CNS inflammation.

IL-10 plays a critical role in the regulation of EAE by controlling auto-pathogenic T cells (Bettelli et al., 1998). IL-10\(^{+/+}\) mice are more susceptible and develop a more severe EAE compared to wild-type mice, and T cells from IL-10\(^{+/+}\) mice exhibit stronger antigen-specific proliferation and produce more IFN-\(\gamma\) and TNF-\(\alpha\) when restimulated \textit{ex vivo} (Bettelli et al., 1998). Previously called cytokine synthesis inhibitory factor (CSIF), IL-10 is capable of inhibiting the synthesis of several cytokines from different cells and impairs the ability of APCs to stimulate Th1-type lymphocytes (Fiorentino et al., 1989, 1991a/b). As such it represents one of the most important immune-regulating cytokines.

In the present study, IL-10 mRNA expression in the spleen was found to decrease to almost half its normal level 7 days after immunization for EAE, before gradually increasing again toward the level observed in control mice. This observation is consistent with the marked upregulation of expression of many pro-inflammatory genes at this time in the spleen. IL-10 mRNA expression in the ILN correlated well with that of TGF-\(\beta\) with a modest increase by day 14 persisting through to day 21. Substantial upregulation of IL-10 mRNA expression
was observed in the brain during the acute and chronic phases of EAE and may be the product of induced or adaptive Treg cells, differentiated in response to TGF-β secretion by neurons, or infiltrating from the peripheral compartment. Indeed, a role for IL-10 has been described in the suppression of pathogenic autoreactive T cells in EAE by CD25⁺CD4⁺ natural Tregs (Zhang et al., 2004). A role for autoantigen-reactive B cell-derived IL-10 in the resolution of EAE has also been described (Fillatreau et al., 2002).

Although IL-10 expression in the spinal cord shows no change in its regulation at the later stage of disease, relative to the concentrations seen in control mice, the pattern of expression at all time-points in mice with EAE almost mirrors that seen elsewhere in the CNS. It is, therefore, likely that the IL-10 mRNA expression observed in the control mice was extraordinarily high, and skewed the relative concentrations of IL-10 observed in mice with EAE.

Before the identification of IL-17, TNF-α and IFN-γ were thought to be the two main pro-inflammatory cytokines responsible for both immunity against infection and mediating pathology across a range of autoimmune disorders. More recently, the focus of much research has switched to the role of IL-17 in mediating autoimmunity, however, each of these cytokines are still considered to play an important role in the pathogenesis of many inflammatory autoimmune disorders.

TNF-α is an inflammatory cytokine produced by T cells and macrophages as well as microglia and astrocytes. It has been demonstrated in the CNS of mice with EAE and in the lesions of MS patients (Merrill et al., 1992; Selmaj et al., 1991). CNS resident glial cells and infiltrating macrophages, stimulated by IFN-γ secreted by infiltrating T cells, are thought to be the main source of TNF-α in MS.
lesions and EAE (Selmaj et al., 1991; Renno et al., 1995). It has also been demonstrated that IL-17-producing T cells can secrete TNF-α (Langrish et al., 2005), and that IL-17 is a potent inducer of TNF-α from local cells in inflamed tissues (Jovanovic et al., 1998). TNF-α, together with IL-1β and IFN-γ, is known to induce VCAM-1 expression on a wide variety of cells, including endothelial cells, DCs and astrocytes, and the interaction between this cell adhesion molecule and VLA-4 – expressed on the surface of all lymphocytes and monocytes – might allow circulating lymphocytes to localize to the CNS (Merrill and Benveniste, 1996).

The present study found only a small increase in TNF-α mRNA expression in the spleen 7 days after induction of EAE, coinciding with the peak of IL-17, IL-23, IL-1β and IL-6 expression in the spleen during the development of disease and may be expressed by Th17 cells. In contrast, TNF-α expression in the ILN and the CNS was not upregulated until after the onset of clinical signs, and peaked in the chronic stage of disease. TNF-α is produced by T cells, but the main source of this cytokine in the CNS during EAE is likely resident glial cells and infiltrating macrophages. Its expression is induced in response to IL-17 signaling, and it is possible that the peak of IL-17 expression in the CNS 14 days after immunization, precedes, and is responsible for, the elevated expression of TNF-α in the later stages of disease.

IFN-γ is also known to induce the expression of TNF-α in microglia, along with a number of other inflammatory cytokines (Renno et al., 1995). This study found substantial upregulation of IFN-γ mRNA in all areas of the CNS 21 days after induction of EAE. Elevated expression was particularly significant in the cerebellum and spinal cord. In contrast, IFN-γ mRNA expression was upregulated early after immunization in the spleen and ILN. Despite the elevated
expression of IFN-γ mRNA 7 days post immunization in the spleen and lymph nodes, a significant increase in MOG-specific IFN-γ secretion by T cells was not observed until 21 days after induction of EAE. This supports the theory that naïve precursor T cells preferentially differentiate to a Th17 phenotype early after induction of EAE, perhaps due to the blocking of Th1 signals by TGF-β. Hence, early elevated IFN-γ mRNA expression may have been partially accounted for innate cells including NK cells and γδ T cells.

There has been much debate recently over a possible protective role for Th1-secreted IFN-γ in EAE. It has been demonstrated that IFN-γ is a potent suppressor of IL-17 production (Harrington et al., 2005; Park et al., 2005). Co-culturing of CD4+ T cells with IFN-γ-deficient APCs enhances production of IL-17 (Harrington et al., 2005). In contrast, addition of exogenous IFN-γ substantially inhibits the development of effector Th17 cells from IFN-γ-deficient precursor cells. Harrington and colleagues also provide evidence that IFN-γ might mediate this effect by down-regulating expression of the IL-23R (Harrington et al., 2005). Additionally, IFN-γ−/−, IFN-γR−/− and IL-12p35−/− mice develop a more severe disease course than wild-type mice (Ferber et al., 1996; Willenborg et al., 1996; Becher et al., 2002). Further support was provided by Das and colleagues (2009) with evidence that IL-6 alone was sufficient to induce Th17 differentiation in the absence of Th1 and Th2 cytokines. They point to an indirect role for TGF-β in Th17 responses through inhibition of Th1 and Th2 cell differentiation by preventing expression of STAT4 and GATA-3, thereby permitting Th17 cell lineage commitment (Das et al., 2009).

However, mice lacking the Th1 transcription factors T-bet and STAT-4, and thus lacking Th1 cells, have very large numbers of IL-17-producing T cells.
and yet are resistant to EAE (Chitnis et al., 2001; Bettelli et al., 2004). In addition, both Th1- and Th17-related cytokines are present in the CNS at the peak of disease. This suggests that both IFN-γ and IL-17 may cooperate to induce the development of CNS-specific autoimmunity, possibly mediating their effects at different stages of disease. Consistent with the data presented here, a report by Korn and colleagues (2007) showed that IL-17 expression peaks before IFN-γ in the CNS of mice with EAE and that IFN-γ persists for a longer period after IL-17 expression has disappeared. Therefore, it is possible that Th17 cells are generated more rapidly than Th1 cells and direct the initial acute inflammation, but that Th1 cells and their associated cytokines function to prolong and perpetuate tissue inflammation (Bettelli et al., 2007).
### Table 3.1 Summary

<table>
<thead>
<tr>
<th>mRNA expression</th>
<th>Post-immunization (12 h)</th>
<th>Pre-clinical (d 7)</th>
<th>Acute (d 14)</th>
<th>Chronic (d 21)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-17</td>
<td>--</td>
<td>↑ LN/Sp</td>
<td>↑ Sp/CNS</td>
<td>↑ CNS</td>
</tr>
<tr>
<td>RORγt</td>
<td>↑ CNS</td>
<td>↑ LN</td>
<td>↑ LN</td>
<td>↑ LN</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>--</td>
<td>↑ LN/Sp</td>
<td>↑ LN/CNS</td>
<td>↑ CNS</td>
</tr>
<tr>
<td>TNF-α</td>
<td>--</td>
<td>↑ Sp/CNS</td>
<td>↑ CNS</td>
<td>↑ LN/CNS</td>
</tr>
<tr>
<td>IL-1β</td>
<td>↑ Sp</td>
<td>↑ LN/Sp/CNS</td>
<td>↑ LN/Sp/CNS</td>
<td>↑ CNS</td>
</tr>
<tr>
<td>IL-23</td>
<td>--</td>
<td>↑ LN/Sp ↓ CNS</td>
<td>↑ LN/CNS</td>
<td>--</td>
</tr>
<tr>
<td>IL-6</td>
<td>--</td>
<td>↑ LN/Sp ↓ CNS</td>
<td>↑ LN/Sp/CNS</td>
<td>--</td>
</tr>
<tr>
<td>TGF-β</td>
<td>--</td>
<td>--</td>
<td>↑ LN/CNS</td>
<td>--</td>
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<tr>
<td>IL-10</td>
<td>--</td>
<td>--</td>
<td>↑ LN/CNS</td>
<td>↑ LN/CNS</td>
</tr>
</tbody>
</table>

* Sp = Spleen, LN = inguinal lymph nodes, CNS = Cerebral cortex, cerebellum and spinal cord
Figure 3.1  EAE in C57BL/6 mice induced with myelin antigens in combination with *Mycobacterium Tuberculosis* emulsified in Freund's adjuvant. EAE was induced in C57BL/6 mice by s.c. injection of 100 μg of MOG₃₅₋₅₅ or PBS emulsified in CFA, supplemented with 4 mg/ml H37 Ra *M. tuberculosis*, or PBS alone. Mice were injected i.p. with 500 ng pertussis toxin (PT) on d 0 and 2. Animals were monitored daily for weight and signs of clinical disease. Disease severity was graded as follows: grade 0 – normal; grade 1 – flaccid tail; grade 2 – wobbly gait; grade 3 – hind limb weakness; grade 4 – hind limb paralysis; grade 5 – tetraparalysis/death. Results are mean score +/- SEM (n = 5/control group and 16 in MOG and CFA group).
Figure 3.2 IL-17A mRNA expression in spleen, inguinal lymph node (ILN) and CNS tissue during the development of EAE. EAE was induced in C57BL/6 mice and at 12 h, 7, 14 and 21 d mice were sacrificed and tissue isolated to RNALater. Total RNA was purified, reverse transcribed into cDNA, and amplified by real-time PCR. The data represent the fold change for IL-17A mRNA expression relative to PBS controls, following normalization to the endogenous control, 18S rRNA. Data are mean and standard errors (n = 4-5/time-point). * P<0.05 vs. controls.
Figure 3.3 MOG-specific IL-17 production by spleen and lymph node cells during the course of EAE. EAE was induced in C57BL/6 mice and at 12 h, 7, 14 and 21 d mice were sacrificed and single cell suspensions prepared from spleens (A) and inguinal lymph nodes (B). Cells were restimulated with medium only, MOG_{35,55} (1, 5 or 25 μg/ml), or PMA and αCD3 as a positive control. After 72 h, supernatants were collected and IL-17 concentrations quantified by ELISA. (n = 4-5/time-point) ** P<0.01; *** P<0.001 vs. controls.
Figure 3.4  RORγt mRNA expression in spleen, inguinal lymph node (ILN) and CNS tissue during the development of EAE. EAE was induced in C57BL/6 mice and at 12 h, 7, 14 and 21 d mice were sacrificed and tissue isolated to RINAlater. Total RNA was purified, reverse transcribed into cDNA, and amplified by real-time PCR. The data represent the fold change for RORγt mRNA expression relative to PBS controls, following normalization to the endogenous control, 18S rRNA. Data are mean and standard errors (n = 4-5/time-point).  * P<0.05; ** P<0.01; *** P<0.001 vs. controls.
Figure 3.5  IFN-γ mRNA expression in spleen, inguinal lymph node (ILN) and CNS tissue during the development of EAE.  EAE was induced in C57BL/6 mice and at 12 h, 7, 14 and 21 d mice were sacrificed and tissue isolated to RINAlater.  Total RNA was purified, reverse transcribed into cDNA, and amplified by real-time PCR.  The data represent the fold change for IFN-γ mRNA expression relative to PBS controls, following normalization to the endogenous control, 18S rRNA.  Data are mean and standard errors (n = 4-5/time-point).  * P<0.05;  ** P<0.01 vs. controls.
Figure 3.6  MOG-specific IFN-γ production by spleen and lymph node cells during the development of EAE. EAE was induced in C57BL/6 mice and at 12 h, 7, 14 and 21 d mice were sacrificed and single cell suspensions prepared from spleens (A) and inguinal lymph nodes (B). Cells were restimulated with medium only, MOG35-55 (1, 5 or 25 µg/ml), or PMA and αCD3 as a positive control. After 72 h, supernatants were collected and IFN-γ concentrations quantified by ELISA. (n = 4-5/time-point) * P<0.05; ** P<0.01 vs. controls.
Figure 3.7  TNF-α mRNA expression in spleen, inguinal lymph node (ILN) and CNS tissue during the development of EAE. EAE was induced in C57BL/6 mice and at 12 h, 7, 14 and 21 d mice were sacrificed and tissue isolated to RNA later. Total RNA was purified, reverse transcribed into cDNA and amplified by real-time PCR. The data represent the fold change for TNF-α mRNA expression relative to PBS controls, following normalization to the endogenous control, 18S rRNA. Data are mean and standard errors (n = 4-5/time-point). * P<0.05; ** P<0.01; *** P<0.001 vs. controls.
Figure 3.8  IL-1β mRNA expression in spleen, inguinal lymph node (ILN) and CNS tissue during the development of EAE. EAE was induced in C57BL/6 mice and at 12 h, 7, 14 and 21 d mice were sacrificed and tissue isolated to RNA later. Total RNA was purified, reverse transcribed into cDNA, and amplified by real-time PCR. The data represent the fold change for IL-1β mRNA expression relative to PBS controls, following normalization to the endogenous control, 18S rRNA. Data are mean and standard errors (n= 4-5/time-point). * P<0.05; ** P<0.01; *** P<0.001 vs. controls.
Figure 3.9 IL-23p19 mRNA expression in spleen, inguinal lymph node (ILN) and CNS tissue during the development of EAE. EAE was induced in C57BL/6 mice and at 12 h, 7, 14 and 21 d mice were sacrificed and tissue isolated to RNA later. Total RNA was purified, reverse transcribed into cDNA, and amplified by real-time PCR. The data represent the fold change for IL-23p19 mRNA expression relative to PBS controls, following normalization to the endogenous control, 18S rRNA. Data are mean and standard errors (n = 4-5/time-point). * P<0.05; ** P<0.01 vs. controls.
Figure 3.10  IL-6 mRNA expression in spleen, inguinal lymph node (ILN) and CNS tissue during the development of EAE. EAE was induced in C57BL/6 mice and at 12 h, 7, 14 and 21 d mice were sacrificed and tissue isolated to RNA later. Total RNA was purified, reverse transcribed into cDNA, and amplified by real-time PCR. The data represent the fold change for IL-6 mRNA expression relative to PBS controls, following normalization to the endogenous control, 18S rRNA. Data are mean and standard errors (n = 4-5/time-point). * P<0.05; ** P<0.01; *** P<0.001 vs. controls.
Figure 3.11  TGF-β mRNA expression in spleen, inguinal lymph node (ILN) and CNS tissue during the development of EAE. EAE was induced in C57BL/6 mice and at 12 h, 7, 14 and 21 d mice were sacrificed and tissue isolated to RNALater. Total RNA was purified, reverse transcribed into cDNA, and amplified by real-time PCR. The data represent the fold change for TGF-β mRNA expression relative to PBS controls, following normalization to the endogenous control, 18S rRNA. Data are mean and standard errors (n = 4-5/time-point). * P<0.05; ** P<0.01 vs. controls.
Figure 3.12 IL-10 mRNA expression in spleen, inguinal lymph node (ILN) and CNS tissue during the development of EAE. EAE was induced in C57BL/6 mice and at 12 h, 7, 14 and 21 d mice were sacrificed and tissue isolated to RNALater. Total RNA was purified, reverse transcribed into cDNA, and amplified by real-time PCR. The data represent the fold change for IL-10 mRNA expression relative to PBS controls, following normalization to the endogenous control, 18S rRNA. Data are mean and standard errors (n = 4-5/time-point). * P<0.05; ** P<0.01 vs. controls.
IL-17-producing T cells that infiltrate the CNS during the development of EAE

Chapter 4
4.1 Introduction

It is clear that both resident cells of the CNS and infiltrating immune cells play vital roles in mediating neuroinflammation in EAE. However, blockade of systemic immune cell infiltration into the CNS, by i.v. administration of antibodies to MAC-1, a β2-integrin involved in transmigration of monocytes across the blood-brain barrier, significantly suppressed the severity of EAE (Huitinga et al., 1993). This study highlighted the requirement for infiltration of peripheral immune cells in the development of disease in the EAE model. Furthermore, Hickey and Kimura (1988) showed that peripheral APC, and not those endogenous to the CNS, were both necessary and sufficient to bring about CNS neuroinflammation. By limiting MHC class II expression to the invading peripheral immune cells, they showed that there was no requirement for MHC class II expression on parenchymal microglial cells for the development of EAE (Hickey & Kimura, 1988). The pathological role of T lymphocytes has long been acknowledged in EAE, following initial studies on the adoptive transfer of T cells specific for CNS autoantigens (Ben-Nun et al., 1981). Furthermore, the anti-α4β1 integrin monoclonal antibody, natalizumab, efficiently blocks adhesion of T cells to vascular cell adhesion molecule-1 (VCAM-1) on inflamed endothelium, preventing the accumulation of leukocytes in the CNS and the development of EAE (Yednock et al., 1992). Taken together, these studies demonstrate an absolute requirement for infiltration of immune cells from the periphery into the CNS for the initiation of neuroinflammation in EAE. As well as myeloid DC, monocytes and activated macrophages and T cells readily cross the blood-brain barrier and are found in large numbers, particularly in perivascular regions, in the brains of diseased mice.

IL-17 has recently emerged as a critical cytokine in T cell-mediated autoimmune diseases. Produced by a T cell subset distinct from Th1 and Th2
cells, IL-17 induces IL-1, TNF-α and IL-6 expression, as well as upregulation of a number of chemokines and matrix metalloproteinases (MMPs) in inflamed tissues. In adoptive transfer studies, IL-17-producing T cells induced EAE, but IFN-γ-producing T cells did not; although both types of cells could cross the blood-brain barrier and infiltrate the CNS (Langrish et al., 2005). In addition to EAE, it has been demonstrated that Th17 cells are the critical cells involved in the pathogenesis of a number of other models of autoimmune disease. In collagen-induced arthritis (CIA; Nakae et al., 2003), allergic airway hypersensitivity (Hellings et al., 2003), experimental myocarditis (Rangachari, 2006) and inflammatory bowel disease (Ogawa et al., 2003), clinical signs are reduced following depletion or knockout of IL-17. Steinman and colleagues have also shown increased transcripts for IL-17 and IL-6 in lesions from patients with MS (Lock et al., 2002). Moreover, IL-17-secreting T cells have been detected in the CSF of MS patients (Matusevicius et al., 1999).

While recent findings have challenged the dogma that EAE is mediated solely by Th1 cells, there is no doubt that Th1 cells are indeed present in the CNS parenchyma of mice displaying clinical signs of EAE. There is strong debate at present regarding the role of Th1 cells in the development of EAE. Recent thinking has focused on a possible protective function for IFN-γ-secreting Th1 cells, following the demonstration that IFN-γ can inhibit the development of Th17 cells (Harrington et al., 2005). Co-culturing of CD4+ T cells with IFN-γ-deficient APC enhanced production of IL-17. In contrast, addition of exogenous IFN-γ substantially inhibited the development of IL-17-producing effector T cells from IFN-γ-deficient precursor cells (Harrington et al., 2005). Weaver and colleagues also provide evidence that IFN-γ might mediate this effect by down-regulating expression of the IL-23R (Harrington et al., 2005). Despite these findings, Th1 cells may yet play a critical role in disease initiation. EAE can still be passively induced in IL-17-deficient mice, although disease progression is
severely attenuated (Komiyama et al., 2006). Furthermore, Segal and colleagues have recently demonstrated that either IL-23 or IL-12 driven myelin-reactive T cells can mediate CNS autoimmunity using disparate immunological pathways, but resulting in a similar clinical outcome (Kroenke et al., 2008). Consequently, CD4\(^+\) Th1 cells may contribute to inflammation in CNS inflammatory disease, as well as having a regulatory role by suppressing differentiation of Th17 cells.

\(\gamma\delta\) T cells are pathogenic in CIA and colitis models in mice (Roark et al., 2007; Nanno et al., 2008), and have been implicated in the pathogenesis of EAE. Depletion of \(\gamma\delta\) T cells immediately prior to disease onset or during the chronic phase significantly reduced clinical and pathological expression of EAE (Rajan et al., 1996). Other studies have shown reduced severity of EAE in mice with a defective \(\delta\) chain of the \(\gamma\delta\) TCR (Tcrd\(^{-}\); Spahn et al., 1999). This small subset of T cells was also found to kill oligodendrocytes in vitro, the cells responsible for axonal myelination in the CNS (Freedman et al., 1991). The pathogenic role of \(\gamma\delta\) T cells in autoimmunity was initially thought to be mediated through IFN-\(\gamma\) production. Recent studies, however, have shown that \(\gamma\delta\) T cells can secrete IL-17 during infection with \(M.\) *tuberculosis* or *E. coli*, where it promotes recruitment of neutrophils (Lockhart et al., 2006; Shibata et al., 2007). However, the role of \(\gamma\delta\) T cells in IL-17-production in MS or EAE has not been addressed. The precise contribution of \(\gamma\delta\) T cells in EAE remains uncertain, although it is thought that they function as a source of inflammatory cytokines and chemokines that bias the cytokine milieu towards the formation of a proinflammatory Th1- or Th17-like environment required for the development of EAE (Gao et al., 2001).

Although EAE is thought to be primarily a CD4\(^+\) T cell-mediated disease, and these cells are considered to be the major source of IL-17, it is interesting to
note that they are not the dominant cell type in MS lesions. In fact, CD8\(^+\) T cells outnumber CD4\(^+\) T cells at all stages of lesion development (Booss et al., 1983). A more recent study by Fugger and colleagues has reported enrichment of CD4\(^+\) and CD8\(^+\) T cells in active lesions from MS patients, and, more importantly, that both cell types actively produce IL-17 (Tzartos et al., 2008).

Unlike activated T cells, B cells do not appear to cross the blood-brain barrier. However, disruption of the blood-brain barrier, which occurs in MS and EAE, may well allow the entry of B cells that could potentially differentiate into antibody-producing plasma cells upon activation in the CNS (Keegan & Noseworthy, 2002).

Several other cell types have been identified in the inflammatory infiltrate of a number of different models of EAE. Some studies have identified infiltrating neutrophils that are both attracted by and produce IL-17 (Brown et al., 1982; Carlson et al., 2008). Astrocytes, and later neutrophils, were shown to be a source of CCL2 (macrophage chemoattractant protein (MCP)-1), CCL3 (macrophage inflammatory protein (MIP)-1\(\alpha\)) and CXCL2 (MIP-2; GRO2), chemokines involved in the trafficking of macrophages and neutrophils into the CNS (Nygårdas et al., 2000). In support of these findings, neutrophils have been detected in the CNS during the preclinical phase of EAE (Carlson et al., 2008). Furthermore, administration of a neutrophil-depleting antibody to mice with EAE prevented the establishment of lymphoid / myeloid infiltrates and the development of neurological deficits, as well as reducing blood-brain barrier breakdown (Carlson et al., 2008).
Natural killer (NK) cells have also been found in the CNS of mice with EAE. NKT cells express the IL-23R and secrete IL-17 after stimulation with αCD3 and IL-23 (Rachitskaya et al., 2008). However, numerous studies have indicated that NK cells have a regulatory rather than a pathogenic role, possibly through killing of myelin antigen-specific encephalitogenic T cells (Xu et al., 2005). Alternatively, NK cells may have an antagonistic effect on Th17 cell differentiation through their production of IFN-γ.

It is clear, therefore, that not only is inflammatory cell infiltration from the periphery required for initiation of neuroinflammation in EAE, but also, many different cell types may be present in the inflammatory infiltrate, each making key contributions at different stages of disease development. Moreover, the cellular infiltrate may differ between MS and many EAE models.

4.1.1 Aims and rationale for study

Given the early elevated expression of IL-17 mRNA and that of its associated cytokines in the lymphoid organs, preceding its upregulation in the CNS, it is probable that IL-17-producing T cells are activated early on in the periphery following induction of EAE, and subsequently migrate to the CNS, where they stimulate local production of pro-inflammatory cytokines and chemokines by resident glial cells. In an effort to identify when and where IL-17-producing T cells are activated and to learn how different cell types infiltrating the CNS might interact during the development of disease, a number of lymphocyte populations from the spleen, inguinal lymph nodes, brain and spinal cord were examined for production of IL-17 and IFN-γ at multiple time-points following the induction of EAE.
Chapter 4

4.2 Results

4.2.1 Clinical course of EAE

EAE was induced in C57BL/6 by s.c. injection of 100 μg of MOG$_{35-55}$ emulsified in CFA, containing 4 mg/ml H37Ra *M. tuberculosis*. Control mice were injected with PBS. Both groups were injected i.p. with 500 ng pertussis toxin (PT) on days 0 and 2. Animals were monitored daily for signs of clinical disease (Fig. 4.1). 97% of mice had developed EAE by day 13 post immunisation, clinical signs beginning with loss of tail tone (grade 1), and progressing through varying degrees of hind limb weakness, leading ultimately to hind limb paralysis (grade 4). None of the control mice developed any clinical signs. Mice were sacrificed 7, 10, 14, 21 and 35 days post-immunization and the spleen, inguinal lymph nodes, whole brain and spinal cords removed for analysis of IL-17 and IFN-γ production by a range of different cell types.

4.2.2 Increased frequency of leukocytes and T cells in the brain and spinal cord of mice with EAE

The frequency of CD45$^+$ leukocytes was markedly higher in the brain and spinal cord of mice with EAE compared with control mice (Fig. 4.2 A & B). This increase was partially due to the elevated frequency of CD3$^+$ T cells found in the CNS tissue of diseased animals (Fig. 4.2 A & B). The elevated frequency of lymphocytes in the brain was reflected by an immediate increase in the total number of CD3$^+$ T cells from around $1 \times 10^4$ in control mice to nearly $5 \times 10^4$ by day 7 of EAE ($p < 0.05$), increasing further by day 10, and to over $2 \times 10^5$ by day 14 ($p < 0.05$; Fig. 4.2 E). A similar pattern of CD3$^+$ T cell infiltration was also observed in the spinal cord, albeit with fewer absolute numbers of T cells infiltrating the tissue (Fig. 4.2 F). While there was large variability in the absolute number of CD3$^+$ T cells in the spleen of mice with EAE, there was little change in
the average total count at all time points compared to control mice (Fig. 4.2 C). There was a small decrease in the total number of CD3⁺ T cells in the spleen 14 days after induction of EAE, but this was not significant. In the ILN, there was a small but non-significant increase in the total number of CD3⁺ T cells on days 14 and 21 (Fig. 4.2 D).

4.2.3 Increased frequency of IL-17-expressing CD3⁺ T cells both in the periphery and the CNS of mice with EAE

There was a significant increase (p < 0.01) in the frequency of IL-17-expressing CD3⁺ T cells in the spleen 7 days after the induction of EAE, compared with control mice (Fig. 4.3A). At all other time points examined, no difference was observed between controls and mice with EAE. There was an incremental increase in the frequency of IL-17-expressing CD3⁺ T cells in the ILN, peaking about three-fold higher than control mice on day 14 (p < 0.01; Fig. 4.3B) and returning to controls levels thereafter. In the brains of control mice, the frequency of IL-17⁺ CD3⁺ T cells is roughly similar to that seen in the periphery – around 0.5% of total CD3⁺ T cells. This rose to 2.5% by day 7 (p < 0.01), in the pre-clinical phase of EAE, and reached its peak of about 5.5% on day 10 (p < 0.001; Fig. 4.3C), around the onset of the first signs of disease. IL-17 production by CD3⁺ T cells gradually declined through the subsequent time points examined but continued to be significantly higher than controls on day 14 (p < 0.01) and 21 (p < 0.01). The average frequency of IL-17-expressing CD3⁺ T cells was slightly higher in the spinal cord than in other tissues of control mice. Despite this, there was a slight increase in the their frequency 10 days after induction of EAE, rising to a two-fold increase over that in control mice by day 14 (Fig. 4.3D), before returning to normal control levels, around 0.5%, by day 21.
4.2.4 Increased frequency of IFN-γ-expressing CD3⁺ T cells both in the periphery and the CNS of mice with EAE

There was a slight increase in the frequency of IFN-γ-expressing CD3⁺ T cells in the spleen of mice 14 days after the induction of EAE (Fig. 4.4A). This had been preceded by a slight decrease in frequency on day 10 and followed by another decrease on day 21. A similar increase was also observed in the ILN on day 14 (Fig. 4.4B), with near to control levels of IFN-γ expression at all other time points. In the brain, there was a linear increase in the frequency of IFN-γ⁺ CD3⁺ T cells, beginning by day 7 and continuing with significant increases at all time points examined up until day 35 (p < 0.001; Fig. 4.4C). A similar pattern of increasing CD3⁺ IFN-γ expression was seen in the spinal cord (Fig. 4.4D), but with a generally lower frequency of IFN-γ-producing CD3⁺ T cells at all time points, in comparison to the brain. In addition, there appeared to be a slight reduction in the frequency of IFN-γ⁺ CD3⁺ T cells on day 21, relative to days 14 and 35.

4.2.5 Increased frequency of IL-17-expressing CD4⁺ T cells both in the periphery and the CNS of mice with EAE

The frequency of IL-17 producing CD4⁺ T cells in the spleen of mice with EAE increased dramatically in the 7 days following immunization (Fig. 4.5A). At day 7, around 12% (p < 0.01) of all CD4⁺ T cells were producing IL-17 compared with less than 2% in control mice. A small increase in expression of IL-17 by CD4⁺ T cells was also observed in the ILN (Fig. 4.5B), which gradually returned to control levels over the course of disease. Similarly, in the spinal cord, there was a small increase in the frequency of IL-17-expressing T cells 7 days after induction of EAE (Fig. 4.5D), which returned to control levels thereafter. IL-17-expressing CD4⁺ T cells were significantly more frequent in the brains of mice with EAE compared with control mice by day 7 (p < 0.01), and up to 18% of
CD4\(^+\) T cells were producing IL-17 by day 10, at the onset of clinical signs (p < 0.001; Fig. 4.5C). This appeared to be the peak of IL-17 production by CD4\(^+\) T cells, dropping slightly by day 14 in the acute phase of disease, although the frequency of IL-17 expression by CD4\(^+\) T cells in the brain remained significantly higher than that in controls at all time points over the remainder of the course of disease.

4.2.6 Increased frequency of IFN-\(\gamma\)-expressing CD4\(^+\) T cells both in the periphery and the CNS of mice with EAE

The frequency of IFN-\(\gamma\) expressing CD4\(^+\) T cells increased in both the spleen (p < 0.01) and ILN from about 5% in control animals to just over 15% by day 14 of EAE (Fig. 4.6A & B). By day 21, the expression of IFN-\(\gamma\) dropped below the level observed in control mice, and, in the spleen, returned to control levels by day 35. A similar pattern was also observed in the CNS, with the frequency of IFN-\(\gamma\) expressing CD4\(^+\) T cells not increasing until 14 days after induction of EAE. In both the brain and spinal cord, this increase was highly significant, with around 70% (p < 0.001; Fig. 4.6C) of all CD4\(^+\) T cells in the brain and 50% (p < 0.001; Fig. 4.6D) in the spinal cord producing IFN-\(\gamma\) by day 14. The frequency of IFN-\(\gamma\) expressing T cells in the CNS decreased after this time point but remained significantly higher than controls in the spinal cord on days 21 and 35 (both p < 0.01).

4.2.7 Increased frequency of IL-17-expressing \(\gamma\delta^+\) T cells in the CNS of mice with EAE

In addition to CD4\(^+\) T cells, \(\gamma\delta\) T cells were also found to infiltrate the CNS of mice with EAE. Whilst these constitute only around 3% of the total CD3\(^+\) population, up to 80% of \(\gamma\delta\) T cells in the brain produced IL-17 at the onset of
clinical signs, around 10 days after induction of EAE (p < 0.001; Fig. 4.7C). The expression of IL-17 by γδ T cells in the brain decreased thereafter, but remained significantly higher than the levels observed in control mice (around 10%) at all time points examined. A slight increase in IL-17 production by γδ T cells from the spinal cord was observed 14 days after induction of EAE, but the main peak of IL-17 expression was not seen until day 21 (p < 0.01; Fig. 4.7D) and remained higher than control levels up to day 35. A significant increase in expression of IL-17 was also observed in γδ T cells isolated from the ILN from day 10 (p < 0.05; Fig. 4.7B) through to day 21 (p < 0.05), returning to control levels by day 35. However, no change in IL-17 production by γδ T cells from the spleen was observed at any point over the course of disease (Fig. 4.7A).

4.2.8 Increased frequency of IFN-γ-expressing γδ T cells in the CNS of mice with EAE

There was a significant increase in the frequency of IFN-γ⁺ γδ T cells in the brain 10 days after induction of EAE (p < 0.05; Fig. 4.8C). Production of IFN-γ by γδ T cells continued to increase, being expressed by 20% of all γδ T cells by day 21 (p < 0.05), and decreasing again by day 35. A similar, but not significant, trend was also observed in the spinal cord of mice with EAE (Fig. 4.8D); however, no change in IFN-γ expression, compared with controls, was observed on day 14. There was a significant increase in IFN-γ production by γδ T cells from the ILN 10 days after induction of EAE (p < 0.05; Fig. 4.8B), returning to control levels by day 21. Whilst not significant, a similar increase in IFN-γ expression was also observed in γδ T cells from the spleen on day 10 (Fig. 4.8A). At all other time points examined, the frequency of IFN-γ-expressing γδ T cells was similar to that seen in the spleens of control mice.
It is demonstrated that γδ T cells infiltrate the CNS during the development of EAE, and account for 2-3% of total CD3+ T cells in the CNS at the onset of clinical signs (Fig. 4.9A). The total number of IL-17+ γδ T cells in brain increased significantly (p <0.001) at the onset of clinical signs of EAE (d 10), and remained in the brain up to 21 days after induction of EAE (Fig. 4.9B). The number of IFN-γ-producing γδ T cells in the brains of mice with EAE was only slightly higher than that seen in control mice. As shown separately in figures 4.7C and 4.8C, a very high proportion of all γδ T cells in the brain produced IL-17 prior to and around the onset of clinical signs of EAE (Fig. 4.9C). In contrast, the frequency of γδ T cells expressing IFN-γ was significantly lower, and there was only a small increase in percentage of γδ+ IFN-γ+ T cells over the course of EAE.

### 4.2.9 IL-17-expressing γδ T cells in the brains of mice with EAE are CD27 and these cells are significantly more frequent in the CNS at the onset of EAE

It was recently reported that the cytokine profiles of peripheral γδ T cells are predetermined during thymic development and are stable, even during infection. Most γδ T cells in the spleen and lymph nodes were shown to express the TNF receptor family member CD27 and secrete IFN-γ, whereas IL-17 production was restricted to CD27+ γδ T cells (Ribot et al., 2009). It was confirmed in the present study that cytokine production by γδ T cells that infiltrate the CNS during autoimmune inflammation in EAE also segregates with CD27 expression. γδ T cells were isolated from the brains of mice with EAE at the onset of clinical signs and analysed by flow cytometry. While practically none of the CD27+ γδ T cells in the brain expressed IL-17, up to 83% of CD27+ γδ T cells produced IL-17 following mitogenic stimulation (Fig. 4.10). Furthermore, it was found that CD27+ IL-17+ γδ T cells preferentially accumulate in the target organ during autoimmune inflammation. Approximately 30% of γδ T cells isolated
from the spleen of perfused mice and analysed by flow cytometry were CD27 IL-17+. The frequency of CD27 IL-17+ cells infiltrating the CNS, however, was significantly greater, representing around 70% of all γδ T cells in the brain at the onset of clinical signs of EAE (Fig. 4.10).

4.2.10 The majority of IL-17+ γδ T-cells in the brain at the onset of EAE are of the Vγ4 subset

This study has demonstrated that γδ T cells taken from the spleen, lymph nodes, brain and spinal cord can produce IL-17 after stimulation with PMA/ionomycin. By comparing γδ T cells expressing the variable chains Vγ4, Vγ5 or Vδ6.3/2 in the TCR, it was found that this potential predominantly occurs within the Vγ4+ T cell subset. Indeed, up to 70% of γδ T cells in the brain at the acute phase of EAE were Vγ4+ and more than 50% of these secreted IL-17 (Fig. 4.11). We also detected Vγ5+ cells and Vδ6.3/2+ cells, but the majority of these were not IL-17-secreting. In the spleen, the overall frequency of IL-17-secreting γδ T cells was lower than that found amongst γδ T cells infiltrating the brain (Fig. 4.11). Approximately 20% of γδ T cells in the spleen expressed IL-17, 10 days after induction of EAE; however, over 90% of these were Vγ4+. Again, Vγ5+ cells and Vδ6.3/2+ cells were detected, but these did not produce IL-17.

This study demonstrates that γδ T cells in the brains of mice at the onset of EAE comprise approximately 60% Vγ4+ T cells, with Vγ5+ cells and other variant subsets, presumably including Vγ1+ T cells, making up the remainder of the population (Fig. 4.12). By gating on IL-17+ γδ T cells, the contribution of the different subsets to the IL-17 pool was determined. It was confirmed that Vγ4+ γδ T cells are the main source of IL-17, contributing approximately 55% of the total
γδ-derived IL-17 in the acute phase of EAE. Vγ5^ T cells account for less than 15% of total γδ-derived IL-17, while other variant subsets contribute the remainder (Fig. 4.12). Vγ4^ T cells comprise over 60% of all γδ T cells in the spleens of mice at the onset of EAE (Fig. 4.13). Furthermore, this population accounts for over 90% of total γδ-derived IL-17 in the spleen at this stage. Vγ5^ T cells account for less than 10% of γδ T cells, and contribute less than 7% of the IL-17 produced by γδ T cells.

4.2.11 IL-1β and IL-23 stimulate IL-17, IL-21 and IL-22 production by γδ T cells without TCR engagement

Previous work from our laboratory has demonstrated that IL-1 and IL-23 production by innate immune cells promotes IL-17 production by T cells (Brereton et al., 2009). Furthermore, IL-1α or IL-1β synergises with IL-23 to promote IL-17 production by CD3^+ T cells from naïve mice, in the presence or absence of T cell receptor engagement (Sutton et al., 2006). Having shown that γδ T cells express IL-17 in vivo, the possibility that IL-1 and IL-23 induce IL-17 production by γδ T cells was investigated. The results showed that IL-1 and IL-23 synergized to induce IL-17A mRNA expression by γδ-depleted CD3^+ T cells, in the absence of anti-CD3 (Fig. 4.14). Stimulation of purified γδ T cells with IL-23 alone induced expression of IL-17A mRNA, which was enhanced by addition of IL-1β. Moreover, this was significantly (p < 0.001) higher than that induced by IL-1 and IL-23 in γδ T cell-depleted CD3^+ T cells.

Consistent with findings at the mRNA level, IL-1 and IL-23 induced IL-17 protein secretion by γδ-depleted CD3^+ T cells (Fig. 4.14). Purified γδ T cells produced IL-17 in response to IL-23, but also a small amount in response to IL-1.
signaling alone; while IL-1 augmented IL-23-induced IL-17 secretion by γδ T cells. IL-17 production by purified γδ T cells was significantly (p < 0.001) higher than that produced by γδ T cell-depleted CD3⁺ T cells.

In addition to IL-17A, Th17 cells produce IL-17F and IL-22. It was found in this study that stimulation of purified γδ T cells with IL-23 also induced expression of IL-17F and IL-22 mRNA, which was enhanced by addition of IL-1β (Fig. 4.15). γδ-depleted CD3⁺ T cells also upregulated IL-17F and IL-22 mRNA expression in response to synergized IL-1 and IL-23 signaling. However, IL-17F and IL-22 expression was significantly (p < 0.001) higher in cultures of purified γδ T cells than that induced by IL-1 and IL-23 in γδ T cell-depleted CD3⁺ T cells.

IL-21 plays a role in the development of murine CD4⁺ Th17 cells (Korn et al., 2007; Nurieva et al., 2007; Zhou et al., 2007). Naïve T cells do not express IL-23R on their surface, and it is thought that IL-21, induced by IL-6, upregulates IL-23R upon ligation of the TCR and thus promotes Th17 cell differentiation in the presence of IL-23. Interestingly, it was found in this study that IL-1β and IL-23, but neither cytokine alone, promoted substantial IL-21 expression in γδ T cells, but not in CD3⁺ T cells depleted of γδ T cells from naïve mice (Fig. 4.16). Furthermore, purified CD4⁺ T cells from mice with EAE, but not from naïve mice, also expressed IL-21 mRNA when stimulated with IL-1β and IL-23 together, in the absence of TCR engagement.
4.2.12 IL-23R and RORγt are constitutively expressed by γδ T cells

IL-23R is required for the terminal differentiation and therefore the function of CD4⁺ Th17 cells (McGeachy et al., 2009). It has previously been reported that IL-23R is expressed on NK cells and memory T cells (Parham et al., 2002). This study found that γδ T cells expressed high constitutive levels of IL-23R, which were enhanced by stimulation with IL-23 alone or in combination with IL-1β (Fig. 4.17). The transcription factor RORγt is also essential for the development of Th17 cells (Ivanov et al., 2006). Its expression has been demonstrated in NKT cells (Rachitskaya et al., 2008). Here, RORγt mRNA was found to be constitutively expressed in γδ T cells, and further enhanced by IL-23.

4.2.13 IL-1β and IL-23 stimulate IFN-γ and TNF-α expression by γδ T cells without TCR engagement

IFN-γ is the archetypal Th1-type cytokine, induced in response to IL-12 and IL-18 signaling. It was shown here that γδ T cells express IFN-γ during the course of EAE (Fig. 4.8). Interestingly, IFN-γ mRNA expression was significantly (p < 0.001) upregulated by stimulation with IL-23, and this is enhanced in the presence of IL-1β (Fig. 4.18). IFN-γ was also induced in CD3⁺ T cells depleted of γδ T cells, by IL-1β and IL-23 signaling. However, IFN-γ expression in γδ T cells stimulated with IL-1β and IL-23 was significantly higher than that induced in γδ T cell-depleted CD3⁺ T cells.

TNF-α is a pro-inflammatory cytokine produced by both Th1 and Th17 cells. Furthermore, IL-17 induces upregulation of TNF-α in inflamed tissues. Here, stimulation with IL-1β or IL-23 alone induced a small, but significant (p < 0.01) increase in TNF-α expression by γδ T cell-depleted CD3⁺ T cells (Fig. 4.18).
Chapter 4

this was further enhanced in the presence of IL-1β and IL-23 together. Purified γδ T cells constitutively expressed a slightly concentration of TNF-α mRNA, compared to γδ T cell-depleted CD3+ T cells. Again, IL-1β or IL-23 alone, but particularly IL-1β and IL-23 together stimulated significant (p < 0.01) upregulation of TNF-α mRNA. Furthermore, the concentration of TNF-α induced in γδ T cells by stimulation with each cytokine, or both together, was significantly (p < 0.05) higher than that induced in γδ T cell-depleted CD3+ T cells.

4.2.14 IL-1β and IL-23 induces substantial IL-17A and IL-17F gene expression in vivo

IL-1 and IL-23 can activate γδ T cells in vitro, upregulating an array of Th17-related molecules. The capacity for IL-1 and IL-23 to stimulate IL-17 production in vivo was also examined. Mice were injected s.c. in the footpad with IL-1β and IL-23 or PBS alone and draining popliteal lymph nodes isolated after 4 or 24 h. Rapid, significant upregulation of IL-17A and IL-17F gene expression (p < 0.01) was observed in the lymph nodes 4 hr after injection of IL-1 and IL-23 into the footpad, which had subsided 24 h later (Fig. 4.19).

4.2.15 IL-1β and IL-23 increases innate IL-17 production by CD3 T cells or γδ T cells in vivo

In addition to analysis of gene expression in lymph node tissue, cell suspensions were analysed by flow cytometry to determine which cells were responding to increased IL-1β and IL-23. There was a small increase in the frequency of CD3+ T cells expressing IL-17, 4 h after injection of IL-1 and IL-23 into the footpad. The percentage of CD3+ IL-17+ T cells in the popliteal lymph nodes returned to normal levels by 24 h. It has been demonstrated (Fig. 4.7;
Jensen et al., 2008) that IL-17-producing \(\gamma\delta\) T cells are found at relatively high frequency in the peripheral lymphoid organs of naïve mice. Consistent with these findings, approximately 25% of \(\gamma\delta\) T cells from PBS administered mice expressed IL-17. This was enhanced, however, 4 h after injection of IL-1 and IL-23 (Fig. 4.25). Although the increase was small, it was observed in all mice in the group, and had returned to levels seen in PBS controls 24 h later. These findings suggest that IL-1 and IL-23 promote rapid IL-17 gene expression and protein production \textit{in vivo} as well as \textit{in vitro} and that \(\gamma\delta\) T cells are a source of innate IL-17.
4.3 Discussion

The critical role of IL-17 in T cell mediated autoimmune diseases has recently become apparent and much effort has been invested in trying to determine what drives production of this cytokine by T cells under conditions of infection or autoimmunity. Most of this work has focused on IL-17-producing CD4^αβ T cells, so called Th17 cells. It has been shown that IL-1 and IL-23 promote the development or expansion of Th17 cells (Sutton et al., 2006), whilst IL-6 (Bettelli et al., 2006) or IL-21 (Korn et al., 2007) in conjunction with TGF-β can drive differentiation of naïve T cells into Th17 cells. Thus, Th17 cells are thought to be the primary source of IL-17 in models of autoimmunity and infection. A number of studies, however, have also demonstrated IL-17 production by CD8^+ T cells (He et al., 2007), γδ T cells (Lockhart et al., 2006) and NKT cells (Kennedy et al., 1996), as well as neutrophils (Ferretti et al., 2003) and microglia (Kawanokuchi et al., 2008).

This study examined the kinetics and organ distribution of IL-17 and IFN-γ production by a number of T cell populations during the course of EAE. A substantial increase in the frequency of CD45^+ leukocytes in the CNS of mice with EAE was observed. This appeared to be at least in part due to the large influx of CD3^+ T cells into the brain and spinal cord of mice during the development of disease. Consistent with previous reports (Wekerle et al., 1987), very few T cells were found in the CNS of naïve mice. There was a small influx of CD3^+ T cells into the brain and spinal cord in the pre-clinical phase of EAE, increasing with the first signs of disease; however, the acute phase of EAE correlated with a massive influx of lymphoid cells to the brain and spinal cord.
CD4⁺ T cells were found to infiltrate the brain and spinal cord prior to the onset of clinical signs of disease. Approximately 16% of these cells in the brain produce IL-17 at the onset of clinical signs, 10 days after induction of EAE. Given that virtually no IL-17 is expressed by CD4⁺ T cells in the CNS in the absence of disease, this is a significant finding. In a similar pattern to total CD3⁺ T cells, IL-17 production by CD4⁺ T (Th17) cells became less frequent as disease progressed, but remained significantly greater in the brains of mice with EAE compared with control mice. Conversely, IL-17 expression by CD3⁺ and CD4⁺ T cells was significantly elevated early in the spleen, during the preclinical phase of EAE. Th17 cells were also more frequent in the lymph nodes prior to disease onset, but continued to increase into the acute phase of disease. IFN-γ-producing CD4⁺ T (Th1) cells were more frequent than Th17 cells in both the brain and spinal cord in the acute phase of EAE. However, the frequency of these cells also decreased rapidly by day 21, returning to levels seen in control mice. At the same time, IFN-γ-producing CD3⁺ T cells continued to expand in the CNS, perhaps indicating that CD8⁺ T cells or NK cells become the main source of IFN-γ in the chronic phase of EAE.

In addition to CD4⁺ αβ T cells, γδ T cells were also found to infiltrate the CNS in the preclinical phase of EAE. At the onset of clinical signs, a very high proportion (up to 80%) of these cells expressed IL-17 in the brain. There was no change in the frequency of IL-17-producing γδ T cells in the spleen and only a slight increase in the ILN, in the acute phase of disease. This is consistent with the idea that γδ T cells are part of the innate immune system and only found in small numbers in lymphoid tissues, but are more abundant in peripheral tissues, where they are activated locally. In contrast, the frequency of γδ T cells producing IFN-γ was significantly lower, with only a small increase in the percentage and absolute numbers of IFN-γ⁺ γδ⁺ T cells in the brain during the course of EAE, and predominantly after the onset of clinical signs. In the spinal
cord, increased cytokine expression by $\gamma \delta$ T cells was not observed until the chronic phase of EAE.

$\gamma \delta$ T cells have been implicated in the pathogenesis of EAE. Deletion of these cells immediately prior to onset of clinical signs or during the chronic phase of disease significantly reduced clinical and pathological expression of EAE (Rajan et al., 1996). Disruption of the TCR $\delta$ chain gene led to reduced severity of disease in one study (Spahn et al., 1999), although not in another study (Clark and Lingenheld, 1998). It is thought that $\gamma \delta$ T cells participate in the development of disease by regulating leukocyte transfer across the blood-brain barrier and acting as a source of proinflammatory molecules. Rajan et al. (1998) showed a marked decrease in IFN-$\gamma$ expression at all stages of disease in $\gamma \delta$-depleted mice, as well as reduced expression of IL-1, IL-6 and TNF-$\alpha$ at disease onset, although expression of these cytokines returned to levels comparable with controls at the height of disease. This is in line with more recent data showing that IL-17 induces expression of IL-1, IL-6 and TNF-$\alpha$ in inflamed tissues (Langrish et al., 2005). $\gamma \delta$ T cells, therefore, may play a role in facilitating the activation and migration of myelin-reactive T cells into the CNS, either directly or indirectly, by providing appropriate proinflammatory cytokine and chemokine signaling. Such effects may not require large numbers of cells and could be facilitated by the relatively small numbers of $\gamma \delta$ T cells we and others have found in the brain and spinal cord of mice with EAE.

While much of the literature to date has focused on Th17 cells, it has become clear that $\gamma \delta$ T cells are also a major source of IL-17. Like $\alpha \beta$ Th17 cells, IL-17-producing $\gamma \delta$ T cells also require the transcription factor ROR$\gamma$T for their development (Ivanov et al., 2006). In bacterial infections, $\gamma \delta$ T cells can rapidly produce IL-17 (Lockhart et al., 2006; Shibata et al., 2007), and in some models
have been demonstrated to be the main source of IL-17 (Umemura et al., 2007). Interestingly, two groups have shown that γδ T cells from the spleens of naïve mice are capable of producing IL-17 (Stark et al., 2005; Romani et al., 2008). Moreover, naïve γδ T cells have been demonstrated to produce IL-17 in response to IL-23 alone, a response similar to that demonstrated by memory T cells (Lockhart et al., 2006; Shibata et al., 2007). Thus, it seems possible that certain, perhaps tissue-specific, subsets of γδ T cells are already differentiated in the periphery, and are capable of rapidly producing IL-17 in response to increased local availability of IL-23 (Roark et al., 2008).

Expression of the TNF receptor family member CD27 has recently emerged as a thymic determinant of the cytokine profile of peripheral γδ T cells. Silva-Santos and colleagues demonstrated that the phenotypes of IFN-γ- and IL-17-producing γδ T cell subsets are established during thymic development, when CD27 acts as a regulator of γδ T cell differentiation (Ribot et al., 2009). They showed that the majority of γδ T cells in the spleen and lymph nodes, as well as peripheral tissues including the lungs and gut, expressed CD27 and secreted IFN-γ. Between 10 and 30% of peripheral γδ T cells were CD27+ and comprised essentially all IL-17-producing γδ cells. Consistent with these findings, this study showed that IL-17+ γδ T cells from the spleen or that had accumulated in the brain by the onset of clinical signs of EAE were CD27+. Furthermore, while the frequency of IL-17+ CD27+ γδ T cells in the spleen was in line with that reported by Silva-Santos and colleagues, they were found at significantly higher frequency in the brain. Indeed the ratio of CD27+ to CD27+ γδ T cells in the brain marginally favored the CD27 non-expressing subset, and approximately 70% of these were IL-17+. This indicates that CD27+ γδ T cells preferentially accumulate in the CNS in the pre-clinical and acute phases of EAE and are highly activated.
IL-17 production by γδ T cells has been shown to play an important role in the induction and propagation of CIA, an autoimmune model similar to rheumatoid arthritis (Roark et al., 2007). Disease severity is markedly reduced in IL-17⁺ mice (Nakae et al., 2003), and mice depleted of the Vγ4⁺ subset of γδ T cells showed reduced incidence and milder clinical signs of disease (Roark et al., 2007). Interestingly, in the later study it appears that expansion of the IL-17-producing Vγ4 γδ T cell population was driven by self molecules that arose during inflammation, rather than the immunizing antigen. Murine γδ T cells are distinguished by their Vγ usage, with Vγ1 and Vγ4 the two main peripheral γδ T cell subsets in the mouse. Vγ4 are the main circulatory γδ T cells, found in the blood, spleen and lymph nodes (Romani et al., 2008). It is demonstrated here that up to 60% of γδ T cells in the brain at the acute phase of EAE were Vγ4⁺ and these were the source of more than half of all γδ T cell-derived IL-17. Vγ5 and Vδ 6.3/2 γδ T cells, normally found in the skin and intestinal epithelium and thought to have potential for autoimmune reactivity, were also detected, but the majority of these did not express IL-17. In the spleen, the overall frequency of IL-17-producing γδ T cells was lower, but over 90% of these were of the Vγ4 subset.

Previous work from our laboratory showed that CD3⁺ but not CD4⁺ or CD8⁺ T cells secrete IL-17 in response to IL-1β and IL-23, in the absence of stimulation with anti-CD3 or anti-CD28 (Sutton et al., 2009). It was found in the present study that stimulation of purified γδ T cells with IL-23 induced secretion of IL-17, which was enhanced by addition of IL-1β. This was significantly higher than that induced in γδ T cell-depleted CD3⁺ T cells, demonstrating that γδ T cells are responsible for the innate IL-17 production by CD3⁺ T cells. The effect of IL-1β and IL-23 on the expression of IL-17 by γδ T cells was confirmed by real time RT-PCR. Other Th17 cell-associated cytokines, including IL-17F, IL-22 and TNF-α, were also upregulated in purified γδ T cells in response to IL-23 signaling,
and this was enhanced by addition of IL-1β. Interestingly, IFN-γ mRNA too was elevated in γδ T cells stimulated with IL-23, and enhanced by IL-1β signaling.

It has recently been demonstrated that IL-21 plays a critical role in generating the Th17 response (Korn et al., 2007; Zhou et al., 2007). Here, IL-1β and IL-23 together, but neither cytokine alone, induced substantial IL-21 production by γδ T cells, but not by γδ T cell-depleted CD3+ T cells. Furthermore, purified CD4+ T cells from mice with EAE, but not from naïve mice, expressed IL-21 in response to synergized IL-1β and IL-23 signaling, consistent with an autocrine role of this cytokine in amplifying more IL-21 and IL-23R in naïve CD4+ T cells, and propagating the Th17 response (Zhou et al., 2007). Interestingly, γδ T cells from IL-21−/− mice have previously shown a 10-fold decrease in IL-17 expression, compared to wild type mice, indicating a role for IL-21 in the development of IL-17-producing γδ T cells as well as CD4+ T cells (Nurieva et al., 2007). IL-21 signaling additionally upregulates expression of RORγt, a key transcription factor in the differentiation of Th17 cells (Zhou et al., 2007; Ivanov et al., 2006). RORγt is expressed by NKT cells (Rachitskaya et al., 2008) and CD4+CD3− lymphoid tissue inducer (LTi)-like cells (Takatori et al., 2008). γδ T cells were found in this study to constitutively express RORγt, and this was enhanced in response to IL-23.

A major function of IL-23 in autoimmunity and infection is considered to involve promoting IL-17 production from memory CD4+ T cells (Higgins et al., 2006; van Beelen et al., 2007). However, studies in an EAE model, involving adoptive transfer of T cells into IL-23−/− mice, showed that IL-23 played a critical role in the development but not in the effector function of encephalitogenic T cells (Thakker et al., 2007). In an experimental autoimmune uveitis model,
neutralization of IL-23 reduced IL-17 production and attenuated autoimmununty when administered immediately before and after induction of disease, but not at the effector stage of the disease (Luger et al., 2008). These findings are consistent with the report that IL-23R is required for the terminal differentiation and therefore effector function of Th17 cells in vivo, rather than expansion or survival of pathogenic Th17 cells (McGeachy et al., 2009).

Naïve T cells do not express IL-23R on their surface, and it is thought that IL-6, possibly by inducing IL-21, upregulates IL-23R upon ligation of the TCR, thus promoting Th17 cell differentiation in the presence of IL-23 (Zhou et al., 2007). It has previously been reported that unconventional T cells, in particular NKT cells express the IL-23R and secrete IL-17 during infection or inflammation or when activated in vitro with anti-CD3 and IL-23 (Rachitskaya et al., 2008; Shibata et al., 2007). It is shown in this study that γδ T cells constitutively express IL-23R at high concentration, and this was enhanced by stimulation with IL-23 alone or in combination with IL-1β. Taken together with the demonstration by Klein and colleagues that γδ T cells also constitutively express IL-1R, and secrete autocrine IL-1 (McCandless et al., 2009), it appears that γδ T cells from naïve mice have all the machinery necessary to rapidly respond to innate IL-23 and IL-1 produced by neighboring cells, resulting in the rapid production and release of IL-17 and other inflammatory cytokines.

Finally, it was found that IL-17A and IL-17F gene expression is rapidly upregulated in the draining lymph nodes 4 h after injection of IL-1 and IL-23 into the footpad. Consistent with earlier data and reports elsewhere, intracellular staining revealed the frequency of IL-17-producing γδ T cells to be relatively high in the lymph nodes of naïve mice (Jensen et al., 2008). IL-17 expression in both CD3⁺ T cells and γδ T cells was enhanced following injection of IL-1β and IL-23.
These findings suggest that IL-1 and IL-23 promote rapid IL-17 gene expression and protein production in vitro and in vivo and that γδ T cells are a source of innate IL-17.

Thus, γδ T cells may behave like IL-17-producing memory αβ T cells. Yoshikai and colleagues demonstrated that resident Vδ1+ γδ T cells in the peritoneal cavity behaved like memory CD4 T cells (Shibata et al., 2007). They showed a rapid increase in IL-17 production following i.p. injection of E. coli, which coincided with upregulation of IL-23p19 mRNA expression. Furthermore, they confirmed that IL-23 production by another cell source was necessary for the activation of γδ T cells and their subsequent production of IL-17 (Shibata et al., 2007). Astrocytes are the most numerous cells in the CNS, comprising up to 80% of all cells. It has recently been shown that astrocytes can secrete IL-23 but not the Th1 driving cytokine, IL-12 (Miljkovic et al., 2007). Furthermore, in a transwell system, astrocytes were able to induce IL-17 secretion by cervical lymph node cells, and block IFN-γ production. This effect was reversed when cell-cell contact was permitted, and both IL-17 and IFN-γ were produced (Miljkovic et al., 2007). Thus, astrocytes appear to be able to restrain the IL-12/IFN-γ axis by their soluble products, an effect that is lost upon TCR engagement.

A number of studies have reported increased expression of the pro-inflammatory cytokines IFN-γ and TNF-α by γδ T cells in the CNS in the acute phase of EAE. Indeed, it was reported that γδ T cells express cytokines differentially in the CNS compared with αβ T cells, and have the potential to modulate the local inflammatory response during EAE (Smith and Barnum, 2008). γδ T cells have been shown to produce IL-17 in the periphery in a number
of models of infection and autoimmunity. The present study, however, is the first to describe IL-17 production by γδ T cells in the CNS during EAE.

O’Brien and colleagues have put forward a hypothesis that Th17-like γδ T cells differentiate early in the life of a mouse in response to environmental, inflammatory or stress-related stimuli and position themselves in both peripheral and CNS tissue, in order that they can mount a rapid response when triggered by IL-23 and/or other APC products, following activation by external pathogens (Roark et al., 2008). We have demonstrated that γδ T cells are present in the CNS during the development of MOG-induced EAE in C57BL/6 mice and, at the onset of clinical signs, a very high percentage of these cells express IL-17. It is tempting to speculate that IL-23 produced by activated astrocytes following induction of EAE or after the initial influx of antigen specific αβ T cells, may stimulate CNS-resident ‘memory’ γδ T cells to produce IL-17, which may then have the potential to regulate the local inflammatory response during the development of EAE.

γδ-derived IL-17 is involved in recruitment of neutrophils, and other leukocytes to the site of inflammation (Shibata et al., 2007; Carlson et al., 2008). It has been suggested that neutrophils may be among the first leukocytes to infiltrate the CNS in some models of EAE (Brown et al., 1982), and have been implicated in blood-brain barrier breakdown (Veldhuis et al., 2003). Interestingly, neutrophil depletion attenuated clinical and pathological signs of EAE (McColl et al., 1998; Carlson et al., 2008). Furthermore, IL-17 has been shown to induce ELRregulated CXC chemokines which are involved in neutrophil recruitment to the CNS (Kolls & Linden, 2004; Carlson et al., 2008). As such, Segal and colleagues have described a possible role for neutrophils in augmenting blood-brain barrier disruption during the period between reactivation of myelin-
specific T cells and the massive influx of non-specific leukocytes at the onset of clinical signs of EAE (Carlson et al., 2008).

Finally, studies by Antel and colleagues have demonstrated that γδ T cells are cytotoxic against brain-derived oligodendrocytes, the cells responsible for the ensheathment of neuronal axons with myelin, the target of pathogenic T cells in MS and EAE (Freedman et al., 1991; Freedman et al., 1997). It is possible that the activation of γδ T cells during the effector phase of disease may result, not only in the recruitment of αβ T cells, neutrophils and other leukocytes to the site of inflammation in the CNS, but also mediate irreversible damage of oligodendrocytes and ultimately lead to blocking of signal conduction and the development of neurological symptoms and signs of MS and EAE.
### Table 4.1 Summary

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<tr>
<th>Cells in CNS</th>
<th>Cytokine</th>
<th>EAE</th>
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<tr>
<td>CD45⁺ Leukocytes</td>
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<td>CD3⁺ T cells</td>
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<tr>
<td>Brain</td>
<td>+</td>
<td>IFN-γ⁺</td>
<td>↑</td>
</tr>
<tr>
<td>Spleen</td>
<td>-</td>
<td>IL-17⁺</td>
<td>←</td>
</tr>
<tr>
<td>Spleen</td>
<td>+</td>
<td>IFN-γ⁺</td>
<td>←</td>
</tr>
<tr>
<td>% of IL-17⁺γδ⁺ T cells</td>
<td>Vγ4⁺</td>
<td>Vγ5⁺</td>
<td>Other</td>
</tr>
<tr>
<td>Brain</td>
<td>54 %</td>
<td>15 %</td>
<td>31 %</td>
</tr>
<tr>
<td>Spleen</td>
<td>93 %</td>
<td>5 %</td>
<td>1 %</td>
</tr>
<tr>
<td>γδ⁺ T cells</td>
<td>IL-17</td>
<td>IL-17F</td>
<td>IL-22</td>
</tr>
<tr>
<td>Medium only</td>
<td>←</td>
<td>←</td>
<td>←</td>
</tr>
<tr>
<td>+ IL-1β</td>
<td>←</td>
<td>←</td>
<td>←</td>
</tr>
<tr>
<td>+ IL-23</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>+ IL-1β + IL-23</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑↑</td>
</tr>
<tr>
<td>+ IL-1β + IL-23 (in vivo)</td>
<td>↑</td>
<td>↑</td>
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</table>

* Constitutively expressed
Figure 4.1 Clinical course of EAE. EAE was induced in C57BL/6 mice by s.c. injection of 100 µg of MOG_{35-55} emulsified in CFA, supplemented with 4 mg/ml H37 Ra *M. tuberculosis*. The control group were injected with PBS. Both groups were injected i.p. with 500 ng pertussis toxin on d 0 and 2. Animals were monitored daily for signs of clinical disease. Disease severity was graded as follows: grade 0 – normal; grade 1 – flaccid tail; grade 2 – wobbly gait; grade 3 – hind limb weakness; grade 4 – hind limb paralysis; grade 5 – tetraparalysis/death. Results are mean score +/- SEM.
Figure 4.2  Infiltration of leukocytes into the brain and spinal cord of mice during the development of EAE. Mononuclear cells were isolated from the spleen, ILN, brain and spinal cord of naive mice and mice 7, 10, 14 and 21 d after induction of EAE. Cells were stained for surface CD45 and CD3 and analysed by FACS. Results are representative dot plots for brain (A) and spinal cord (B) from one control mouse and one mouse with EAE (d 14). The total number of cells in the spleen (C), ILN (D), brain (E) and spinal cord (F) are shown in the bar chart (n = 4/time point). * P<0.05 vs. controls.
Figure 4.3  Increased frequency of IL-17-expressing CD3^ T cells both in the periphery and the CNS of mice with EAE. Mononuclear cells were isolated from the spleen (A), ILN (B), brain (C) and spinal cord (D) of naive mice and mice 7, 10, 14, 21 and 35 d after induction of EAE. Cells were re-stimulated with PMA and ionomycin with brefeldin A for 14 h, stained for surface CD45 and CD3, and intracellular IL-17, and analysed by FACS. Results are representative dot plots for one control mouse and one mouse with EAE, with the mean frequency of cells in the CD3^ population in the bar chart (n = 4/time point). * P<0.05; ** P<0.01; *** P<0.001 vs. controls.
### Figure 4.4
Increased frequency of IFN-γ-expressing CD3+ T cells both in the periphery and the CNS of mice with EAE.

Mononuclear cells were isolated from the spleen (A), ILN (B), brain (C) and spinal cord (D) of naive mice and mice 7, 10, 14, 21 and 35 d after induction of EAE. Cells were re-stimulated with PMA and ionomycin with brefeldin A for 14 h, stained for surface CD45 and CD3, and intracellular IFN-γ, and analysed by FACS. Results are representative dot plots for one control mouse and one mouse with EAE, with the mean frequency of cells in the CD3+ population in the bar chart (n = 4/time point). * P<0.05; ** P<0.01; *** P<0.001 vs. controls.
A. Spleen

Control | d7
---|---
CD4 | 2.7% | 7.2%

B. ILN

Control | d7
---|---
CD4 | 0.3% | 2.6%

C. Brain

Control | d10
---|---
CD4 | 0.0% | 17.0%

D. Spinal cord

Control | d10
---|---
CD4 | 1.2% | 2.0%

Figure 4.5 Increased frequency of IL-17-expressing CD4+ T cells both in the periphery and the CNS of mice with EAE. Mononuclear cells were isolated from the spleen (A), ILN (B), brain (C) and spinal cord (D) of naïve control mice and mice 7, 10, 14, 21 and 35 d after induction of EAE. Cells were re-stimulated with PMA and ionomycin with brefeldin A for 14 h, stained for surface CD45, CD3 and CD4, and intracellular IL-17, and analysed by FACS. Results are representative dot plots for one control mouse and one mouse with EAE, with the mean frequency of cells in the CD4+ population in the bar chart (n = 4/time point). ** P<0.01; *** P<0.001 vs. controls.
Figure 4.6  Increased frequency of IFN-γ-expressing CD4+ T cells both in the periphery and the CNS of mice with EAE. Mononuclear cells were isolated from the spleen (A), ILN (B), brain (C) and spinal cord (D) of naïve control mice and mice 7, 10, 14, 21 and 35 d after induction of EAE. Cells were re-stimulated with PMA and ionomycin with brefeldin A for 14 h, stained for surface CD45, CD3 and CD4, and intracellular IFN-γ, and analysed by FACS. Results are representative dot plots for one control mouse and one mouse with EAE, with the mean frequency of cells in the CD4+ population in the bar chart (n = 4/time point). ** P<0.01; *** P<0.001 vs. controls.
Figure 4.7 Increased frequency of IL-17-expressing γδ T cells in the CNS of mice with EAE. Mononuclear cells were isolated from the spleen (A), ILN (B), brain (C) and spinal cord (D) of naïve control mice and mice 7, 10, 14, 21 and 35 d after induction of EAE. Cells were re-stimulated with PMA and ionomycin with brefeldin A for 14 h, stained for surface CD45, CD3 and γδ TCR, and intracellular IL-17, and analysed by FACS. Results are representative dot plots for one control mouse and one mouse with EAE, with the mean frequency of cells in the γδ⁺ population in the bar chart (n = 4/time point). * P<0.05; ** P<0.01; *** P<0.001 vs. controls.
Figure 4.8 Increased frequency of IFN-γ-expressing γδ T cells in the CNS of mice with EAE. Mononuclear cells were isolated from the spleen (A), ILN (B), brain (C) and spinal cord (D) of naïve control mice and mice 7, 10, 14, 21 and 35 d after induction of EAE. Cells were re-stimulated with PMA and ionomycin with brefeldin A for 14 h, stained for surface CD45, CD3 and γδ TCR, and intracellular IFN-γ, and analysed by FACS. Results are representative dot plots for one control mouse and one mouse with EAE, with the mean frequency of cells in the γδ+ population in the bar chart (n = 4/time point). * P<0.05; ** P<0.01; *** P<0.001 vs. controls.
Figure 4.9 Increased frequency and absolute number of IL-17-secreting γδ T cells in the brain at the onset of clinical signs of EAE. Mononuclear cells were isolated from the brain of naïve control mice and mice 7, 10, 14, 21 and 35 d after induction of EAE and re-stimulated with PMA and ionomycin with brefeldin A for 14 h. Cells were stained for surface CD45, CD3 and γδ TCR, and intracellular IL-17 and IFN-γ, and analysed by FACS. Results are representative dot plots for one control mouse and one mouse with EAE at the onset of clinical signs (d 10; A). Numbers on dot plots refer to percentage of gated CD45+ leukocytes. The absolute number of IL-17+ and IFN-γ+ γδ T cells (B) and a summary of the mean percentage of IL-17+ and IFN-γ+ cells (as previously shown in Fig. 4.7C and 4.8C) in the γδ population (C) are shown in the bar charts (n = 4/time point). * P<0.05; ** P<0.01; *** P<0.001 vs. controls.
Figure 4.10  A high frequency of IL-17^CD27^y\delta T cells are present in the CNS at the onset of EAE. Mononuclear cells were isolated from the brain and spleen of perfused naive control mice or mice 10 d after induction of EAE and re-stimulated with PMA and ionomycin with brefeldin A for 14 h. Cells were stained for surface CD3, y\delta TCR and CD27, and intracellular IL-17, and analysed by FACS. Representative dotplots show that IL-17-expressing y\delta T cells in the brains of mice with EAE are CD27^ (A), while the mean percentage +/- SEM of IL-17^ y\delta T cells among the CD27^ population in the spleen and brain is shown, with representative dot plots, in (B).
Figure 4.11 The majority of IL-17$^+$ γδ T-cells in the brain and spleen at the onset of EAE are of the Vγ4 subset. Mononuclear cells were isolated from the brain and spleen of perfused mice 10 d after induction of EAE and re-stimulated with PMA and ionomycin with brefeldin A for 14 h. Cells were stained for surface CD3, γδ TCR, Vγ4, Vγ5 and Vδ6.3/2, and intracellular IL-17, and analysed by FACS. Dot plots are representative of 4 mice. Numbers on dot plots refer to percentage of gated γδ T cells.
Figure 4.12  Vγ4 T cells account for the majority of γδ-derived IL-17 in the brain at the onset of EAE. Mononuclear cells were isolated from the brains of perfused mice 10 d after induction of EAE and re-stimulated with PMA and ionomycin with brefeldin A for 14 h. Cells were stained for surface CD3, γδ TCR, Vγ4, Vγ5 and Vδ6.3/2, and intracellular IL-17, and analysed by FACS. Dot plots are representative of 4 mice. Numbers on dot plots refer to percentage of IL-17⁺ γδ T cells.
Figure 4.13 Vγ4 T cells account for almost all of the γδ-derived IL-17 in the spleen at the onset of EAE. Spleen cells were isolated from perfused mice 10 d after induction of EAE and re-stimulated with PMA and ionomycin with brefeldin A for 14 h. Cells were stained for surface CD3, γδ TCR, Vγ4, Vγ5 and Vδ6.3/2, and intracellular IL-17, and analysed by FACS. Dot plots are representative of 4 mice. Numbers on dot plots refer to percentage of IL-17⁺ γδ T cells.
Figure 4.14  IL-1β and IL-23 stimulate IL-17A production by γδ T cells without TCR engagement. γδ-depleted CD3^+ T cells or γδ T cells from the spleen of naïve mice were stimulated with medium only, IL-1β, IL-23, or IL-1β and IL-23 together, in the absence of αCD3, for 48 h. Supernatants were harvested and IL-17A concentrations quantified by ELISA. Total RNA was purified from cells, reverse transcribed into cDNA, and IL-17A mRNA expression evaluated by real-time PCR, following normalization to the endogenous control, 18S rRNA. ** P<0.01; *** P<0.001 vs. medium only γδ-depleted CD3^+ T cells; +++ P<0.05 vs. similarly stimulated γδ-depleted CD3^+ T cells.
Figure 4.15  IL-1β and IL-23 stimulate IL-17F and IL-22 expression by γδ T cells without TCR engagement. γδ-depleted CD3⁺ T cells or γδ T cells from the spleen of naïve mice were stimulated with medium only, IL-1β, IL-23, or IL-1β and IL-23 together, in the absence of αCD3, for 48 h. Total RNA was purified, reverse transcribed into cDNA, and IL-17F and IL-22 mRNA expression evaluated by real-time PCR, following normalization to the endogenous control, 18S rRNA. *** P<0.001 vs. medium only γδ-depleted CD3⁺ T cells; +++ P<0.05 vs. similarly stimulated γδ-depleted CD3⁺ T cells.
Figure 4.16 IL-1β and IL-23 synergise to induce IL-21 expression by γδ T cells and memory CD4 T cells without TCR engagement. γδ-depleted CD3⁺ T cells, γδ T cells, or CD4 T cells from naïve mice and mice with EAE (d 7) were stimulated with medium only, IL-1β, IL-23, or IL-1β and IL-23 together, in the absence of TCR engagement, for 48 h. Total RNA was purified, reverse transcribed into cDNA, and IL-21 mRNA expression evaluated by real-time PCR, following normalization to the endogenous control, 18S rRNA. *** P<0.001 vs. medium only γδ-depleted CD3⁺ T cells. +++ P<0.001 vs. similarly stimulated γδ-depleted CD3⁺ T cells.
Figure 4.17  IL-23R and RORγt are constitutively expressed by γδ T cells. γδ-depleted CD3⁺ T cells or γδ T cells from the spleen of naïve mice were stimulated with medium only, IL-1β, IL-23, or IL-1β and IL-23 together, in the absence of TCR engagement, for 48 h. Total RNA was purified, reverse transcribed into cDNA, and IL-23R and RORγt mRNA expression evaluated by real-time PCR, following normalization to the endogenous control, 18S rRNA. * P<0.05; ** P<0.01; *** P<0.001 vs. medium only γδ-depleted CD3⁺ T cells. + P<0.05 vs. similarly stimulated γδ-depleted CD3⁺ T cells.
Figure 4.18 IL-1β and IL-23 stimulate IFN-γ and TNF-α expression by γδ T cells without TCR engagement. γδ-depleted CD3⁺ T cells or γδ T cells from the spleen of naïve mice were stimulated with medium only, IL-1β, IL-23, or IL-1β and IL-23 together, in the absence of TCR engagement, for 48 h. Total RNA was purified, reverse transcribed into cDNA, and IFN-γ and TNF-α mRNA expression evaluated by real-time PCR, following normalization to the endogenous control, 18S rRNA. ** P<0.01; *** P<0.001 vs. medium only γδ-depleted CD3⁺ T cells. + P<0.05; +++ P<0.001 vs. similarly stimulated γδ-depleted CD3⁺ T cells.
Figure 4.19  IL-1β and IL-23 administration in vivo results in significant but transient upregulation of IL-17A and IL-17F gene expression. C57BL/6 mice (n=5) were injected s.c. in the footpad with either PBS or with IL-1β and IL-23 (both 100 ng/mouse). Draining popliteal lymph nodes were isolated after 4 or 24 h and IL-17 mRNA expression evaluated by real-time PCR, following normalization to the endogenous control, 18S rRNA. Results are mean fold increase +/- SEM for 5 mice/timepoint, relative to PBS alone. ** P<0.01; *** P<0.001 vs. PBS alone.
Figure 4.20  IL-1β and IL-23 increases innate IL-17 production by CD3 T cells *in vivo*. C57BL/6 mice (n=5) were injected s.c. in the footpad with either PBS or with IL-1β and IL-23 (both 100 ng/mouse). Draining popliteal lymph nodes were isolated after 4 and 24 hr and re-stimulated with PMA and ionomycin with brefeldin A for 6 h. Cells were stained for surface CD3 and intracellular IL-17, and analysed by FACS. Results are mean percentage of total CD3 T cells +/- SEM for 5 mice, with representative dot plots. Numbers on dot plots refer to percentage of gated CD3 T cells.
Figure 4.21   IL-1β and IL-23 administration in vivo increases the frequency of IL-17 producing γδ T cells. C57BL/6 mice (n=5) were injected s.c. in the footpad with either PBS or with IL-1β and IL-23 (both 100 ng/mouse). Draining popliteal lymph nodes were isolated after 4 and 24 hr and re-stimulated with PMA and ionomycin with brefeldin A for 6 h. Cells were stained for surface CD3 and γδ TCR, and intracellular IL-17, and analysed by FACS. Results are mean percentage of total γδ T cells +/- SEM for 5 mice, with representative dot plots. Numbers on dot plots refer to percentage of gated γδ T cells.
The effect of caspase-1 inhibition on Th1 and Th17 cell activation and the development of EAE

Chapter 5
5.1 Introduction

MS, EAE and other T cell mediated autoimmune diseases are driven by autoantigen-specific T helper cells. The polarization, and ultimately the pathogenicity, of these cells is dictated by the cytokine environment created by cells of the innate immune system, in particular DC, during the initiation of an immune response. Upon capture and processing of antigen into peptide-MHC complexes, DC migrate to lymph nodes where they present antigen to naive T cells via TCR-MHC interaction. DC provide a second signal to the T cell through the interaction of adhesion and costimulatory molecules, such as CD80 and CD86, with CD28 on the surface of T cells, inducing activation and clonal expansion of the antigen-specific T cell. Finally, the innate immune system helps to direct the expansion of naive T cells into the different effector T cell subtypes, including CD8$^+$ killer lymphocytes, an array of CD4$^+$ T helper cells, including Th1, Th2 and Th17 cells, as well as the different subsets of Treg cells. This is achieved through the secretion of cytokines into the surrounding environment during antigen presentation that specifically drive differentiation of one subset while inhibiting differentiation of another. IL-1 is one of a number of inflammatory cytokines that links the innate and adaptive immune responses.

There are 11 members of the IL-1 family (IL-1F) of ligands; most are involved in local or systemic inflammation, but some are anti-inflammatory and protective against excessive inflammation. IL-1$\alpha$ (IL-1F1) and IL-1$\beta$ (IL-1F2), together with IL-1 receptor antagonist (IL-1Ra; IL-1F3) and IL-18 (IL-1F4), are the archetypal family members and have been extensively studied in humans and animal models of disease. IL-1Ra is a specific inhibitor of IL-1$\alpha$ and IL-1$\beta$ by competitively binding the type I IL-1 receptor (IL-1RI) with high affinity, while IL-1F5 and IL-1F7 appear to exert their anti-inflammatory effects non-specifically (Dinarello, 2009). IL-33 (IL-1F11), the latest addition to the IL-1 family, plays a
role in mast cell functions and drives allergic and Th2 responses (Schmitz et al., 2005).

Though IL-1α and IL-1β are only two of a family of eleven IL-1 proteins, the term IL-1 is widely used to describe these two members of the IL-1 family. IL-1α and IL-1β are encoded by two different genes and synthesized as 31 kDa proteins (pro-IL-1α and pro-IL-1β). On binding to their receptors, both IL-1α and IL-1β induce similar effects. Both ligands, as well as the antagonist IL-1Ra, bind IL-1RI and IL-1RII, of which only IL-1RI transduces a signal (Warabi, 2009). On binding of IL-1 to IL-1RI, the accessory protein IL-1RACP is recruited to form a high affinity heterodimeric receptor complex, initiating the IL-1 signaling cascade (Braddock and Quinn, 2004). The type II receptor (IL-1RII) acts as a decoy receptor, without transmitting the IL-1 signal (Warabi, 2009).

IL-1α is a cell-associated protein, believed to function in an autocrine manner. It is expressed by a variety of somatic cells, contributing to normal homeostasis. IL-1α is also constitutively expressed by neurons in the rat brain (Lemke et al., 1999). In severe illness IL-1α is found at high levels in the circulation, thought to be released by dead and dying cells and cleaved by extracellular calpain proteases (Wakabayashi et al., 1991). Consequently, IL-1α is also thought to be a damage-associated molecular pattern molecule (DAMP) or an alarmin.

IL-1β is primarily produced by cells of the monocytic lineage, including monocytes, macrophages and DC. It plays an important role in host protection against microbial infection, but can also promote tissue damage in chronic inflammatory diseases. Treatment with rhIL-1Ra (Kineret/Anakinra) has proved
beneficial in systemic-onset juvenile idiopathic arthritis, osteoarthritis, acute pancreatitis, and several autoinflammatory disorders (Dinarello, 2009). Activation of a number of signaling pathways can lead to IL-β gene expression, including those induced by IL-1β itself, IL-17 and TNF-α, as well as PAMPs such as LPS and DAMPs such as ATP. These PAMPs and DAMPs are recognized through membrane-bound TLR and NLR receptors (Braddock and Quinn, 2004). IL-1β is not constitutively expressed by monocytes, macrophages or DC, however stimulation via TLR and NLR results in IL-1β mRNA expression and its translation to pro-IL-1β protein (Eder, 2009). Pro-IL-1β is subsequently cleaved by active caspase-1, also known as IL-1β-converting enzyme, to generate mature 17 kDa IL-1β. Caspase-1 is a cysteine protease composed of two 10 kDa subunits and two 20 kDa subunits. Caspase-1 itself requires activation in a proteolytic cascade following assembly and activation of an inflammasome.

The inflammasome is a large multi-protein complex comprising the intracellular adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD (caspase-activating recruitment domain)), which links caspase-1 with an NLR acting as an intracellular sensor (Franchi et al., 2009a). There are three different types of inflammasome, involving different NLRs. The NLRP1 inflammasome plays a crucial role in the innate immune response to the anthrax lethal toxin from Bacillus anthracis (Hsu et al., 2008). The NLRP3 inflammasome is involved in caspase-1 activation in response to a variety of microbial stimuli, including LPS, muramyl dipeptide (MDP), bacterial RNA and double stranded viral RNA (Hise et al., 2009; Martinon et al., 2004; Kanneganti et al., 2006), as well as endogenous danger signals such as ATP and urate crystals (Mariathasan et al., 2006; Martinon et al., 2006). The NLRC4 or Ipaf inflammasome regulates caspase-1 activation in response to cell infection with intracellular bacteria, including Salmonella typhimurium, Legionella pneumophila and Pseudomonas aeruginosa (Franchi et al., 2006; Amer et al., 2006; Sutterwala
et al., 2007). The mechanism of activation of the inflammasomes is poorly understood, but K⁺ efflux is thought to be an important initiating event, at least for NLRP1 and NLRP3 containing complexes, with downstream involvement of the pore-forming pannexin-1 protein in NLRP3 inflammasomes (Pelegrin and Surprenant, 2006; Eder, 2009).

The majority of secretory proteins are externalized via the classical pathway - across the endoplasmic reticulum, through the Golgi apparatus to the plasma membrane and released either directly or via secretory granules. However the release of IL-1β, along with some other proteins that lack a conventional hydrophobic signaling sequence, is not well understood, but thought to involve a non-classical pathway, independent of the endoplasmic reticulum and the Golgi apparatus (Halban and Irminger, 1994). Following synthesis and processing to its biologically active form by caspase-1, IL-1β is postulated to be exported to the extracellular milieu either in secretory lysosomes, in plasma membrane microvesicles or upon cell lysis (Eder, 2009).

Like IL-1β, IL-18 is also dependent upon cleavage by caspase-1 for activation from its inactive precursor (24 kDa) to its mature, 18 kDa form. IL-18 functions with IL-12 and IL-15 to promote IFN-γ production, thereby contributing to the Th1 response. In the absence of IL-12 however, IL-18 can also induce IL-4, driving a Th2 response (Nakanishi et al., 2001). It has been demonstrated that production of both IFN-γ and the Th2 related cytokines IL-4, IL-5 and IL-13 was significantly increased in transgenic mice expressing high levels of IL-18 (Hoshino et al., 2001). IL-18 can also act synergistically with IL-23 to induce Th17 cytokine production independently of TCR stimulation (Weaver et al., 2005). However, IL-18 has been shown to have a less dominant role in Th17 cell activation when compared with its role in Th1/Th2 differentiation in vivo. van de
Loo and colleagues (2009) have demonstrated that blockade of IL-18 signaling with a soluble form of the IL-18R accessory protein (sIL-18RP) led to reduced IFN-γ and IL-4 production by splenic CD3+ T cells, and decreased circulating CD4+CD25+Foxp3+ Treg cells, but enhanced Th17 differentiation and increased IL-17 production in CIA (Veenbergen et al., 2009). These findings support those of Harrington, Park and others that cytokines produced by Th1 and Th2 cells are antagonistic to Th17 cells, as well as to each other (Harrington et al., 2005; Park et al., 2005). Finally, the role of IL-18 in the development of EAE is controversial. It has been reported that IL-18 deficient mice are resistant to disease, being unable to mount autoreactive Th1 responses (Shi et al., 2000). This resistance was suggested to result from diminished IFN-γ production by NK cells. Conversely, Becher and colleagues (2006) demonstrate that IL-18−/− mice are fully susceptible to MOG-induced EAE, with T cell responses and IFN-γ levels comparable to those in WT mice. However, deletion of IL-18Rα conferred resistance to development of EAE, and the authors argue that an alternative, as yet unidentified IL-18Rα ligand induces production of IL-23 and consequent expansion of Th17 cells (Gutcher et al., 2006).

IL-1 is an upstream mediator of the innate immune response. It has pleiotropic effects, including induction of expression of various growth and trophic factors, inflammatory mediators, adhesion molecules, chemokines and other cytokines, either directly or indirectly, as well as upregulating itself in an autocrine manner. Thus, it is an important molecule at the onset of many inflammatory, autoimmune or infectious responses. Furthermore, IL-1 is known to activate microglia, induce astrogliosis and recruit macrophages and PMN from the periphery (Shaftel et al., 2007). Therefore, elevated IL-1 signaling can amplify inflammation in the target organ by inducing production of further inflammatory cytokines, as well as other toxic molecules, including reactive
Chapter 5

oxygen species, peroxides, nitric oxide, and lipid mediators of inflammation, such as prostaglandins, leukotrienes and platelet-activating factor (Warabi, 2009).

Research from our laboratory has previously shown that IL-1 acts upstream of IL-17 signaling (Sutton et al., 2006). It was demonstrated that IL-1α or IL-1β synergizes with IL-23 to promote IL-17 production by murine T cells in the presence or absence of T cell receptor engagement (Sutton et al., 2006). Moreover, IL-1RI−/− mice were found to be resistant to EAE, and this was associated with deficient IL-17 production by T cells. Adoptive transfer of encephalitogenic T cells into IL-1RI−/− mice, however, could induce disease (Sutton et al., 2006). These findings were corroborated in a recent study that suggests IL-1 synergizes with IL-6 and IL-23 to induce expression of the transcription factors IRF4 and RORγt, thereby driving Th17 polarization (Chung et al., 2009). Iwakura and colleagues (2003) have also demonstrated that IL-1Ra deficient mice, that spontaneously develop autoimmune arthritis due to excessive IL-1 signaling, exhibited vastly elevated expression of IL-17. Furthermore, the spontaneous development of arthritis did not occur in IL-1Ra−/− mice also deficient in IL-17 (Nakae et al., 2003a). In addition, mutations in the Nlpr3 gene causing inflammasome hyperactivation and excess IL-1β production by APC, can result in augmented Th17 cell differentiation and consequent IL-17-dominant immunopathology (Meng et al., 2009). These studies suggest that IL-1 is required for the induction of Th17 cells, possibly via a cascade of IL-1-induced IL-6 and IL-23, and is therefore critical during the development of EAE, but not at its effector stage.
5.1.1 Aims and rationale for the study

Data in the previous chapters have shown that peripheral levels of IL-1β correlate with the differentiation of naïve precursor T cells to Th17 cells, and that expression of IL-1β in the CNS correlates with the clinical course of disease in EAE. Expression of IL-1β and IL-1RαcP are significantly elevated in sera and CSF of MS patients compared to controls (Dujmovic et al., 2009). Genetic studies have identified a relationship between IL-1 gene polymorphisms and disease severity in some cases of MS (Schrijver et al., 2003). Recently, it has been demonstrated that treatment with IFN-β results in a non-selective inhibitory effect on IL-1β expression in MS patients and mice with EAE (Ramgolam et al., 2009). Thus, IL-1 might be a suitable therapeutic target in EAE and MS. Moreover, because IL-1 is an upstream mediator of the innate immune response, both peripherally and in the CNS, therapeutic targeting of IL-1 in MS may be more effective than blocking any other single inflammatory mediator. There are a number of different routes available to target the IL-1 pathway. Broadly, these include inhibition of IL-1 synthesis or maturation, buffering of IL-1 with binding proteins or soluble receptors, antagonism of IL-1 receptors with recombinant IL-1Ra or other peptide antagonists, or inhibition of IL-1 signal transduction.

The IL-1Ra mimetic Anakinra (Kineret; Amgen) is approved for the treatment of rheumatoid arthritis (RA) and is in Phase II/III trials for other inflammatory diseases, including osteoarthritis (OA) and type 1 diabetes mellitus. Anti-IL-1β antibodies and soluble IL-1R II molecules are also in development for the treatment of a range of inflammatory diseases. In addition, a number of small-molecule inhibitors of IL-1β processing and release, including the caspase-1 inhibitor, VX-740 (Pralnacasan; Vertex Pharmaceuticals) and the second-generation compound, VX-765, have been investigated in Phase II clinical trials for RA, OA and psoriasis. Both Pralnacasan and VX-765 have shown efficacy in animal models of RA (CIA), OA (STR/1N spontaneous OA), psoriasis and
contact dermatitis (oxazalone-induced DTH dermatitis) (Braddock and Quinn, 2004).

In EAE, several studies have examined the effect of blocking IL-1 signaling. IL-1Ra given i.p. every day from induction of EAE to day 6, suppressed disease in DA rats immunized with rat spinal cord homogenate (Badovinac et al., 1998). This was associated with diminished encephalitogenic capacity of LN cells, which were shown to have lower proliferative responses to antigen and mitogen and decreased expression of IL-2 receptors. Subcutaneous administration of rhIL-1Ra every day in the effector stage, starting nine days post-immunization, delayed the onset and reduced the severity of clinical signs and weight loss in Lewis rats immunized with MBP (Martin and Near, 1995). Gene therapy, by delivery of an IL-1Ra producing non-replicative Herpes simplex virus-1 derived vector to mice at induction of EAE and again seven days later, delayed disease onset and significantly reduced severity (Furlan et al., 2007). This correlated with reduced macrophage infiltration in the perimeningeal space. Treatment with soluble IL-1R every day from one day before induction of EAE to day 11 post-induction also resulted in delayed onset and reduced severity of paralysis in Lewis rats immunized with guinea pig myelin (Jacobs et al., 1991).

It has previously been demonstrated that caspase-1−/− mice are less susceptible to MOG-induced EAE compared to wild type C57BL/6 mice, at low doses of the encephalitogenic peptide (Furlan et al., 1999). There was no impairment in the proliferation potential of LN cells in caspase-1-deficient mice; however, T cells were defective in Th1 development. Continuous administration of the caspase-1 inhibitor Z-VAD-fmk, at high concentrations, also suppressed EAE, in a preventative but not therapeutic protocol, in two different mouse models (Furlan et al., 1999). This correlated with decreased infiltration of CD3+
T cells and macrophages to the CNS, together with reduced demyelination and axonal loss.

Because most of these studies were carried out before IL-17 was appropriately recognized for its role in autoimmune disease, none have examined the effect of IL-1 pathway blockade on IL-17 production by T cells in EAE. The precursors of both IL-1β and IL-18 have been identified as substrates of caspase-1; thus, blocking caspase-1 might have a more pronounced effect on disease than inhibiting either IL-1β or IL-18, alone. This study examined the ability of Ac-YVAD-cmk, an irreversible inhibitor of caspase-1, to block the differentiation of IL-17-producing T cells and attenuate the clinical signs of EAE.
5.2.1 *M. tuberculosis* can activate the NLRP3 inflammasome

The NLRP3 inflammasome is involved in caspase-1 activation in response to a variety of microbial stimuli and endogenous danger signals (Martinon & Tschopp, 2006). NLRP3 associates with inactive caspase-1 through the adapter protein ASC. Recognition of an appropriate microbial or danger pattern by the NLR leads to oligomerization of ASC and activation of caspase-1. ASC-YFP^+ macrophages (a kind gift from Dr. Eicke Latz, University of Massachusetts, USA) were used to assess activation of the NLRP3 inflammasome *in vitro*. In resting cells, ASC-YFP is stably expressed at low enough levels to be polydispersed throughout the cytoplasm, as visualised using confocal microscopy. As a positive control, stimulation of ASC-YFP^+ macrophages with LPS for 4 h, in the presence of ATP for the final 1 h, resulted in aggregation of the ASC protein into large cytosolic aggregates with a complete loss of the cytoplasmic distribution of ASC-YFP, indicative of inflammasome formation in these cells (Fig. 5.1 A). Evidence of ASC contraction towards specific poles was visible after just 20 min stimulation with ATP.

EAE induction in mice typically requires CFA which includes killed *M. tuberculosis* (Mtb), a powerful stimulus for priming the innate immune response. In order to test the ability of Mtb to induce IL-1β production early in this process, it was important to show that Mtb can activate the inflammasome, leading to caspase-1 activation. Complete loss of cytoplasmic ASC and formation of a single complex was observed when macrophages were incubated with Mtb (50 μg/ml) for 1 h (Fig. 5.1 B). Mtb, therefore, is capable of activating the NLRP3 inflammasome in these cells.
5.2.2 *M. tuberculosis* induces pro-IL-1β expression by DC and processing to its mature form

Having shown that Mtb can activate the inflammasome in macrophages, it was important to demonstrate that this lead to efficient processing of pro-IL-1β to its mature form. ASC-YFP⁺ macrophages are useful to observe inflammasome activation *in situ*. However, DC are the principal APC *in vivo* and, therefore, all further experiments were performed using BMDC. BMDC were stimulated with Mtb (10 or 50 μg/ml) for 18 h, LPS for 3 h +/- ATP for 1 h, or medium only as controls. Supernatants were collected and IL-1β expression quantified by ELISA and Western blot. Unstimulated DC did not express pro-IL-1β (Fig. 5.2 B; lane 1). Stimulation with LPS induced production of pro-IL-1β, but without conversion to its mature form. Addition of ATP to the LPS-primed cells for 1 h lead to processing of pro-IL-1β and secretion of mature IL-1β (Fig. 5 A & B; lane 2). Incubation of BMDC with Mtb (at both the higher and lower concentrations) also led to upregulation of pro-IL-1β and cleavage to its active form, as detected by ELISA (Fig. 5 A) and western blot (Fig. 5 B). Thus, Mtb can induce transcription and translation of pro-IL-1β and processing to its active form.

5.2.3 *M. tuberculosis* or LPS and ATP induced IL-1β secretion by DC is caspase-1-dependent

Having established that Mtb can induce inflammasome formation and processing of pro-IL-1β into its active form, the role of caspase-1 was examined. BMDC were stimulated with Mtb (2, 10 or 50 μg/ml) or medium alone for 18 h, or with LPS for 3 h +/- ATP for 1 h, with or without caspase-1 inhibitor (8-40 μM), or an equivalent concentration of DMSO. Supernatants were collected and IL-1β concentrations quantified by ELISA. Mtb, at 10 and 50 μg/ml, induced IL-1β production by DC, as did LPS, which was augmented by the addition of ATP to the cell culture (Fig. 5.3 A). Pre-incubation of DC with YVAD-cmk for 1 h
before stimulation significantly (p < 0.01) reduced the concentration of IL-1β detected in the supernatants of treated cultures. DC pre-incubated with a concentration of DMSO equivalent to that present in the lower and higher concentrations of YVAD-cmk cultured DC as a control, had no effect on production of IL-1β at these concentrations (Fig. 5.3 B).

5.2.4 *M. tuberculosis* induced IL-18, but not IL-23, production by DC is caspase-1-dependent

Like IL-1β, IL-18 is also dependent upon cleavage by caspase-1 for activation from its inactive precursor (24 kDa) to its mature, 18 kDa form. BMDC were stimulated with Mtb (2, 10 or 50 µg/ml) or medium alone for 18 h, or with LPS for 3 h +/- ATP for 1 h, with or without caspase-1 inhibitor (8-40 µM). Mtb (10 and 50 µg/ml) induced IL-18 production by DC at comparative concentrations to that induced by LPS (Fig. 5.4 A). The addition of ATP to LPS-stimulated DC had no effect on the concentration of IL-18 processed. Pre-incubation of DC with the higher concentration of YVAD-cmk for 1 h prior to stimulation significantly reduced IL-18 secretion (p < 0.01), confirming a role for caspase-1 in the induction of IL-18 by DC.

IL-23 is an important inflammatory cytokine, with a critical role in the differentiation or expansion of Th17 cells. IL-23 expression was upregulated in response to Mtb (10 and 50 µg/ml) to greater levels than that observed with LPS. The addition of ATP to LPS stimulated cells appeared to block expression of IL-23 by DC. Pre-incubation with YVAD-cmk failed to block IL-23 production, although there was a small, but significant decrease in IL-23 concentration in the supernatants of cells stimulated with Mtb (50 µg/ml) that had been pre-incubated with YVAD-cmk at the higher concentration.
5.2.5 IL-1β or IL-18 can synergise with IL-23 to promote IL-17 production by T cells

IL-1 and IL-23 are known to drive IL-17 production by memory CD4⁺ T cells and γδ T cells (van Beelen et al., 2007; Sutton et al., 2009). IL-1R and IL-23R are not expressed on naïve CD4⁺ T cells but are upregulated in response to IL-1, IL-6, IL-21 and IL-23 signaling (Zhou et al., 2007; Korn et al., 2008; McCandless et al., 2009). These receptors are constitutively expressed in γδ T cells (Sutton et al., 2009; McCandless et al., 2009). CD3⁺ and CD4⁺ T cells were purified by negative selection using magnetic separation (purity was routinely >95%), from the spleens of naïve mice or mice with EAE, 10 days post immunization, and cultured with IL-1β, IL-18 and IL-23, or combinations thereof, without TCR stimulation. IL-1 and IL-23 synergized to stimulate IL-17 expression in purified CD3⁺ T cells from mice with EAE and naïve mice (Fig. 5.5). IL-23 alone induced a small amount of IL-17 from purified CD4⁺ T cells from mice with EAE, without TCR stimulation, and this was augmented by addition of IL-1 to the culture. Neither IL-1 and IL-23 or PMA and anti-CD3 could induce IL-17 production by CD4⁺ T cells from naïve mice. Interestingly, IL-18 was also able to synergize with IL-23 to induce IL-17 secretion by CD3⁺ and CD4⁺ T cells from mice with EAE, but also from CD3⁺ T cells from naïve mice.

5.2.6 IL-18 promotes IFN-γ production in antigen-experienced CD4 T cells from mice with EAE and synergizes with IL-23 to induce IFN-γ expression in CD3 T cells from naïve mice

IL-18 is an important cytokine driving the Th1 response to infection and autoimmunity. IL-18 functions with IL-12 and IL-15 in the induction of IFN-γ production from T cells and NK cells (Nakanishi et al., 2001). CD3⁺ and CD4⁺ T cells were purified by negative selection using magnetic separation (purity was
routinely >95%), from the spleens of naïve mice or mice with EAE, 10 days post immunization, and cultured with IL-1β, IL-18 and IL-23, or combinations thereof, without TCR stimulation. This study found that CD4\(^+\) T cells from mice with EAE produced high concentrations of IFN-\(\gamma\) in response to stimulation with IL-18 alone (Fig. 5.6). IL-1 or IL-23 together with IL-18 induced IFN-\(\gamma\) expression in CD4\(^+\) and CD3\(^+\) T cells from mice with EAE. IL-1 synergized with IL-23 to induce a small amount of IFN-\(\gamma\) from both purified CD4\(^+\) and CD3\(^+\) T cells. IL-1, IL-18 and IL-23 also synergized to induce IFN-\(\gamma\) production by CD3\(^+\) T cells from naïve mice, without TCR engagement.

5.2.7 \textit{M. tuberculosis}-activated DC promote IL-17 and IFN-\(\gamma\) production by purified CD3\(^+\) T cells

Because \(\text{Mt}b\) can stimulate DC to produce active IL-1β, IL-18 and IL-23, and these cytokines can induce IL-17 and IFN-\(\gamma\) production by T cells, the ability of \(\text{Mt}b\)-stimulated DC to drive IL-17 or IFN-\(\gamma\) expression in purified CD3\(^+\) T cells was examined. BMDC were cultured with \(\text{Mt}b\) (10 \& 50 \(\mu\)g/ml) or medium only for 24 h, washed and added to CD3\(^+\) T cells purified by negative selection using magnetic separation (purity was routinely >95%), at a ratio of 1:4, DC to T cells. CD3\(^+\) T cells from both naïve mice and mice with EAE (d10), cultured with DC that had been stimulated with \(\text{Mt}b\) at both concentrations, secreted large amounts of IL-17 and IFN-\(\gamma\) (Fig. 5.7). Cytokine production was inducible in T cells from naïve mice as well as mice with EAE, although antigen-experienced T cells produced more IL-17 than T cells from naïve mice.
5.2.8 Inhibition of caspase-1 reduces the ability of *M. tuberculosis*-activated DC to induce IL-17 production by T cells from naïve mice

IL-1 is critically required for the early differentiation of Th17 cells, and, together with IL-23, drives IL-17 production from γδ T cells (Chung *et al.*, 2009; Sutton *et al.*, 2009). If IL-17 production by T cells cultured with Mtb-stimulated DC is IL-1β-dependent, then inhibition of caspase-1 might block this effect. Surprisingly, CD3⁺ T cells from naïve mice cultured with unstimulated DC upregulated IL-17 expression, compared to T cells cultured in medium only. Pretreatment of DC for 1 h with YVAD-cmk failed to block this effect, indicating that IL-1β was not involved. Co-culture of T cells with DC that had been stimulated with Mtb (10 and 50 μg/ml) further upregulated IL-17 expression; this was IL-1β-dependent, as pre-treatment of DC with YVAD-cmk significantly (*p < 0.05*) inhibited IL-17 production in a dose-dependent manner.

5.2.9 Therapeutic administration of a caspase-1 inhibitor attenuates the development of acute MOG-induced EAE and this is associated with decreased Th17 responses

Having shown that inhibition of caspase-1 blocks IL-1β and IL-18 secretion by DC and restrains Th1 and Th17 responses, the possibility that inhibiting caspase-1 may be effective against EAE was examined. C57BL/6 mice were immunized with MOG in CFA, which we have found develop a chronic progressive form of disease (Fig. 4.1). YVAD-cmk (25 μg/mouse) or vehicle (DMSO) was administered s.c. at the site of immunization, every second day from day 5 to day 21 post-induction. Treatment with the caspase-1 inhibitor induced significant attenuation of clinical signs of EAE compared to DMSO-treated control mice (Fig. 5.9). The mean day of onset was comparable between the two groups, however clinical signs in the treatment group never progressed past a mild ataxic gait. Therefore, blocking caspase-1 therapeutically had a clear beneficial
effect on disease progression in EAE. 21 days after induction of EAE, animals were sacrificed and spleen and draining inguinal lymph node cells restimulated with increasing concentrations of MOG. Caspase-1 inhibition resulted in significantly reduced IL-17 production (p < 0.05) by antigen-specific T cells in the spleen \textit{ex vivo} (Fig. 5.10). Antigen-specific IL-17 was undetectable in lymph node cells from the treated group, but was detected in a concentration-dependent manner in lymph node cells from DMSO-treated control mice. Interestingly, IL-17 produced in response to PMA and anti-CD3 by lymph node cells from mice treated with the caspase-1 inhibitor was also reduced, perhaps indicating deficient proliferation of Th17 cells in the absence of active caspase-1.

5.2.10 Inhibition of caspase-1 from induction and throughout the course of disease almost ameliorates clinical signs of MOG-induced EAE

Having shown that inhibition of caspase-1 immediately prior to the appearance of signs reduced but did not ameliorate EAE, the effect of inhibition at the induction phase and throughout the course of EAE was examined. Consistent with the previous experiment, shown in Fig. 5.9, therapeutic administration of caspase-1 inhibitor prior to the onset of clinical signs prevented severe paralysis (Fig. 5.11 A). However, administration of YVAD-cmk at the induction of EAE (days 0, 1 and 2) and continuing every second day from day 5 to day 21, almost ameliorated clinical signs of disease in these mice (Fig. 5.11 A). Although mean day of onset was similar to control groups treated with DMSO at the same time points, most mice never developed more than paralysis of the tail. In addition to the significant amelioration of clinical signs (p < 0.01), mice in the treatment group showed greater recovery after the initial weight loss at the onset of clinical signs (Fig. 5.11 B). Administration of YVAD-cmk at the induction phase only, on days 0, 1 and 2, conferred little therapeutic benefit and did not reduce the severity of clinical signs of EAE, although these mice showed more rapid recovery after the acute phase of disease (Fig. 5.11 A).
5.2.11 Inhibition of caspase-1 at induction of EAE significantly reduced antigen-specific pro-inflammatory cytokine production in the lymph nodes when tested 5 days post induction

Pre-incubation of DC with a caspase-1 inhibitor prevented their ability to induce Th17 differentiation, following activation with Mtb in vitro. To test whether blocking caspase-1 activity in vivo could also reduce Th17 differentiation, YVAD-cmk was administered to mice at induction of EAE, on day 0, 1 and 2, and inguinal lymph node cells isolated 5 days post-immunization and cultured in the presence of increasing concentrations of MOG. Mice from the treatment group produced significantly less antigen-specific IL-17 and IFN-γ (both p < 0.05), compared to mice treated with DMSO at the same time points (Fig. 5.12). Cytokine production induced by PMA and anti-CD3 was comparable between groups, indicating no defect in proliferation or viability of cells from treated mice. Antigen-specific IL-10 was not detected from these cells at this stage of disease.

5.2.12 Caspase-1 inhibition significantly reduced antigen-specific pro-inflammatory cytokine production at the onset of clinical signs of EAE

To further investigate the effect of caspase-1 blockade in the early stages of the pathogenesis of EAE, one group of mice were administered YVAD-cmk, s.c. into the site of immunization for EAE, on days 0, 1, 2, 5, 7 and 9 (a protocol similar to that used to achieve the most significant suppression of clinical signs in Fig. 5.11), and a control group given DMSO at the same time points. Mice were sacrificed 10 days after induction of EAE, and inguinal lymph node cells restimulated with increasing concentrations of MOG. A significant decrease in antigen-specific IL-17 (p < 0.01) was observed in cells from the mice treated with the caspase-1 inhibitor (Fig. 5.13). TNF-α was also significantly reduced (p < 0.01) in supernatants of cells from treated compared with control mice. IFN-γ
production was only slightly reduced compared to control mice at the onset of clinical signs, but there was a very significant increase in non-antigen-specific IL-10 in lymph node cultures from treated compared with control mice.

5.2.13 Caspase-1 inhibition had no effect on the frequency of Th1 or Th17 cells in the spleen or lymph nodes of treated mice at the onset of clinical signs of EAE

Following immunization with myelin peptides in CFA, DC present processed antigen, complexed to MHC-II, to autoantigen-specific T cells in the peripheral lymphoid organs. DC also secrete inflammatory cytokines that direct differentiation to the different Th cell subsets. IL-1, together with IL-6 and IL-23, plays an important role in driving differentiation of naïve precursors to a Th17 phenotype. As such, inhibition of caspase-1 during the pathogenesis of EAE may result in decreased Th17 or Th1 cells in the spleen or lymph nodes, and account for the decreased antigen-specific cytokine production observed in previous results. Mice were administered YVAD-cmk or DMSO on days 0, 1, 2, 5, 7 and 9, and cells from the spleen and lymph node analyzed for CD4^+ IL-17 and IFN-γ expression, 10 days after induction of EAE. The frequency of IL-17^+ CD4 (Th17) cells and IFN-γ^+ CD4 (Th1) cells was comparable between control and treatment groups, both in the spleen and lymph nodes, at the onset of clinical signs of EAE (Fig. 5.14).

5.2.14 Caspase-1 inhibition resulted in a significantly lower frequency of IL-17^+ γδ T cells in the spleens of treated mice at the onset of clinical signs of EAE

γδ T cells have been shown to be an important source of IL-17 early in the course of EAE and have a pathogenic role during the development of disease. IL-
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23 can induce IL-17 production by γδ T cells, in the absence of TCR engagement, and this is significantly augmented in the presence of IL-1. In line with these findings, the frequency of IL-17-expressing γδ T cells was significantly reduced (p < 0.05) in the spleens of mice that were treated with YVAD-cmk on days 0, 1, 2, 5, 7 and 9, compared with control mice at the onset of clinical signs (Fig. 5.15). The frequency of IL-17⁺ γδ T cells was only slightly reduced in lymph nodes from the same mice. The expression of IFN-γ by γδ T cells in the spleen or lymph nodes was not affected by treatment with the caspase-1 inhibitor, at this stage in the development of EAE.

5.2.15 Caspase-1 inhibition resulted in a lower frequency of IL-17⁺ and IFN-γ⁺ γδ T cells in the CNS of treated mice at the onset of clinical signs of EAE

Data in the previous chapter have shown that γδ T cells infiltrate the CNS before the onset of clinical signs and a significant proportion of these express IL-17. At the onset of clinical signs, 10 days after induction of EAE, MNCs were isolated from the brains and spinal cords of mice that had been treated with YVAD-cmk on days 0, 1, 2, 5, 7 and 9, and control mice that had received DMSO at the same time points. Brains were processed individually and spinal cords pooled for each group. Analysis of cytokine production revealed a slight reduction in the frequency of IL-17⁺ and IFN-γ⁺ γδ T cells in the brains of treated compared with control mice (Fig. 5.16). In the spinal cord, the frequency of IL-17⁺ γδ T cells was approximately 50% lower in mice administered the caspase-1 inhibitor, compared to controls, but no change in expression of IFN-γ by γδ T cells was observed, at the onset of clinical signs of EAE.
5.2.16 Caspase-1 inhibition resulted in a lower frequency of Th1 and Th17 cells in the CNS of treated mice at the onset of clinical signs of EAE

In MS and EAE, autoantigen-specific T cells are activated in the periphery, and invade the CNS leading to organ-specific autoimmune disease. Myelin-specific Th17 cells are thought to be involved early in the development of CNS inflammation in EAE. Analysis of MNCs isolated from the CNS of mice that had been treated with YVAD-cmk on days 0, 1, 2, 5, 7 and 9, showed a 50% reduction in the frequency of CD4+ Th17 cells in both the brain and spinal cords at the onset of clinical signs of EAE, when compared with control mice that had received DMSO (Fig. 5.17). A marked decrease in the frequency of IFN-γ-secreting Th1 cells was also observed, particularly in the spinal cord, but also in the brain.

5.2.17 Attenuation of clinical signs of EAE due to caspase-1 inhibition correlates with decreased macrophage and PMN infiltration into the brain

Following infiltration and reactivation in the CNS, myelin-specific T cells release soluble cytokines and other inflammatory mediators that directly attack cells and structures in the target organ. In addition, T cells secrete or induce IL-1, IL-6, IL-8 and an array of chemokines and adhesion molecules involved in PMN and macrophage recruitment to the CNS and mediating disruption of the blood-brain barrier. In order to examine the effect of blocking caspase-1 activity on leukocyte infiltration to the CNS, MNCs were isolated from the brains of mice treated with YVAD-cmk or DMSO at days 0, 1 and 2 and every second from day 5 to day 21 after induction of EAE, and analyzed by flow cytometry. Leukocytes were gated based on CD45 expression and the frequency of macrophages (F4/80+) and PMN (Ly6G+) were determined. Macrophages are present in large numbers in the CNS of mice with EAE, and it is highly likely that these cells play a role in
the development of tissue damage and subsequent neurological dysfunction. A marked decrease in the frequency of macrophages was observed in the brains of YVAD-cmk treated mice, reduced approximately 50% relative to DMSO-treated control mice (Fig. 5.18). PMN have been shown to appear in the CNS shortly before the onset of clinical signs, and their absolute numbers increased in proportion to the growing inflammatory infiltrate. PMN accounted for only a small percentage of total CD45$^+$ leukocytes in the brains of mice with EAE, 21 days post-immunization, but were less frequent in mice that received the caspase-1 inhibitor, compared with control mice.
5.3 Discussion

EAE is mediated by myelin-specific T cells, which are activated in the periphery and translocate into the CNS. Upon recognition of their cognate antigen through the TCR, T cells are reactivated by local and infiltrating APC, initiating a cascade of inflammatory events that results ultimately in demyelination and axonal damage. Th17 cells are now acknowledged to play a central role in the development and pathogenesis of EAE. Th17 cells are thought to be generated more rapidly than Th1 cells and direct the initial acute inflammation within the target organ. IL-1 signaling is critical for Th17 development \textit{in vivo}; IL-1R1\textsuperscript{−/−} mice have defective Th17 responses and are resistant to the induction of EAE (Sutton \textit{et al.}, 2006). Furthermore, IL-1β synergizes with IL23 to promote IL-17 production from \(\gamma\delta\) T cells, without TCR stimulation (Sutton \textit{et al.}, 2009). Thus, IL-1, in addition to its myriad effects within the CNS, is an important upstream mediator of autoimmune inflammation, and potentially an important target for the treatment of neurological disorders with an inflammatory component.

Caspase-1 is required early in the IL-1 signaling pathway for proteolytic cleavage of pro-IL-1β to its mature form. Recognition of an appropriate microbial or danger signal by NLRP3, leads to oligomerization of the adapter molecule ASC to form the inflammasome, a multi-protein complex involving the NLR and inactive caspase-1, which mediates activation of the enzyme. Caspase-1 is required for processing of pro-IL-1β and its secretion as an active protein. Previous studies have found that mice deficient in caspase-1, or when it was inhibited pharmacologically, were less susceptible to EAE (Furlan \textit{et al.}, 1999). This was associated with impaired Th1 cell development and reduced perivascular infiltrates. The present study aimed to determine the effect of caspase-1 inhibition on Th17 cell differentiation, and how this impacts on the development of EAE.
Mycobacterium tuberculosis (Mtb) is a Gram-neutral bacterial pathogen due to its wax-like cell wall, but bears certain characteristics of Gram-positive bacteria. It is the causative agent of most cases of Tuberculosis (TB). Mtb is required in the CFA emulsion with myelin peptide for the efficient induction of EAE. This study found that Mtb was capable of activating the NLRP3 inflammasome in macrophages. Incubation of macrophages that were stably transfected with a ASC-YFP^ gene, with Mtb for 1 h resulted in contraction of ASC from the cytoplasm and oligomerization, indicative of inflammasome formation. ASC oligomerization was comparable to that observed on stimulation of ASC-YFP^ macrophages with LPS and ATP, known to induce NLRP3 inflammasome formation in macrophages (Kahlenberg et al., 2005). Stimulation of BMDC with LPS alone lead to upregulation of pro-IL-1β, but not secretion of the mature protein, as detected by both ELISA and western blot. Addition of ATP to LPS pre-stimulated cells induced processing of pro-IL-1β and secretion into the supernatant. Stimulation of BMDC with Mtb also lead to induction of pro-IL-1β and maturation to its active 17 kDa form; a process dependent on proteolytic cleavage of pro-IL-1β by activated caspase-1. This was confirmed by studies showing that pre-incubation of BMDC with a caspase-1 inhibitor blocked IL-1β and IL-18 secretion into the supernatant. IL-23 is another cytokine critical for IL-17 production by Th17 cells. IL-23 was also upregulated by DC in response to Mtb stimulation but was not dependent on caspase-1, or affected by its inhibition, in the same cultures. Taken together, these results indicate that Mtb possess the necessary machinery to induce active IL-1β secretion by DC, including TLR or NLR ligands that induce IL-1β transcription and translation and pore-forming molecules required for K^+ efflux and inflammasome formation.

IL-1 can act directly on murine T cells to enhance IL-17 production, either alone or in the presence of IL-23. Previous work in our laboratory has shown that IL-1β synergizes with IL-23 to promote IL-17 production by murine T cells in the
presence or absence of TCR engagement, whereas T cells from IL-1R1−/− mice failed to induce IL-17 production in response to IL-23 (Sutton et al., 2006). Human memory Th17 cells, but not naïve T cells, secrete IL-17 in vitro in response to stimulation with IL-1 and IL-23 (van Beelen et al., 2007). Furthermore, it was demonstrated in the previous chapter that γδ T cells produce IL-17 in response to IL-1 and IL-23 in the absence of TCR engagement, and these cells increased susceptibility to EAE (Sutton et al., 2009). Thus, the DC-derived cytokines IL-1 and IL-23 play an important role promoting IL-17 production in inflammation.

This study found that CD4+ T cells, purified from the spleens of mice with EAE, produced low concentrations of IL-17 in response to IL-23 signaling, which was dramatically augmented in the presence of IL-1β. Importantly, IL-18 could also synergize with IL-23 to induce IL-17 expression by CD4 T cells, presumably memory Th17 cells. Consistent with findings in chapter 4, IL-1β and IL-23 synergized to induce IL-17 production by CD3+ T cells, likely γδ T cells, from naïve mice in the absence of anti-CD3. IL-18 was also found to synergize with IL-23 to induce IL-17 production from these cells, revealing an additional mechanism by which caspase-1 can regulate IL-17 production.

IL-18 plays a role in both innate and adaptive immunity by activating neutrophils and enhancing T cell and NK cell maturation. IL-18 has been shown to function with IL-12 to promote IFN-γ production by NK cells and CD4+ T cells and Th1-type responses. However, in the absence of IL-12, IL-18 drives a Th2 response (Nakanishi et al., 2001). The present study found that CD4+ T cells from mice with EAE, most likely memory Th1 cells, produced high concentrations of IFN-γ when stimulated in vitro with IL-18. IL-18 together with IL-1β or IL-23 also induced IFN-γ production by CD4 T cells from mice with EAE, albeit in
lower concentrations. This might indicate antagonistic effects of these Th17-associated cytokines on IL-18, in a reciprocal manner to that of IL-18 on IL-1β, as has been described (Andoh et al., 2008). Surprisingly, IL-23, in synergy with either IL-18 or IL-1β, also induced IFN-γ expression by unconventional CD3^ T cells. NKT cells express IL-23R (Rachitskaya et al., 2008) and secrete IFN-γ upon stimulation (Godfrey et al., 2000). γδ T cells also constitutively express IL-23R (Fig. 4.21) and IL-1R (McCandless et al., 2009). Data from the previous chapter demonstrates that γδ T cells produce IFN-γ (Fig. 4.8) and upregulate IFN-γ mRNA in response to IL-23 signaling, in the presence or absence of IL-1β (Fig. 4.22). IL-18 has also been shown to induce IFN-γ expression by γδ T cells (Haas et al., 2009). Thus, cells of the innate immune system, including NK cells and γδ T cells, may be primed to respond rapidly to inflammatory cytokines, such as IL-23 and IL-1 or IL-18, and may play a role in driving adaptive Th1 responses.

Immature DC express low levels of MHC class II and costimulatory molecules, and cannot prime T cell responses. Upon internalization and processing of exogenous antigens, however, they migrate to the lymph nodes and undergo a maturation process, shutting off their antigen capturing and processing capacity. Mature DCs express high levels of MHC and costimulatory molecules, rendering them capable of priming naïve T cells (Pulendran, 2004). The cytokines secreted by DC help determine the phenotypic lineage of effector T cells. Here, BMDC cultured with Mtb for 24 h, and then washed, induced IL-17 secretion by purified CD3^ T cells from naïve mice in an Mtb concentration-dependent manner, without addition of exogenous anti-CD3. Mtb-activated DC also induced IFN-γ production by T cells, which was maximal at the lower concentration of Mtb used to stimulate DC. Thus, Mtb-induced maturation of DC results in expression of molecules sufficient to generate IL-17 and IFN-γ production by T cells. Furthermore, the ability of DC to induce IL-17 production in T cells is dependent on active caspase-1, since this effect was lost when DC
were pre-treated with increasing concentrations of YVAD-cmk. This data indicates a crucial role for IL-1β, or IL-18, secreted by mature DC following processing of Mtb, for the expression of IL-17 by CD3+ T cells.

In light of this data on the role of caspase-1 in IL-17 expression by T cells, together with the critical early requirement of IL-1 for the differentiation of Th17 cells and induction of EAE (Sutton et al., 2006; Chung et al., 2009), the effect of caspase-1 inhibition was examined in vivo in the EAE model. Sub-cutaneous administration of YVAD-cmk, every second day starting 5 days after induction of EAE, significantly attenuated the clinical signs of disease. The mean day of disease onset and development of initial clinical signs were comparable between the treated and control mice. However, mice that received the caspase-1 inhibitor failed to progress beyond an ataxic gait, whilst control mice given DMSO went on to develop severe paralysis of the hind limbs. The reduced paralysis observed in treated mice, despite a similar early progression of disease, may point to decreased IL-1β-induced IL-17, or decreased IL-18-induced IFN-γ, or both, even though Th1 and Th17 cell differentiation may have already occurred in the periphery. Activated T cells can enter the CNS, but only persist if reactivated upon recognition of their cognate antigen, presented in as MHC-II complex by CNS-resident or infiltrating APC. Therefore, decreased IL-1β and IL-18 production by DC, due to blockade of caspase-1 at this stage, may be sufficient to dampen the inflammatory response even after the onset of initial clinical signs. Interestingly, analysis of MOG-specific IL-17 production in the chronic phase of disease, revealed a significant reduction in IL-17 production in cells from both the spleen and inguinal lymph nodes in mice treated with caspase-1 inhibitor, even at this late stage of disease.
To determine the effect of caspase-1 inhibition at different stages of disease, mice were administered YVAD-cmk or DMSO at the induction phase and then every second day from day 5 to day 21, or prior to and during disease from 5 days post-immunization. Mice given the caspase-1 inhibitor from day 5 onward exhibited early signs similar to controls, and a similar mean day of onset, but failed to progress beyond an ataxic gait throughout the course of disease. Significantly, however, mice that received the inhibitor from the induction of disease, through to day 21 post-immunization, displayed a mean clinical score of less than grade 1 over the course of disease, and in some mice complete amelioration of clinical signs of EAE.

Mice that had been treated with YVAD-cmk on days 0, 1 and 2 produced significantly less IL-17 and IFN-γ (both p < 0.05) in cultures of lymph node cells 5 days after induction of EAE, compared with mice that had received DMSO. This may indicate impaired differentiation of naïve T cells to Th17 and Th1 cells, respectively. Antigen-specific IL-10 was not detected from lymph node cells at this early stage. When tested 10 days post-immunization, IL-17 production by MOG-specific Th17 cells remained significantly less than in control mice (p < 0.01), as were concentrations of TNF-α, another Th17 cell cytokine (p < 0.01). Antigen-specific IFN-γ expression was also lower at this stage in mice given the caspase-1 inhibitor, although not significantly so. This is consistent with findings in previous chapters that indicate Th17 cells, and the cytokines that drive their activity, are the more prominent Th subset around the onset of disease and that blocking caspase-1 results more in a deficit of soluble IL-1β than the Th1-driving IL-18, at this stage. Conversely, IL-18 may be redundant in this role if IL-12 is also produced by DC. Again, MOG-specific IL-10 was not evident in cultures of lymph node cells from either treatment group; however, there was a significant increase (p < 0.001) the concentration of non-antigen-specific IL-10 in lymph node cells of mice administered the caspase-1 inhibitor.
Despite the reduced antigen-specific IL-17 and IFN-γ produced by cells from the spleen and lymph nodes of treated mice at the onset of clinical signs of EAE, no difference in the frequency of either Th17 or Th1 cells was observed when analyzed by intracellular cytokine staining. However, the frequency of IL-17⁺ γδ T cells was significantly lower in the spleens of mice treated with the caspase-1 inhibitor \( (p < 0.05) \). This provides evidence that IL-17 production by circulating γδ T cells is dependent on IL-1β production during the development of EAE. It was demonstrated in the previous chapter that IL-17⁺ γδ T cells are present at high frequency in the CNS around the onset of clinical signs of EAE. However, systemic administration of the caspase-1 inhibitor failed to reduce the frequency of IL-17-producing γδ T cells in the brain or spinal cord, when tested 10 days after induction of EAE. Conversely, inhibition of caspase-1 during the pathogenesis of EAE did result in a 50% reduction of CD4⁺ Th17 cells in both the brain and spinal cords at the onset of clinical signs. A marked decrease in the frequency of IFN-γ-secreting Th1 cells was also observed, particularly in the spinal cord, but also in the brain. Thus, despite the fact that the frequency of myelin-specific Th17 and Th1 cells was not reduced in the periphery, fewer pathogenic T cells accumulated in the CNS around the onset of disease in treated mice.

Recent data indicates that IL-23R engagement is critical for the acquisition of encephalo-tropism by differentiated Th cells \emph{in vivo} and is essential for the production of IL-17 by CNS-infiltrating Th17 cells (Gyulveszi \emph{et al.}, 2009). It was demonstrated that the lack of IL-23R engagement does not interfere with the expansion of autoreactive T cells, but does compromise their capacity to leave secondary lymphoid organs and to access their target tissue. These findings were supported in a report by Cua and colleagues that found that IL-23 was not required for lineage commitment but was critical for terminal differentiation of Th17 cells and their capacity to leave the lymph nodes and home to target tissues.
(McGeachy et al., 2009). Naïve T cells do not express IL-23R on their surface, and it is thought that IL-6, possibly by inducing IL-21, upregulates IL-23R upon ligation of the TCR, thus promoting full differentiation of Th17 cells in the presence of IL-23 (Zhou et al., 2007). IL-1 enhances production of IL-6 (Simi et al., 2007), and it was shown in chapter 3 that the expression of IL-6 in the inguinal lymph nodes closely followed that of IL-1β. Therefore, it is possible that decreased production of IL-1β following caspase-1 inhibition leads to reduced IL-6 production in the peripheral lymphoid organs and ultimately lower expression of IL-23R by T cells. According to the report by Becher and colleagues, this would not affect expansion of Th17 cells in the spleen and lymph nodes, but would impede their capacity to leave these organs and enter the CNS (Gyulveszi et al., 2009). This may help explain the findings in this study of no reduction in development and expansion of Th17 cells in the spleen and lymph nodes of mice administered the caspase-1 inhibitor. Conversely, MOG-specific IL-17 was decreased in ex vivo cultures of antigen-specific lymph node and spleen cells, and infiltration of Th17 cells into the CNS was reduced at the onset of clinical signs of disease.

Within the CNS, myelin-specific T cells are reactivated in the perivascular space where they directly, or via activation of glia, induce chemokine expression that results in further leukocyte recruitment and blood-brain barrier breakdown (Cross et al., 1990). Expression of chemokines such as CCL2 (macrophage chemoattractant protein (MCP)-1), CCL3 (macrophage inflammatory protein (MIP)-1α), CCL4 (MIP-1β), CCL5 (regulated upon activation, T cell expressed and secreted (RANTES)), CXCL1 (KC; GRO1), CXCL2 (GRO2; MIP-2) and CXCL10 (IFN-γ-inducible protein (IP)10), results in accumulation of their receptor expressing cells, including macrophages and PMN, from the periphery. This leads to the formation of organized inflammatory infiltrates during the chronic phase of EAE, promoting disease progression and/or clinical relapse.
IL-1β is a powerful inducer of CXCL1 and CXCL2 in the CNS, and subsequent recruitment of PMN (Anthony et al., 1998). It was recently demonstrated that IL-1β is produced by CD4⁺ and CD8⁺ T cells, but also by IL-17⁺ γδ T cells that infiltrate the CNS (McCandless et al., 2009). Furthermore, IL-17 produced by T cells was shown to induce recruitment of neutrophils via upregulation of CXCL1 and CXCL2 (Ferretti et al., 2003). Consistent with these findings, and the data showing reduced Th17 cell infiltration of the CNS around the onset of EAE, the present study found that the frequency of PMN in the brains of mice treated with the caspase-1 inhibitor was lower at the chronic phase of disease, when compared with DMSO-treated control mice. Although PMN are among the first leukocytes to infiltrate the CNS in some models of EAE, they are scarce in mature infiltrates (Carlson et al., 2008). Indeed, it is thought that PMN may mediate or augment blood-brain barrier breakdown during the period between the reactivation of myelin-specific T cells in the CNS and the massive influx of nonspecific leukocytes that heralds the onset of clinical EAE (Carlson et al., 2008). Therefore, it will be of interest to assess the effect of caspase-1 inhibition on PMN recruitment to the CNS in the pre-clinical and acute phases of EAE.

Macrophages are known to be prominent in the chronic phase of disease and their depletion has been shown to suppress the development of clinical signs of EAE (Huitinga et al., 1995). IFN-γ is a potent inducer of CNS chemokine expression, and particularly important in the recruitment of macrophages to sites of inflammation (Lin et al., 2009). The frequency of macrophages in the brain was strikingly lower in the chronic phase of disease in mice treated with YVAD-cmk, compared with DMSO-treated control mice. This may result from the reduced frequency of Th1 cells infiltrating the CNS of treated mice, together with the lower expression of the inflammatory cytokines IFN-γ, TNF-α and IL-1β in these mice. These findings are consistent with those of Adorini and colleagues, who showed that pharmacological blockade of caspase-1 with a different inhibitor.
also lead to reduced numbers of infiltrating macrophages and CD3\(^+\) T cells (Furlan et al., 1999). Importantly, macrophages and T cells are known to express CXCR4 (Lee et al., 2003), the receptor for the secondary lymphoid chemokine CXCL12 expressed on the parenchymal surface of the CNS endothelium, where it functions to restrict parenchymal infiltration by leukocytes. Unique to MS and EAE, however, CXCL12 has been reported to relocate to the luminal side of the microvasculature (McCandless et al., 2008). The process was shown to correlate with disease severity in MS patients. Klein and colleagues further demonstrated that loss of perivascular CXCL12 and relocation to the luminal side of the blood vessels occurred early in EAE and was IL-1\(\beta\) mediated (McCandless et al., 2009). Moreover, they found that infiltrating T cells, in particular IL-17\(^+\) \(\gamma\delta\) T cells, were the main source of IL-1\(\beta\) in this relocation. This highlights an alternative mechanism by which disruption of IL-1\(\beta\) signaling might attenuate the severity of autoimmune inflammation in the CNS; crucially, in a T cell-dependent manner.

IL-1\(\beta\) is a critical component of disease pathogenesis in MS and EAE. An imbalance in the IL-1\(\beta\) to IL-1Ra ratio predisposes to MS (de Jong et al., 2002) and IL-1RI-deficient mice are resistant to EAE (Sutton et al., 2006). IL-1\(\beta\) cooperates with IL-23 to induce IL-17 production by memory T cells and innate IL-17 production by \(\gamma\delta\) T cells, and there is some evidence that it is required for the differentiation of Th17 cells. IL-1\(\beta\) induces blood-brain barrier disruption and accumulation of leukocytes within the CNS (Shaftel et al., 2007). IL-1\(\beta\) also activates microglia, resulting in the release of a variety of inflammatory cytokines and other toxic molecules including reactive oxygen species, nitric oxide, peroxides, prostaglandins and leukotrienes (Simi et al., 2007).

This study addressed the role of caspase-1 in IL-17 and IFN-\(\gamma\) production by T cells and its impact on disease progression in EAE. It was demonstrated that
Mtb induces inflammasome formation and activation of caspase-1, resulting in the secretion of mature IL-1β, IL-18, and other cytokines by DC. DC activated in this manner stimulated IL-17 expression in T cells, which was dependent on active caspase-1. Administration of a caspase-1 inhibitor \textit{in vivo} significantly attenuated the clinical signs of EAE, when given over the course of development of disease. Although not affecting the development of Th17 and Th1 cells in the peripheral lymphoid organs, inhibition of caspase-1 lead to reduced antigen-specific cytokine production by these cells and decreased infiltration into the CNS at the onset of clinical signs of EAE. Additionally, accumulation of PMN and macrophages was reduced in the brains of treated mice, in the chronic phase of disease. Numerous studies have examined the effects of blocking IL-1 signaling in a variety of inflammatory diseases. The effect of blocking IL-1 by caspase-1 inhibition on IL-17 production by T cells in EAE has not been reported. This study confirms previous findings that inhibition of caspase-1 may be beneficial in EAE and consequently in MS, and demonstrates that the therapeutic effect of caspase-1 inhibition may be partially mediated by reducing the CNS-infiltrating capacity of auto-reactive Th17 cells and decreased IL-17 production upon reactivation.
Chapter 5 Summary

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<th>Process (in vitro)</th>
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**In vivo**

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Effect of YVAD treatment throughout EAE

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Figure 5.1 *M. tuberculosis* can activate the NLRP3 inflammasome, required for caspase-1 activation, in macrophages. YFP\(^{+}\)ASC-reporter macrophages were stimulated with LPS for 3 h and ATP for 1 h, Mtb (10–50 \(\mu\)g/ml) for 1 h or medium only. YFP\(^{+}\) macrophages were imaged on an Olympus FV1000 laser scanning confocal microscope (40 X objective with 1.0 or 2.4 zoom).
Figure 5.2  *M. tuberculosis* induces pro-IL-1β expression by DC and processing to its mature form. BMDC (1 x 10^6 cells/ml) were stimulated with Mtb (10 or 50 µg/ml) for 18 h, LPS for 3 h +/- ATP for 1 h, or medium only, as controls. Supernatants were collected and IL-1β expression quantified by ELISA (A) and Western blot (B).
Figure 5.3 *M. tuberculosis* or LPS and ATP induced IL-1β secretion by DC is caspase-1-dependent. BMDC (1 x 10^6 cells/ml) were stimulated with Mtb (2, 10 or 50 
µg/ml) or medium alone for 18 h, or with LPS (100 ng/ml) for 3 h +/- ATP (5 mM) for 1 h, with or without caspase-1 inhibitor (8-40 µM; A) or an equivalent concentration of DMSO as a control (B). Supernatants were collected and IL-1β concentrations quantified by ELISA. Activated DC versus DC + YVAD: ** P < 0.01, *** P < 0.001 by two-way ANOVA with repeated measures.
Figure 5.4  *M. tuberculosis*-induced IL-18, but not IL-23, production by DC is caspase-1-dependent. BMDC (1 x 10^6 cells/ml) were stimulated with Mtb (2, 10 or 50 μg/ml) or medium alone for 18 h, or with LPS (100 ng/ml) for 3 h +/- ATP (5 mM) for 1 h, with or without caspase-1 inhibitor (8-40 μM). Supernatants were collected and IL-18 (A) and IL-23 (B) concentrations quantified by ELISA. Activated DC versus DC + YVAD: ** P < 0.01, *** P < 0.001 by two-way ANOVA with repeated measures.
Figure 5.5 IL-1β or IL-18 can synergise with IL-23 to promote IL-17 production by T cells. CD3 (A) and CD4 (B) T cells were purified by negative selection using magnetic separation, from the spleens of naïve mice or mice with EAE (d 10) and cultured with IL-1β, IL-18 and IL-23, or combinations thereof (all 10 ng/ml) in the absence of αCD3, or cells stimulated with medium only or PMA and αCD3 acted as negative and positive controls, respectively. Supernatants were harvested after 72 h and IL-17 concentrations quantified by ELISA.
Figure 5.6  IL-18 promotes IFN-γ production from antigen-experienced CD4 T cells from mice with EAE and synergizes with IL-23 to induce IFN-γ secretion by CD3 T cells from naïve mice. CD3 (A) and CD4 (B) T cells were purified by negative selection using magnetic separation, from the spleens of naïve mice or mice with EAE (d 10) and cultured with IL-1β, IL-18 and IL-23, or combinations thereof (all 10 ng/ml, or cells stimulated with medium only or PMA and αCD3 acted as negative and positive controls, respectively. Supernatants were harvested after 72 h and IFN-γ concentrations quantified by ELISA.
Figure 5.7  *M. tuberculosis*-activated DC stimulate IL-17 and IFN-γ production by purified CD3⁺ T cells. BMDC were stimulated for 24 h with Mtb (10–50 μg/ml) or medium only. DC were washed and cultured with CD3 T cells purified from the spleens of naïve mice or mice with EAE (d 10), in the absence of αCD3. T cells were also cultured with PMA and αCD3 or medium only, as controls. Supernatants were harvested after 72 h and IL-17 and IFN-γ concentrations quantified by ELISA.
Figure 5.8 Inhibition of caspase-1 reduces the ability of *M. tuberculosis*-activated DC to induce IL-17 production by T cells from naïve mice. BMDC were stimulated for 24 h with Mtb (10–50 µg/ml) or medium only, with or without YVAD (2-50 µM). DC were washed and cultured with CD3 T cells purified from the spleens of naïve mice. T cells were also cultured with PMA and αCD3 or medium only, as controls. Supernatants were harvested after 72 h and IL-17 concentrations quantified by ELISA. * P < 0.05 YVAD versus T cells cultured with stimulated DC in the absence of YVAD by unpaired t-test.
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**Figure 5.9** Therapeutic administration of a caspase-1 inhibitor attenuates the development of acute MOG-induced EAE. EAE was induced in C57BL/6 mice by s.c. injection of 100 μg of MOG35-55 emulsified in CFA, supplemented with 4 mg/ml H37 Ra *M. tuberculosis*. Mice were injected i.p. with 500 ng pertussis toxin on d 0 and 2. YVAD-cmk (25 μg/mouse) or vehicle (DMSO) was administered s.c. at the site of immunization, every second day from day 5 to day 21 post-induction. Animals were monitored daily for weight and signs of clinical disease. Disease severity was graded as follows: grade 0 - normal; grade 1 - flaccid tail; grade 2 - wobbly gait; grade 3 - hind limb weakness; grade 4 - hind limb paralysis; grade 5 - tetraparalysis/death. EAE control versus EAE + YVAD: * P < 0.05 by two-way ANOVA with repeated measures. Results are mean score +/- SEM (n = 6/group).
Figure 5.10  Inhibition of caspase-1 suppresses MOG-specific IL-17 production in lymph node and spleen cells. EAE was induced in C57BL/6 mice and mice were treated with caspase-1 inhibitor or DMSO as described in Fig. 5.8. Inguinal lymph node and spleen cells were recovered 21 d post-immunization and re-stimulated in vitro with MOG_{35-55} (2-50 μg/ml), PMA and αCD3 or medium only. Supernatants were recovered after 72 h and IL-17 concentrations quantified by ELISA. EAE control versus EAE + YVAD: * P < 0.05 by unpaired t-test. Results are mean score +/- SEM (n = 6/group).
Figure 5.11 Inhibition of caspase-1 from induction and through the course of disease almost ameliorates clinical signs of MOG-induced EAE. EAE was induced in C57BL/6 mice as described in Fig. 5.8. One group of mice were treated with YVAD (25 μg/mouse) by mixing it with the MOG/CFA emulsion on d 0, and then injecting it s.c. at the site of immunization on d 1 and 2. Another group were administered YVAD s.c. every second day from d 5 onward. A third group were administered YVAD on d 0, 1 and 2 and every second day from d 5 onward. Control animals were administered DMSO. Animals were monitored daily for weight (B) and signs of clinical disease (A). Disease severity was graded as described in Fig. 5.8. EAE control versus EAE + YVAD on d 0, 1, 2 and every second day from d 5: ** P < 0.01 by two-way ANOVA with repeated measures. Results are mean score or weight +/- SEM (n = 6/group).
Figure 5.12 Inhibition of caspase-1 at induction of EAE significantly reduced antigen-specific pro-inflammatory cytokine production in the LN when tested at d 5 post induction. EAE was induced in C57BL/6 mice as described in Fig. 5.8. Mice that were treated with the caspase-1 inhibitor were immunized with MOG/CFA containing YVAD (25 µg/mouse) in the emulsion on d 0, and then injected s.c. at the site of immunization on d 1 and 2. Inguinal lymph node cells were recovered 5 d post-immunization and re-stimulated in vitro with MOG35-55 (2-50 µg/ml), PMA and αCD or medium only. Supernatants were recovered after 72 h and IL-17, IL-10 and IFN-γ concentrations quantified by ELISA (n = 4/group, in triplicate). EAE control versus EAE + YVAD: + P < 0.05; * P < 0.05 by unpaired t-test.
Figure 5.13 Caspase-1 inhibition significantly reduced antigen-specific pro-inflammatory cytokine production at the onset of clinical signs of EAE. EAE was induced in C57BL/6 mice as described in Fig. 5.8. Mice that were treated with the caspase-1 inhibitor were immunized with MOG/CFA containing YVAD (25 μg/mouse) in the emulsion on d 0, and then injected s.c. at the site of immunization on d 1, 2, 5, 7 and 9. Inguinal lymph node cells were recovered 10 d post-immunization and re-stimulated in vitro with MOG_{35-55} (2-50 μg/ml), PMA and αCD3 or medium only. Supernatants were recovered after 72 h and IL-17, IL-10, TNF-α and IFN-γ concentrations quantified by ELISA (n = 4/group, in triplicate). EAE control versus EAE + YVAD: ** P < 0.01, *** P < 0.001; +++ P < 0.001 by unpaired t-test.
Figure 5.14 Caspase-1 inhibition had no effect on the frequency of Th1 or Th17 cells in the spleen or lymph nodes of treated mice at the onset of clinical signs of EAE. EAE was induced in C57BL/6 mice as described in Fig. 5.8. Mice that were treated with the caspase-1 inhibitor were immunized with MOG/CFA containing YVAD (25 μg/mouse) in the emulsion on d 0, and then injected s.c. at the site of immunization on d 1, 2, 5, 7 and 9. Spleen and inguinal LN cells were isolated 10 d post-immunization and re-stimulated with PMA and ionomycin in the presence of brefeldin A for 14 h. Cells were stained for surface CD3 and CD4, and intracellular IL-17 and IFN-γ, and analyzed by FACS. Results are mean percentage of total CD4 T cells +/- SEM (n = 4/group).
Figure 5.15 Caspase-1 inhibition resulted in a significantly lower frequency of IL-17^\textsuperscript{+} \(\gamma\delta\) T cells in the spleens of treated mice at the onset of clinical signs of EAE. EAE was induced in C57BL/6 mice as described in Fig. 5.8. Mice that were treated with the caspase-1 inhibitor were immunized with MOG/CFA containing YVAD (25 \(\mu\)g/mouse) in the emulsion on d 0, and then injected s.c. at the site of immunization on d 1, 2, 5, 7 and 9. Spleen and inguinal LN cells were isolated 10 d post-immunization and re-stimulated with PMA and ionomycin with brefeldin A for 14 h. Cells were stained for surface CD3 and \(\gamma\delta\) TCR, and intracellular IL-17 and IFN-\(\gamma\), and analyzed by FACS. Results are mean percentage of total \(\gamma\delta\) T cells +/- SEM (n = 4/group), plus representative dot plots for IL-17^\textsuperscript{+} and IFN-\(\gamma^+\) \(\gamma\delta\) T cells from the spleens of control and treated mice. Numbers on dot plots refer to percentage of total gated \(\gamma\delta\) T cells. EAE control versus EAE + YVAD: * P < 0.05.
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Figure 5.16  Caspase-1 inhibition resulted in a lower frequency of IL-17⁺ and IFN-γ⁺ γδ T cells in the CNS of treated mice at the onset of clinical signs of EAE. EAE was induced in C57BL/6 mice as described in Fig. 5.8. Mice that were treated with the caspase-1 inhibitor were immunized with MOG/CFA containing YVAD (25 µg/mouse) in the emulsion on d 0, and then injected s.c. at the site of immunization on d 1, 2, 5, 7 and 9. Leukocytes were recovered from the brain and spinal cords of perfused mice 10 d post-immunization and re-stimulated with PMA and ionomycin with brefeldin A for 14 h. Cells were stained for surface CD3 and γδ TCR, and intracellular IL-17 and IFN-γ, and analyzed by FACS. Results are mean percentage of total γδ T cells +/- SEM (n = 4/group for brains and pooled spinal cords).
Figure 5.17  Caspase-1 inhibition resulted in a lower frequency of Th1 and Th17 cells in the CNS of treated mice at the onset of clinical signs of EAE. EAE was induced in C57BL/6 mice as described in Fig. 5.8. Mice that were treated with the caspase-1 inhibitor were immunized with MOG/CFA containing YVAD (25 μg/mouse) in the emulsion on d 0, and then injected s.c. at the site of immunization on d 1, 2, 5, 7 and 9. Leukocytes were recovered from the brain and spinal cord of perfused mice 10 d post-immunization and re-stimulated with PMA and ionomycin with brefeldin A for 14 h. Cells were stained for surface CD3 and CD4, and intracellular IL-17 and IFN-γ, and analyzed by FACS. Results are mean percentage of total CD4 T cells +/- SEM (n = 4/group for brains and pooled spinal cords), plus representative dot plots for IL-17+ and IFN-γ+ CD4 T cells from the brains of control and treated mice. Numbers on dot plots refer to percentage of total CD4 T cell subset.
Figure 5.18 Attenuation of clinical signs of EAE correlated with decreased macrophage and PMN infiltration into the brains of mice administered the caspase-1 inhibitor. EAE was induced in C57BL/6 mice as described in Fig. 5.8. Mice that were treated with the caspase-1 inhibitor were immunized with MOG/CFA containing YVAD (25 μg/mouse) in the emulsion on d 0, and then injected s.c. at the site of immunization on d 1 and 2 and every second day from d 5 onward. Leukocytes were recovered from the brains of perfused mice 21 d post-immunization, surface stained for CD45, Ly-6G (PMN) and F4/80 (MΦ) and analyzed by FACS. Results are mean percentage of total gated CD45+ leukocytes +/- SEM (n = 6/control group and 2 mice pooled/treatment group; A), with representative dot plots (B). Numbers on dot plots refer to percentage of total gated CD45+ leukocytes.
General Discussion

Chapter 6
6.1 General Discussion

MS is a chronic, progressive inflammatory disorder characterised by the destruction of CNS myelin and often the underlying axons. Studies demonstrating the presence of inflammatory cells and their products in the brain lesions of MS patients, supported by findings in animal models, have led to the generally accepted hypothesis that disease is mediated by pathogenic T cell responses against myelin antigens, followed by a broader neurodegenerative process (Compston et al., 2008). It is thought that some environmental trigger activates myelin-specific T cells in genetically predisposed individuals, allowing them to cross the blood-brain barrier. CNS-resident APC or infiltrating perivascular macrophages and DC presenting myelin antigens reactivate these T cells, resulting in the release of inflammatory mediators that recruit cells of the innate immune system which ultimately bring about demyelination and axonal damage. The animal model EAE has proved invaluable in testing hypotheses, and generating new ones, about the development of the inflammatory plaque and subsequent neurological dysfunction in chronic MS.

It was previously believed that Th1 cells were the pathogenic T cells involved in MS and EAE. T cells reactivated ex vivo with the immunizing antigen produced large amounts of IFN-γ but not IL-4 (Zamvil & Steinman, 1990). Furthermore, mice deficient in the Th1-driving cytokine IL-12 were resistant to EAE (Segal et al., 1998). However, IFN-γ^- or STAT1^- mice that lack Th1 cells were found to develop more severe EAE (Ferber et al., 1996; Bettelli et al., 2004). This apparent contradiction began to unravel with the discovery of IL-23, a heterodimer of the IL-23p19 subunit with the IL-12p40 subunit (Oppmann et al., 2000). It was then demonstrated that only IL-23p19^- mice and IL-12p40^- mice were resistant to EAE, but that mice defective in the IL-12-restricted p35 chain developed a hyperacute form of disease (Cua et al., 2003; Gran et al., 2002).
Soon after, it was found that IL-23 promotes the production of IL-17 by activated CD4 T cells, and that IL-23p19−/− mice resistant to EAE had very few CD4 T cells capable of producing IL-17 (Murphy et al., 2003; Langrish et al., 2005). It was quickly established that these IL-23-dependent CD4+ T cells were a distinct lineage, separate from Th1 and Th2 cells, that expressed IL-17A, IL-17F and TNF-α and were subsequently called Th17 cells (Langrish et al., 2005; McKenzie et al., 2006; Weaver et al., 2006). That Th17 cells were the key pathogenic T cells in EAE was confirmed when it was shown that adoptive transfer of memory CD4 T cells activated with IL-23 could induce disease, and that both IL-12 and IFN-γ suppressed IL-17 production, concomitantly explaining the paradox that IFN-γ−/− mice develop more severe EAE (Langrish et al., 2005; Murphy et al., 2003).

The orphan nuclear receptor RORγt was found to be the key transcription factor orchestrating Th17 cell differentiation. RORγt is required for the induction of IL-17A, IL-17F and IL-23R in Th17 cells in vitro and, moreover, in the absence of endogenous RORγt, mice lacked tissue-infiltrating Th17 cells and were significantly less susceptible to EAE (Ivanov et al., 2006a). IL-6-deficient mice are resistant to EAE (Eugster et al., 1998), and Littman and colleagues found that this may result from defective Th17 generation by demonstrating that IL-6 upregulates RORγt and IL-23R in vivo (Ivanov et al., 2006a). Indeed, given that naïve T cells do not express the IL-23R it became accepted that IL-6 and TGF-β were the critical cytokines involved in commitment to the Th17 lineage, and that IL-23 was required for maintenance or expansion of memory Th17 cells (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006). However, direct induction of de novo Th17 cells by IL-23 had been demonstrated when IFN-γ and IL-4 were blocked (Harrington et al., 2005; Park et al., 2005). Recent evidence by Das and colleagues that TGF-β blocks expression of STAT4 and GATA-3, thus preventing Th1 and Th2 cell differentiation, supports these findings.
Furthermore, in mice deficient in both in T-bet and STAT-6, and therefore also deficient in Th1 and Th2 cells, IL-6 alone was sufficient to induce differentiation of Th17 cells (Das et al., 2009).

In the absence of IFN-$\gamma$ and IL-4 antagonism, IL-6 efficiently drives Th17 cell differentiation by inducing IL-21 production, which together with IL-6 upregulates ROR$\gamma$t and IL-23R on activated T cells, bestowing responsiveness to IL-23 (Zhou et al., 2007; Korn et al., 2007a). In addition, IL-6 appears to negate the regulatory effect of Foxp3 on ROR$\gamma$t function, thereby allowing differentiation and expansion of effector Th17 cells (Zhou et al., 2008). Previous work from our laboratory has shown a critical role for IL-1 signaling in Th17 cell development. IL-1RI$^{-/}$ mice have defective Th17 responses and are resistant to the induction of EAE (Sutton et al., 2006). Dong and colleagues further demonstrated that IL-1 synergizes with IL-6 and IL-23 to induce expression of ROR$\gamma$t, thereby driving Th17 polarization (Chung et al., 2009). Thus, IL-1, IL-6, IL-21 and TGF-$\beta$, either directly or indirectly, lead to upregulation of IL-23R and confer activated Th17 cells with the ability to respond to IL-23. IL-23 is absolutely required for the development of encephalitogenic T cells and it was recently reported that IL-23, although not required for lineage commitment, is essential for the terminal differentiation and full pathogenic activity of Th17 cells (Thakker et al., 2007; McGeachy et al., 2009).

Consistent with these findings in EAE, IL-23 and IL-17 appear to have a pathogenic role in MS. The levels of IL-23 expression in monocyte-derived DC are higher in MS patients than in healthy controls (Vaknin-Dembinsky et al., 2006). Elevated frequencies of Th17 cells in the peripheral blood of MS patients have been associated with disease activity (Matusevicius et al., 1999; Durelli et al., 2009). IL-17$^+$ CD4$^+$ and CD8$^+$ T cells as well as IL-17-producing glial cells

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have been detected in active lesions in the brains of MS patients, but not in inactive areas (Tzartos et al., 2008). In addition, Prat and colleagues demonstrated that receptors for IL-17 and IL-22 are present on inflamed endothelium in MS lesions and that these cytokines may disrupt blood-brain barrier tight junctions, potentiating inflammatory T cell migration into the CNS (Kebir et al., 2007). It was also reported in this study that Th17 cells produce granzyme B and are cytotoxic to neurons. These results suggest that Th17 cells play an important role in the pathology of MS.

Although CD4+ Th17 cells are the major source of IL-17, they are not the only cells to secrete this cytokine. Neutrophils and microglia were found to secrete IL-17 (Ferretti et al., 2003; Kawanokuchi et al., 2008). IL-17 production by both murine and human CD8+ T cells has been demonstrated (Shin et al., 1999; Happel et al., 2003). Unconventional T cells can also secrete IL-17. NKT cells constitutively express the IL-23R and produce IL-17 on stimulation with anti-CD3 and IL-23 (Rachitskaya et al., 2008). LTi-like cells also constitutively express IL-23R as well as RORγt and rapidly produce IL-17 and IL-22 in response to IL-23 stimulation (Ivanov et al., 2006b; Takatori et al., 2009). Additionally, IL-17 production by γδ T cells has been described in numerous models of infection and autoimmunity (Lockhart et al., 2006; Shibata et al., 2007; Roark et al., 2007). Therefore, T cells other than CD4+ Th17 cells secrete IL-17, and may contribute to CNS pathology in EAE and MS.

There is substantial evidence that γδ T cells may play a role in both MS and EAE. Oligoclonal expansion or activation of γδ T cells has been found in acute MS brain lesions and in the cerebrospinal fluid of MS patients with recent disease onset, suggesting that γδ T cells contribute to neuroinflammation (Selmaj et al., 1991; Wucherpfennig et al., 1992; Shimonkevitz et al., 1993; Hvas et al.,
In the EAE model, however, the role of γδ T cells is more controversial, where both protective and pathogenic functions have been described (Kobayashi et al., 1997; Ponomarev et al., 2004; Ponomarev & Dittel, 2005; Rajan et al., 1996; Rajan et al., 1998; Spahn et al., 1999; Odyniec et al., 2004; Cardona et al., 2002; Clark & Lingenheld, 1998). There are a number of factors that might explain some of the conflicting results in these studies which have used a number of different mouse strains in combination with either depleting antibodies or genetic manipulation of γδ T cells. The frequency of γδ subtypes varies between mouse strains, and since different functions have been ascribed to various subtypes, this could account for many of the discordant results in different mouse strains. It has also been suggested that antibody depletion using either the GL3 or the UC7-13D5 γδ antibodies, which accelerate the onset of EAE, rather than depleting may activate γδ T cells by cross-linking the receptor (Koenecke et al., 2009). Finally, the Tcrd- mice used in some studies are defective only in the δ TCR chain and, thus, γδ T cells are still present, although unable to respond to TCR stimulation. This suggests that TCR independent activation of γδ T cells could still occur in these mice, potentially confounding the interpretation of experiments.

The results of the present study demonstrate that γδ T cells are an important source of innate IL-17 in EAE. It is shown that γδ T cells constitutively express IL-23R, and this was enhanced by stimulation with IL-23 alone or in combination with IL-1β. It was also found that γδ T cells constitutively express the critical transcription factor for Th17 cell differentiation, RORyt, and expression was further enhanced in the presence of IL-23. In addition, IL-23 induced IL-17A, IL-17F and IL-22 expression, without any additional stimulation. Expression of these cytokines was augmented in the presence of IL-1β. IL-21 expression is induced in naive T cells by stimulation with anti-CD3 and anti-CD28, in the presence of IL-6 (Korn et al., 2007a). Interestingly, it was found
that γδ T cells and primed CD4+ T cell populations, but not CD4+ T cells from naive mice, expressed IL-21 after stimulation with IL-1β and IL-23 together, without TCR engagement. In expressing RORγt and IL-23R, γδ T cells from naive mice appear to possess the signature profile of memory Th17 cells, and may be primed to rapidly respond to endogenous inflammatory mediators (Fig. 6.1). This ability was demonstrated in vivo by the elevated gene expression of IL-17A and IL-17F and the increased frequency of IL-17+ γδ T cells in the popliteal lymph nodes, following injection of IL-1β and IL-23 subcutaneously into the footpad.

This study found that γδ T cells infiltrate the CNS during the development of EAE, and a very high frequency of these cells produced IL-17. Consistent with findings from the CIA model of autoimmune arthritis (Roark et al., 2007), the majority of γδ T cells infiltrating the CNS were of the Vγ4 subset and these accounted for more than half of γδ-derived IL-17 in the brain at the onset of disease. Furthermore, it was demonstrated that CD27 γδ T cells, with the ability to produce IL-17, preferentially accumulated in the CNS compared with the spleen. Therefore, only a small number of γδ T cells expressed IFN-γ in the CNS. In contrast, a similar frequency of IL-17+ and IFN-γ+ CD4 T cells were detected in the CNS at the onset of clinical symptoms, and although total numbers are greater, a significantly lower frequency of CD4+ T cells than γδ T cells expressed IL-17. These findings correlated with elevated IL-17 mRNA expression in the cerebral cortex, cerebellum and spinal cord in the acute phase of EAE. IL-1β, IL-23 and IL-6 mRNA was upregulated in the spleen and lymph nodes in the preclinical phase of EAE, but was enhanced in the CNS in the acute phase, coinciding with the detection of IL-17-producing γδ T cells in the brain and spinal cord. This study demonstrates, therefore, that γδ T cells are an important source of IL-17, and that IL-1β and IL-23 might be important stimuli for early production of innate IL-17 during the development of EAE (Fig. 6.1).
The finding that γδ T cells rapidly express IL-17 *in vitro* and *in vivo* are consistent with a previous report that a high frequency of γδ T cells secrete IL-17 in the draining lymph nodes early after immunization with MOG and CFA, and preceding the generation of MOG-specific CD4 T cells (Jensen *et al.*, 2008). Taken together, this indicates that innate IL-17 production by γδ T cells may have a role early in disease pathogenesis, preceding the adaptive Th17 response. In support of this hypothesis, separate work from our laboratory has demonstrated that clinical symptoms of EAE are attenuated in mice with a defective TCR δ chain (Tcrδ−/−; Sutton *et al.*, 2009). Transfer of CD3+ T cells from naive WT mice into IL-1R1−/− mice, which are resistant to EAE, prior to immunization with MOG and CFA confers susceptibility to disease. However, depletion of γδ T cells from the transferred cells reduced the ability to confer susceptibility to EAE (Sutton *et al.*, 2009). In addition, γδ T cells were found to cooperate with antigen-specific CD4+ and CD8+ T cells in mediating the development of EAE. Furthermore, it was demonstrated that both CD4 T cells and DC express the IL-17R, and that IL-17 and IL-21 in the supernatants from activated γδ T cells could induce IL-17 production by CD4+ T cells (Sutton *et al.*, 2009). This was consistent with ex vivo findings that attenuation of EAE in the Tcrδ−/− mice was associated with decreased IL-17 mRNA expression in lymph nodes and reduced MOG-specific IL-17 secretion, which is primarily produced by CD4+ T cells. Together, these findings support the recent demonstration that IL-21 plays a critical role in IL-17 production by Th17 cells (Korn *et al.*, 2007a; Nurieva *et al.*, 2007; Zhou *et al.*, 2007). In addition, these findings reveal a positive feedback role of IL-17 production and indicate that early IL-17 production by γδ T cells may promote the induction or activation of Th17 cells. Furthermore, the expression of IL-17R on DC and their upregulation of IL-23, as well as IL-1β, IL-6, TGF-β and a number of Th17-related chemokines, in response to IL-17 reveals another potential mechanism whereby γδ T cells may indirectly promote development, expansion or recruitment of Th17 cells (Sutton *et al.*, 2009). Taken together with findings from the present study that γδ T cells infiltrate the CNS prior to the onset of disease and
a large percentage of these secrete IL-17, this data suggests that IL-17-producing γδ T cells have a pathogenic role early in EAE development, not only by substantially contributing to the pool of IL-17 in the target organ and thereby mediating further leukocyte recruitment, but possibly also by controlling the generation or re-activation of αβ Th17 cells.

This study demonstrates that γδ T cells are an important source of innate IL-17 which may be pathogenic in the neuroinflammation associated with MS and EAE by facilitating the activation and migration of myelin-reactive T cells into the CNS. Such effects may not require large numbers of cells and could be facilitated by the kind of numbers of γδ T cells found in the spinal cord and brain of mice with EAE by this study and others (Rajan et al., 1998; Smith & Barnum, 2008). Furthermore, γδ T cells represent a smaller and more discrete population uniquely defined by their restricted TCR usage and tissue specific localization. Thus, compared to the pan T cell therapeutics currently in use for the treatment of MS, targeting γδ T cells might be a more attractive approach, leaving the remainder of the immune system relatively intact (Wohler et al., 2010).

The phenotypic lineages of autoantigen-specific T cells that mediate autoimmune disorders are determined by the cytokine environment created by cells of the innate immune system, in particular DC. The cytokine IL-1, produced by a variety of innate immune cells, is involved in inflammatory pathology, but also plays a role in activating the adaptive immune response. Previous research from our laboratory demonstrated that IL-1RI<sup>-/-</sup> mice were resistant to EAE, and this was associated with defective development of antigen-specific Th17 cells (Sutton et al., 2006). Adoptive transfer of wild-type encephalitogenic T cells into IL-1RI<sup>-/-</sup> mice, however, could induce disease, indicating a critical role for IL-1 in the induction of antigen-specific Th17 cells, rather than in the effector stage of
disease. Elsewhere, it was shown that IL-1 synergizes with IL-6 and IL-23 to induce expression of the transcription factors IRF4 and RORγt, thereby driving Th17 cell polarization (Chung et al., 2009).

A number of studies in other models of autoimmunity also support a role for IL-1-induced IL-17 in disease pathogenesis. Iwakura and colleagues (2003) demonstrated that IL-1Ra-deficient mice, with unrestrained IL-1 signaling, exhibited vastly elevated expression of IL-17 (Nakae et al., 2003b). In addition, mutations in the \textit{Nlrp3} gene causing inflammasome hyperactivation and excess IL-1β production by innate immune cells resulted in augmented Th17 cell differentiation and consequent IL-17-dominant immunopathology (Meng et al., 2009). Data presented in the present study demonstrate that levels of IL-1β in the peripheral lymphoid organs correlate with the differentiation of naïve precursor T cells to Th17 cells, and that elevated expression of IL-1β in the CNS is associated with severity of clinical signs of disease in EAE. In addition, it is shown here that IL-1, together with IL-23, acts directly on γδ T cells to promote early IL-17 production (Fig. 6.1). These studies suggest that IL-1 is required for the induction or activation of IL-17-producing T cells, possibly via a cascade of IL-1-induced IL-6 and IL-23, and may therefore be a suitable target for pharmacological intervention in EAE and MS.

Several studies have demonstrated positive outcomes upon blockade of the IL-1 signaling pathway in EAE. This study examined the ability of Ac-YVAD-cmk, an irreversible inhibitor of caspase-1, to block the differentiation of Th17 cells and attenuate the clinical symptoms of EAE. Caspase-1 is required for processing of pro-IL-1β, prior to its secretion as an active protein. Previous studies have found that mice deficient in caspase-1, or when it was inhibited pharmacologically, were less susceptible to EAE and that this was associated with
impaired Th1 cell development and reduced perivascular infiltrates (Furlan et al., 1999). It was demonstrated in the present study that Mtb, present in the CFA used to induce EAE, is capable of activating the NLRP3 inflammasome, leading to the secretion of active IL-1β; a process dependent on proteolytic cleavage of pro-IL-1β by activated caspase-1 (Fig. 6.1). Furthermore, the ability of Mtb-stimulated DC to induce production of IL-17 in CD3+ T cells was dependent on active caspase-1, since this effect was lost when DC were pre-treated with YVAD-cmk.

Clinical signs of EAE were ameliorated in mice treated with the caspase-1 inhibitor throughout the course of disease. This correlated with a significant reduction in antigen-specific IL-17 and IFN-γ production by lymph node cells upon ex vivo restimulation, when examined at both 5 and 10 days after induction of EAE. Moreover, fewer pathogenic T cells accumulated in the CNS around the onset of disease. Inhibition of caspase-1 during the pathogenesis of EAE resulted in approximately 50% less CD4+ Th17 cells in both the brain and spinal cord at the onset of clinical symptoms, as well as a reduced frequency of Th1 cells, particularly in the spinal cord. The fact that Th17 cells were found at similar frequencies in the peripheral lymphoid organs of both treated and control mice might indicate a role for IL-1β in egress of differentiated T cells from the lymph nodes and/or their ability to home to target organs. This hypothesis is supported by recent findings highlighting the absolute requirement for IL-23R engagement on Th17 cells for their terminal differentiation and ability to leave the lymph nodes and home to target tissues, but not in the initial commitment to the lineage (Gyulveszi et al., 2009; McGeachy et al., 2009). IL-1 enhances production of IL-6 which is thought to upregulate IL-23R expression, possibly via IL-21 and RORγt (Simi et al., 2007; Zhou et al., 2007). In addition, it was demonstrated that IL-23 is upregulated in response to IL-1 and is over-expressed in the spleens of IL-1Ra-deficient mice (Cho et al., 2006). Therefore, decreased availability of IL-1β following caspase-1 inhibition may result in diminished IL-6 and IL-23
production in the peripheral lymphoid organs and reduced expression of IL-23R on T cells.

Consistent with the established role of IL-1 and IL-17 in PMN recruitment, the reduced accumulation of Th17 cells in the CNS correlated with lower frequency of PMN in the brains of mice treated with the caspase-1 inhibitor, in the chronic phase of disease. More strikingly, the frequency of macrophages in the brain was substantially lower in treated mice at this stage. This may reflect the reduced infiltration of Th1 cells to the CNS, and the lower expression of the inflammatory cytokines IFN-γ, TNF-α and IL-1β - molecules known to induce local chemokine expression required for the recruitment of macrophages to sites of inflammation - in these mice (Lin et al., 2009). These findings are consistent with those of a related study showing reduced numbers of infiltrating macrophages and CD3⁺ T cells following pharmacological blockade of caspase-1 (Furlan et al., 1999).

IL-1β is an important cytokine in autoimmune disorders with a neuroinflammatory component. Clearly it has pleiotropic effects, not least within the CNS and in regulating immune cell trafficking across the blood-brain barrier. Amongst its many functions, IL-1β is involved, either directly or indirectly, in the induction of various growth and trophic factors, inflammatory mediators, adhesion molecules, chemokines and other cytokines, as well as upregulating itself in an autocrine manner. Binding of IL-1 to endothelial IL-1R activates endothelial cells, facilitating recruitment of leukocytes into the brain (Ching et al., 2005; Hubbard and Rothlein, 2000). Furthermore, IL-1 is known to activate microglia and induce astrogliosis (Shaftel et al., 2007). Thus, IL-1 is an important molecule that may play a significant role during the inflammatory response within the CNS. The focus of the present investigation into the effects of caspase-1
inhibition, however, has been on the role IL-1 in the development and activation of Th17 cells, which appears to occur in the periphery during development of EAE. The results of this study reveal that the beneficial effect of caspase-1 inhibition may be partially mediated by reducing the capacity of auto-reactive Th17 cells to infiltrate the CNS, together with inhibiting IL-17 production upon reactivation. As such, the findings described here may point toward potential targets for drug intervention in MS.
Figure 6.1 Schematic illustrating the role of caspase-1 and IL-1β in Th17 and γδ T cell-mediated autoimmune inflammation. Pro-IL-1β is induced via activation of TLR and NLR signaling pathways and is processed by caspase-1 after activation of the NALP3 inflammasome by endogenous DAMPs or exogenous PAMPs. Activation of the TLR and NLR pathways also leads to the production of proinflammatory cytokines, including IL-23. IL-1 and IL-23 bind their respective receptors on Th17 and γδ T cells and induce production of IL-17, IL-21 and IL-22. IL-17 binds receptors expressed on a variety of cell types and promotes production of inflammatory cytokines, chemokines and matrix metalloproteases (MMPs) that mediate tissue-specific autoimmune inflammation. (Figure adapted from Mills and Dunne, Nature Medicine 15, 1363-1364, 2009).
Appendix
MATERIALS

A.1 Cell Culture Medium

Roswell Park Memorial Institute (RPMI)-1640 medium (BioSera) or Dulbecco's Modified Eagle Medium (DMEM; Sigma) were supplemented with 8% heat-inactivated (56°C for 30 min) foetal calf serum (FCS; BioSera), 100 mM L-Glutamine (Gibco), and 100 μg/ml penicillin/streptomycin (Gibco).

A.2 Krebs Solution

A.2.1 Krebs

3.975g NaCl
0.095g KCl
0.08g KH₂PO₄
0.135g MgSO₄
0.67g NaHCO₃
0.9g Glucose

Make up to 500 ml with dH₂O and pH to 7.3
Appendix

A.2.2 Calcium (stock)

2.94g CaCl₂ in 20 ml dH₂O  (Store at 4°C)

A.2.3 Krebs/Calcium

Added 200 μl CaCl₂ to 100 ml Krebs solution (1:500 dilution) just before use, for washing or short term storage. For long term storage, add 10% DMSO to Krebs/CaCl₂, snap freeze tissue in liquid N₂ and store at -80°C.

A.3 Ammonium Chloride Lysis Solution

0.87% (w/v) NH₄Cl dissolved in endotoxin-free dH₂O and filtered sterilized.  (Stored at 4°C)

A.4 Phosphate-Buffered Saline (PBS; 20 X)

320g Sodium chloride (NaCl, 1.4M)
46g Sodium hydrogen phosphate (Na₂HPO₄, 0.08M)
8g Potassium di-hydrogen phosphate (KH₂PO₄, 0.01M)
8g Potassium chloride (KCl, 0.03M)

Dissolved in 2 L of dH₂O and adjusted to pH 7.0
A.5  ELISA Reagents

A.5.1  ELISA Wash Buffer

500ml 20X PBS
9.5L dH2O

5 ml Tween 20

A.5.2  Phosphate Citrate Buffer

20.38g Citric acid (C₆H₈O₇)
73.8g di-Sodium hydrogen orthophosphate dodecahydrate
(Na₂HPO₄·12H₂O)

Made up to 2 L with dH₂O, pH 5.0
(Stored at 4°C)

A.5.3  ELISA Substrate Solution

1 o-Phenylenediamine dihydrochloride (OPD) tablet (Sigma)
25 ml Phosphate Citrate Buffer

7 μl H₂O₂
Appendix

A.5.4 ELISA Stopping Solution (1M H$_2$SO$_4$)

26.74 ml 18M H$_2$SO$_4$

473.26 ml dH$_2$O

A.6 FACS Buffer

2% FCS

0.1% Sodium azide (NaNO$_3$)

Made up in 1X PBS

A.7 Stock Isotonic Percoll

Percoll is made isotonic by diluting 9:1 with 10X PBS (Sigma). The different density gradients can be calculated using the equations described in the Percoll Manual (Sigma website). Briefly, for 1.088 g/ml: if you require ~100 ml,

\[
70 \times \frac{1.123 - 1.088}{1.088 - 1.0046} = 29.38
\]

Therefore, 70 ml SIP + 29.38 ml 1X PBS = 99.38 ml 1.088 g/ml Percoll.
A.8 Western Blot Reagents

A.8.1 Tris Gel Buffer: 1.5 M Tris-HCl pH 8.8

18.15 g Tris Base

75 ml dH₂O

Made up to 100 ml with dH₂O, pH to 8.8 with 1 M HCl.

(Stored at 4°C)

A.8.2 0.5 M Tris-HCl pH 6.8

6 g Tris Base

60 ml dH₂O

Made up to 100 ml with dH₂O, pH to 6.8 with 1 M HCl

(Stored at 4°C)

A.8.3 10% (w/v) Sodium Dodecyl Sulphate (SDS)

0.1 g SDS in 1 ml dH₂O

(Store at 4°C for up to 1 week)
Appendix

A.8.4 10% (w/v) Ammonium Persulphate (APS)

0.05g in 500 µl dH₂O

(Made up daily)

A.8.5 Protein Loading Buffer (5 X)

20 ml dH₂O

5 ml Tris-HCl, pH 6.8

4 ml Glycerol

8 ml 10% SDS

~ 100 mg bromophenol blue

A.8.6 Running Buffer (5 X)

15 g Tris Base

72 g Glycine

5 g SDS

1 L dH₂O

(Stored at 4°C)
A.8.7 Transfer Buffer (10 X)

30 g Tris Base

144 g Glycine

5 g SDS

1 L dH₂O

(Stored at 4°C)

Added before use: 20% MeOH

A.8.8 PBS-Tween

0.05% Tween 20 in 1 X PBS (A.4)
Appendix
## Table A.1 Polyacrylamide Gel Components

<table>
<thead>
<tr>
<th>Stocks</th>
<th>15% Separating gel</th>
<th>5% Stacking gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>dH$_2$O</td>
<td>1.7 ml</td>
<td>1.35 ml</td>
</tr>
<tr>
<td>30% (w/v) acrylamide/Bis solution</td>
<td>3.75 ml</td>
<td>335 μl</td>
</tr>
<tr>
<td>1 M Tris pH 6.8</td>
<td>---</td>
<td>250 μl</td>
</tr>
<tr>
<td>1.5 M Tris pH 8.8</td>
<td>1.9 ml</td>
<td>---</td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
<td>75 μl</td>
<td>20 μl</td>
</tr>
<tr>
<td>10% (w/v) APS*</td>
<td>75 μl</td>
<td>20 μl</td>
</tr>
<tr>
<td>TEMED*</td>
<td>3 μl</td>
<td>3 μl</td>
</tr>
</tbody>
</table>

* APS and TEMED were added to stock separating and stacking gel solutions just before pouring.
## Table A.2   Fluorochrome-labeled Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Fluorochrome</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>30-F11</td>
<td>PE-Cy7</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>CD3ε</td>
<td>145-2C11</td>
<td>APC</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td></td>
<td>145-2C11</td>
<td>FITC</td>
<td>eBioscience</td>
</tr>
<tr>
<td></td>
<td>17A2</td>
<td>APC-AlexaFlour 750</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD4</td>
<td>RM4-5</td>
<td>PE-Cy7</td>
<td>eBioscience</td>
</tr>
<tr>
<td></td>
<td>GK1.5</td>
<td>PE</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td></td>
<td>H129.19</td>
<td>FITC</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>CD8β</td>
<td>eBio H35-17.2</td>
<td>PE-Cy5</td>
<td>eBioscience</td>
</tr>
<tr>
<td>γδ</td>
<td>GL3</td>
<td>FITC</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td></td>
<td>GL3</td>
<td>APC</td>
<td>eBioscience</td>
</tr>
<tr>
<td>NK</td>
<td>DX5</td>
<td>FITC</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD27</td>
<td>LG.3A10</td>
<td>PE</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>Vγ4</td>
<td>UC3-10A6</td>
<td>FITC</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>Vγ5</td>
<td>536</td>
<td>FITC</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>V86.3/2</td>
<td>8F4H7B7</td>
<td>PE</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>Ly-6G</td>
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<td>PE</td>
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### Table A.3 Origin of Recombinant Cytokines used in Cell Culture or as ELISA Standards

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### Table A.4 Real Time RT-PCR Primers

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signaling, acting independently of the TH17 pathway, is critical for initiation of


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