Helena Sofia de Azevedo Domingues

Tese de Doutoramento

ROLE OF TH1 AND TH17 CD4$^+$ T CELL SUBSETS IN THE PATHOGENESIS OF EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS

PAPEL DOS LINFÓCITOS CD4$^+$ TH1 E TH17 NA PATOGÉNESE DA ENCEFALOMIELITE AUTOIMUNE EXPERIMENTAL

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Viagem

"Aparelhei o barco da ilusão
E reforcei a fé de marinheiro.
Era longe o meu sonho, e traiçoeiro
O mar...
(Só nos é concedida
Esta vida
Que temos;
E é nela que é preciso
Procurar
O velho paraíso
Que perdemos).

Prestes, larguei a vela
E disse adeus ao cais, à paz tolhida.
Desmedida,
A revolta imensidão
Transforma dia a dia a embarcação
Numa errante e alada sepultura...
Mas corto as ondas sem desanimar.
Em qualquer aventura,
O que importa é partir, não é chegar."

Miguel Torga - 1962

TABLE OF CONTENTS

ABBREVIATIONS

aa – Amino acid
ADCC – Antibody dependent complement cytolysis
Ahr – Aryl hydrocarbon receptor
APC – Allophycocyanin
APCs – Antigen presenting cells
BBB – Blood brain barrier
BCR – B cell receptor
CCL / CXCL – Chemokine ligands
CCR / CXCR – Chemokine receptor
CD – Cluster differentiation
CFA – Complete Freund´s adjuvant
DBD – DNA binding domain
CNS – Central nervous system
CSF – Cerebrospinal fluid
CTLA-4 – Cytotoxic T lymphocyte-associated antigen 4
DCs – Dendritic cells
EAE – Experimental autoimmune encephalomyelitis
EAU – Experimental autoimmune uveitis
ELISA – Enzyme linked immunosorbent assay
FACS – Fluorescence-activated cell sorting
FITC – Fluorescein isothiocyanate
Foxp3 – Forkhead box P3
GATA-3 – GATA binding protein 3
GFAP – Glial fibrillary acidic protein
GFP – Green fluorescent protein
GM-CSF - Granulocyte-macrophage colony-stimulating factor
HLA – human leukocyte antigen
IFN – Interferon
Igs – Immunoglobulins
IL – Interleukin
IRES – internal ribosome entry site
iTregs – Induced regulatory T cells
LBD – Ligand binding domain
MAG – Myelin-associated glycoprotein

MBP – Myelin basic protein

MCP-1 – Monocyte chemoattractant protein-1

MCS – Multiple cloning site

MHC – Major histocompatibility complex

MOG – Myelin oligodendrocyte glycoprotein

MRI – Magnetic resonance imaging

MS – Multiple clerosis

NK – Natural killer

NHR – Nuclear hormone receptor

NR2F6 – Nuclear orphan receptor subfamily 2, group F, member 6

PCR – polymerase chain reaction

PLP – Proteolipid protein

PNS – Peripheral nervous system

PPAR – Peroxisome proliferator-activated receptor

PE – Phycoerythrin

PercP - Peridinin chlorophyll protein

RAG – Recombination-activating gene

RAR – Nuclear retinoic acid receptor-alpha

RFP – Red fluorescent protein

ROR – Retinoic acid receptor-related orphan receptor

RORE – ROR responsive element

SPF – Specific pathogen-free

STAT – Signal transducer and activator of transcription

Tbet – T box expressed in T cells

TCR – T cell receptor

Tg – Transgenic

TGF – Transforming growth factor

Th – Helper T cell

TLRs – Toll-like receptors

TNF – Tumor necrosis factor

Tregs – Regulatory T cells

VCAM-1 – Vascular cell adhesion molecule-1

VLA4 – Very late antigen 4

PUBLICATIONS

During the development of the PhD thesis, I participated in other group projects, which led to significant publications in international peer-reviewed journals:

- Krishnamoorthy G, Saxena A, Mars LT, **Domingues HS**, Mentele R, Ben-Nun A, Lassmann H, Dornmair K, Kurschus FC, Liblau R and Wekerle H. (2009). Myelin-specific T cells also recognize neuronal autoantigen in a transgenic mouse model of multiple sclerosis. Nature Medicine 15: 626-632.

- Pöllinger B, Krishnamoorthy G, Berer K, Lassmann H, Bösl MR, Dunn M, **Domingues HS**, Holz A, Kurschus FC and Wekerle H (2009). Spontaneous relapsing-remitting EAE in the SJL/J mouse: MOG-reactive transgenic T cells recruit endogenous MOG-specific B cells. Journal of Experimental Medicine 206: 1303-1316.

In addition, the results described in chapters 3 and 4 of this thesis are being prepared for publication submission:

- **Domingues HS**, Mues M, Lassmann H, Krishnamoorthy G and Wekerle H. Differential pathogenic competence of Th1 and Th17 cells in experimental autoimmune encephalomyelitis.

THESIS PLANNING

The present thesis is organized in six Chapters, where Chapters 3 to 5 correspond to different scientific studies.

In Chapter 1, a general introduction to the different themes is presented. It includes a review of the literature focused in the field of the autoimmune disease of Multiple Sclerosis, the corresponding animal model, Experimental Autoimmune Encephalomyelitis and the cells and molecules that contribute to the pathogenesis.

In Chapter 2, all the material and methods used and developed in this thesis are described.

In Chapter 3, entitled "*IN VIVO* ANALYSIS OF TH1 AND TH17 CELLS IN ADOPTIVE TRANSFER EAE", the pathogenic roles of auto-reactive Th1 and Th7 CD4 subsets are discussed in the context of the autoimmune model of EAE.

In Chapter 4, entitled, "EVALUATION OF CYTOTOXIC POTENTIAL OF HELPER $CD4^+$ T CELL SUBSETS ON BRAIN RESIDENT ASTROCYTES", auto-reactive Th1 and Th17 cells are characterized in terms of cytotoxicity to astrocytes, important CNS resident cells.

In Chapter 5, entitled "MICROARRAY ANALYSIS IN T CELLS: Th1 *VS.* TH17", it is addressed the validation of one gene, Rev-Erbα, found to differently regulated by Th1 and Th17 cells in a microarray analysis.

In chapter 6, a general discussion and conclusion is established of the three described studies.

SUMMARY

Multiple sclerosis (MS) is an autoimmune disease that affects the central nervous system (CNS) and is characterized by inflammation, demyelination and axonal destruction, consequently leading to neuronal death. The processes that are involved in triggering the onset and driving inflammation, cellular composition and distribution of autoimmune lesions during the course of MS are not completely understood. Thus far, the animal model representing human MS, experimental autoimmune encephalomyelitis (EAE) has proved a central role for myelin-autoimmune $CD4^+$ helper T cells playing in the initiation of inflammatory demyelination of the CNS. In the past recent years, the scientific community has witnessed a real revolution regarding the knowledge of $CD4^+$ T cell biology and this had, of course, an important impact in the understanding of many immune-related diseases. Since the discovery in 2005 of the new Th17 cell subset, a big debate took place concerning the true pathogenic $CD4^+$ T cell subset in EAE and MS. Th1 cells, which produce IFNγ as their signature cytokine, have for many years been considered to be the pathogenic effector T cells in CNS autoimmunity. However, unexpected data obtained together with the discovery of Th17 cells have led the researchers to believe that Th17 cells but not Th1 cells were pathogenic in EAE. Nevertheless, evidence coming from spontaneous EAE models developed in this lab suggested that both $CD4^+$ T cell subsets, Th1 and Th17, may contribute to the pathogenesis, probably with different roles. Therefore, the main aim of this thesis was to address the roles of Th1 and Th17 cell subsets in the context of autoimmune disease of the CNS.

Initially, by establishing an adoptive transfer EAE model, the individual pathogenicity of myelin-specific Th1 and Th17 cells was evaluated *in vivo* and it was observed that both T cell lineages were able to induce EAE, but with different clinical features. While Th1 cells induced only classical paralytic EAE, many animals receiving Th17 cells developed an ataxic, non-classical EAE phenotype. Interestingly, when Th1 and Th17 cells were co-transferred induced more severe EAE with earlier onset, indicating that these two $CD4^+$ subsets are both pathogenic and synergize to trigger autoimmune inflammation in the

CNS. Finally, it was found that transferred Th17 cells can convert to a Th1 phenotype in the host, suggesting plasticity in the Th17 cell subset and emphasizing a pathogenic role for Th1 cells.

Next, taking into account our data and earlier findings previous to the Th17 cell discovery that demonstrated a cytotoxic capacity of auto-antigenic T cells over astrocytes, important CNS resident glial cells, we asked which of the $CD4^+$ T cell subset was cytotoxic to astrocytes. By establishing an *in vitro* co-culture of myelin-specific Th1 and Th17 cells with GFP positive-astrocytes, it was possible to pursue cytotoxicity by fluorescent time-lapse microscopy. It was found that Th1 but not Th17 cells were cytotoxic to astrocytes, further emphasizing the pathogenic role of Th1 cells.

Finally, in order to identify molecules that are differently regulated in Th1 and Th17 cells and to understand which different roles these cells might play in EAE, a transcriptome analysis by microarrays of both populations was performed. We found the nuclear receptor Rev-Erbα to be expressed in Th17 but not in Th1 cells. A pathway analysis revealed a relationship of Rev-Erbα with RORα, an important transcription factor for Th17 differentiation, but no definite role for this molecule in regulating Th17 differentiation could be established.

In conclusion, this thesis demonstrates, contrary to initial evidence, that both myelin-specific Th1 and Th17 $CD4^+$ T cell subsets are able to induce pathogenicity in EAE, though with different capabilities to mediate disease. Also, Th1 cells are true cytotoxic effector cells destroying astrocytes, important neuronal buffer cells. A new gene was also discovered, Rev-Erbα, which is differently regulated by Th1 and Th17 cells. Though no impact of Rev-Erbα in Th17 differentiation could be determined, a possible role in Th17 biology will need to be further addressed.

RESUMO

A Esclerose Múltipla (EM) é uma doença autoimune que afecta o Sistema Nervoso Central (SNC) e é caracterizada pela presença de inflamação, desmielinização e destruição axonal, levando, consequentemente, à morte neuronal. Os processos que despoletam e conduzem a resposta inflamatória, composição celular no SNC e distribuição das lesões autoimunes no curso da doença não são completamente conhecidos. Até ao momento, o modelo animal que representa a EM, a Encefalomielite Autoimune Experimental (EAE), tem sido essencial na identificação dos linfócitos $CD4^+$ autoimunes, específicos para a mielina, como células centrais na iniciação das desmielinização inflamatória do SNC. Nos últimos anos, a comunidade científica tem assistido a uma revolução no conhecimento acerca da biologia das células T $CD4^+$ ajudantes. Consequentemente, este facto teve um impacto extremamente importante na compreensão de muitas doenças relacionadas com o sistema imunitário. Desde a descoberta em 2005 do novo subtipo de células $CD4^+$ ajudantes, as células Th17, uma grande controvérsia cresceu na discussão sobre o verdadeiro subtipo de células $CD4^+$ patogénico na EAE e EM. As células Th1, que produzem IFNγ como principal citocina, foram durante muitos anos consideradas as células $CD4^+$ patogénicas na EM e EAE. No entanto, evidências científicas paradoxais obtidas em paralelo com a descoberta das células Th17 levaram os investigadores a acreditar que, afinal, as células Th17, e não as Th1, eram as patogénicas na EAE. Em todo o caso, observações feitas nos modelos espontâneos para a EAE desenvolvidos no nosso laboratório sugeriram que, talvez, ambos os subtipos sejam importantes e contribuam para a patogénese, provavelmente com diferentes papéis. Assim, o principal objectivo desta tese consistiu na elucidação dos papéis individuais das células Th1 e Th17 no contexto da autoimunidade do SNC.

Inicialmente, ao estabelecer um modelo de EAE por transferência adoptiva, avaliámos *in vivo* a patogenicidade individual das células Th1 e Th17 específicas para a mielina e descobrimos que ambos os subtipos são capazes de induzir EAE, embora com diferentes características clínicas. Ao passo que as células Th1 induziram apenas EAE clássica, muitos animais transferidos com células Th17 desenvolveram um fenótipo de

EAE não-clássico, atáxico. Curiosamente, quando as células Th1 e Th17 foram transferidas em conjunto induziram EAE mais severa e com início de doença antecipado, indicando que estes dois subtipos de células $CD4^+$ são ambos patogénicos e cooperam sinergicamente para despoletar inflamação autoimune no SNC. Finalmente, observámos que as células Th17 transferidas se converteram para o fenótipo Th1 no animal recipiente, sugerindo plasticidade das células Th17.

Em seguida, tendo em conta os nossos resultados e observações anteriores à descoberta das células Th17 que demonstraram uma capacidade citotóxica de células T ajudantes e auto antigénicas, mediadoras de EAE, sobre astrócitos, questionou-se qual subtipo seria o responsável por tal citotoxicidade. Após desenvolvimento de um sistema de co-cultura de astrócitos GFP positivos com células Th1 e Th17 específicas para a mielina, foi possível avaliar a citotoxicidade por microscopia de fluorescência em tempo real. Descobrimos que são as células Th1, e não as Th17, citotóxicas para os astrócitos, enfatizando assim um papel patogénico das células Th1.

Finalmente, com o objectivo de identificar moléculas reguladas de modo diferente pelas células Th1 e Th17 e que, de certo modo, possam explicar os diferentes papéis que estes subtipos possam ter na EAE, foi realizado uma análise do transcriptoma das duas populações por *microarrays*. O nosso estudo focou-se no receptor nuclear Rev-Erbα, expresso nas células Th17 mas não nas Th1. Uma análise das vias celulares e relações moleculares revelou uma associação do Rev-Erbα com o RORα, um importante factor de transcrição para a diferenciação das células Th17. No entanto, não foi possível definir um papel para este receptor na diferenciação das células Th17.

Em suma, nesta tese de doutoramento foi possível demonstrar que, ao contrário das evidências iniciais, no nosso modelo de EAE ambos os subtipos de células T $CD4^+$ específicos para a mielina, Th1 e Th17, são capazes de induzir patogenicidade na EAE. Ainda, mostrou-se que as células Th1, mas não as Th17, são citotóxicas para os astrócitos, importantes células protectoras dos neurónios no SNC. Descobrimos também que o receptor Rev-Erbα é regulado de modo diferente pelas células Th1 e Th17. Apesar de não termos encontrado um papel relevante do Rev-Erbα na diferenciação das células Th17, é possivel que exerça uma função importante na biologia das mesmas, que terá de ser explorada no futuro.

OBJECTIVES

The main aim of this thesis was to address the individual roles of myelin-specific Th1 and Th17 cell subsets in the context of CNS autoimmunity. Specific aims were established and are interconnected. The respective results were divided in chapters 3 to 5:

- *In vivo* evaluation of the pathogenic potential and the individual functions of myelin-specific Th1 and Th17 $CD4^+$ T cell subsets in the adoptive transfer EAE model.
- *In vitro* assessment of Th1 and Th17 cell cytotoxicity to astrocytes.
- Identification and validation of molecules differently regulated in Th1 and Th17 cells by transcriptome microarray analysis.

CHAPTER 1 INTRODUCTION

The field of Neuroimmunology has developed enormously in the last decades and now we finally start to better understand the complexity of inflammation in the nervous system. For a long time, the CNS was considered an immuneprivileged site. However, experimental evidence has shown that this is not the case and that the CNS is constantly being surveyed by the immune system both in health and disease.

1.1 - MULTIPLE SCLEROSIS (MS): THE STRUGGLE BETWEEN THE IMMUNE AND THE NERVOUS SYSTEM

MS is a chronic autoimmune demyelinating disease of the CNS, leading to disability at the level of physical and cognitive functions (Sospedra and Martin, 2005). It affects primarily young people, over 500,000 in Europe and 2 million worldwide and is more frequent in females than in males (Flachenecker and Stuke, 2008). The majority of patients develop MS as a relapsing-remitting (RR) clinical course, with alternated periods of acute disease and recovery. Relapses can last for days, weeks or months with recovery varying between slow and gradual or seemingly instantaneous. Over time, some people in this category develop secondary-progressive MS, which does carry a risk of disability. In a minority of patients, however, the disease develops gradual but progressively from the beggining, designated primary-progressive MS.

MS symptoms are determined by the particular CNS areas that have been demyelinated and how much neural tissue has been damaged. There is no universal pattern for the course of MS and every patient has a different, unique set of symptoms. These may be single or multiple and may range from mild to severe in intensity and from short to long in duration. MS symptoms may include motor deficits such as visual disturbances, muscle weakness with or without difficulties with coordination and balance, muscle spasms, fatigue, loss of sensation, speech impediment, tremors, dizziness, mental changes such as decreased concentration, attention deficits, some degree of memory loss, inability to perform sequential tasks, or impairment in judgment.

By mechanisms that are still not clearly understood, in MS and other autoimmune diseases, such as Type 1 Diabetes, Crohn´s disease or Rheumatoid Arthritis, the immune system recognizes self-antigens and mounts an inflammatory response in a tissue-specific manner, that is, in the organ where the autoantigen is expressed. In the case of MS, the immune system reacts components of the myelin sheath, produced by the oligodendrocytes, leading to axonal damage and, ultimately, to neuronal death.

Though the etiology remains complex, it is generally believed that there is an environmental and genetic contribution to the pathogenesis of MS. In the first case, the geographical difference is a susceptibility factor, with Northern European and American countries being the more affected. In addition, it was proposed that viral infections, like the Epstein-Barr virus, could trigger MS through the molecular mimicry mechanism but so far there are no definite evidences. In the second case, genetic population studies have shown that MS prevalence is higher in people related to MS patients. Also, some susceptibility genes have been correlated with MS. The main genetic factor is the major histocompatibility complex class II (HLA), which includes the HLA-DR and the HLA-DQ genes, in particular the HLA-DR15 haplotype in Caucasians encoding the HLA-DR alleles *DRB1*1501* and *DRB5*0101*. HLA-DR15 has been associated with the transforming growth factor beta (TGFβ) family members, the cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), and the tumor necrosis factor (TNF) cluster, among others. IL-7 and IL-2 cytokines have also been identified as conferring susceptibility to the disease (Sospedra and Martin, 2005;Weiner, 2009). These facts have reinforced the importance of the role of the immune cells in the pathogenesis of MS, in particular the $CD4^+$ T lymphocytes which are activated by the antigen presenting cells (APCs) in a HLA class II-dependent manner. In a healthy individual, some T cells can be found in intact CNS but only those capable of reacting with a CNS antigen, by means of tolerance break, remain and trigger an autoimmune inflammatory response (Hickey et al., 1991).

1.2 - THE ANIMAL MODEL FOR MS: EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS (EAE)

Due to the inaccessibility to patient's brain, the study of MS in humans is restricted to the use of peripheral blood, cerebrospinal fluid (CSF) and post-mortem CNS tissue samples. Clinically, magnetic resonance imaging (MRI) is the non-invasive technique of choice that provides more specific information on the disease process in MS patients. Nevertheless, a profound understanding in human diseases requires the support of an animal model, extensively used in biomedical research. As biggest advantage, animal models provide the possibility of genetic manipulation, most developed in mice. There are transgenic and knockout strains available and the possibility to transfer manipulated cells to recipient hosts, as well. The use of molecular, imaging and immunological tools in all these possible models promotes the knowledge of immunogenetic, histopathological and therapeutic studies and builds a bridge for possible translations to the respective human case.

EAE, the primary model for MS, was first described by Thomas Rivers in 1933 (Rivers et al., 1933). These authors were able to induce encephalomyelitis in monkeys by injecting brain extracts from rabbits. Nowadays, there are several models of EAE depending on the animal strain (mainly rodents) and antigen used for induction and, virtually, it is possible to mimic almost all clinical features of MS (**Table 1.1**).

Each EAE model can differ in sensitivity, clinical course and pathology, determined by the strain and specie used, age, sex, antigen, dose and route of administration (Krishnamoorthy and Wekerle, 2009). Clinically, the classical EAE actively induced by immunization or adoptive cell transfer results in a characteristic ascending paralysis that starts with loss of tail tonus and then is followed, from tail to head, by limb weakness and paralysis. It is associated with a higher inflammation in the spinal cord. In addition, non-classical or atypical EAE phenotypes have been described in some models, characterized by ataxia, inability to walk on a straight line and sometimes continuous rolling unless supported. This phenotype is associated more with brain than spinal cord inflammation (Wensky et al., 2005).

The importance of the EAE model is emphasized by the existence of several models that display different clinical features overlapping with the ones of MS. The use of the EAE model promises, therefore, a deeper understanding of the pathogenic mechanisms of action so improvement of old therapies and/or development of new ones targeting the same pathogenic mechanisms can be established.

Table 1.1 – Clinical Phenotypes of EAE models [adapted from (Weiner, 2009)]

Animal strain	Induction	Disease Type
C57BL/6	MOG	Relapsing-remitting
SJL/J	PLP	Relapsing-remitting
NOD	MOG	Chronic progressive
MOG TCR Tg X MOG BCR Tg	MOG	Devic's disease (Krishnamoorthy et al., 2006)
MOG TCR Tg (SJL/J)	MOG	Relapsing-remitting (Pollinger et al., 2009)
C57BL/6	Monocytes	Progressive
SJL/J	Theiler's virus	Relapsing/progressive
Lewis rat	MBP	Acute relapse ADEM

1.2.1 INDUCED EAE MODELS

By far, the most common EAE method to induce EAE is by active immunization, "active EAE". The animals are immunized subcutaneously with encephalitogenic myelin proteins or peptides in the presence of complete Freund's adjuvant (CFA), composed of mineral oil and heat-killed *Mycobacterium tuberculosis* strain H37RA, to boost the innate immune response and facilitate the antigen's gradual and constant release into the lymph nodes; and peritoneal injection of pertussis toxin to help open the BBB and promote access of myelin specific T cells to CNS. The most common myelin antigens used comprise myelin oligodendrocyte glycoprotein (MOG), proteolipid protein (PLP), myelin basic protein (MBP) and myelin-associated glycoprotein (MAG). The drawback of this model concerns the use of adjuvant and pertussis toxin that may distort the global immune response and, therefore, lead to erroneous evaluations.

EAE can also be induced by adoptive transfer of auto-reactive CD4$^+$ T lymphocytes injected intravenously into susceptible recipients, denominated as "passive EAE", the method of choice for this thesis. It is possible to induce adoptive transfer EAE by either transfer of *in vitro* activated myelin-specific CD4$^+$ T cells freshly isolated from lymphoid organs (spleen or lymph nodes) of immunized wild-type animals, or from myelin-specific T cell receptor (TCR) transgenic mice. This model allows, in a more transparent way, the evaluation of the role of the transferred cells in the absence of adjuvant. EAE develops faster and more homogeneously, permitting the study of the effector phase independently from the induction phase (Wekerle et al., 1994).

1.2.2 SPONTANEOUS EAE MODELS

The induced EAE models, however, do not escape from the artificial nature of the method and might not reflect all the initial pathogenic mechanisms of MS. In the past years, several spontaneous EAE models have been developed, to which our group has contributed significantly (Krishnamoorthy et al., 2006;Pollinger et al., 2009). These models offer special advantages over induced EAE models due to the more "natural" disease induction and the closer resemblance to the human disease. The most interesting examples include the TCR transgenic animal model. These models express a transgenic TCR specifically recognizing a brain auto-antigen, such as MOG, in the context of the MHC class II molecule (Bettelli et al., 2003). Krishnamoorthy and colleagues in parallel with Betelli and colleagues described a model of T and B cell cooperation in the C57BL/6 background by crossing MOG-TCR and MOG-BCR (B cell receptor)transgenic mice (which carries a rearranged heavy chain from a MOG-specific antibody (Litzenburger et al., 1998)), resulting in spontaneous EAE with close similarities to the MS-related Neuromyelitis Optica (NMO) disease (Krishnamoorthy et al., 2006;Bettelli et al., 2006a). Further, Pollinger and colleagues described a spontaneous EAE mouse model in the SJL/J background that bears a MOG-specific TCR and clinically develops relapsing-remitting phenotype. In addition, this model describes

the recruitment of the endogenous B cell repertoire that cooperates with the auto-reactive T cell population to promote EAE pathogenesis (Pollinger et al., 2009).

1.3- THE IMMUNE PLAYERS IN MS AND EAE

Early studies in MS identified the immune component of this disease correlating the cellular composition of brain lesions and CSF infiltrating cells with data from EAE models. These studies have substantiated the contribution of the $CD4^+$ T cells (McFarland and Martin, 2007;Compston and Coles, 2002). For a long time, MS was considered a $CD4^+$ T helper type 1 (Th1) cells-mediated disease and the involvement of these cells is consistent with the HLA association to genetic susceptibility. But today we know that the pathogenic mechanisms are by far more complex and almost all immune cell types have at least some contribution, both from the acquired and innate immune system.

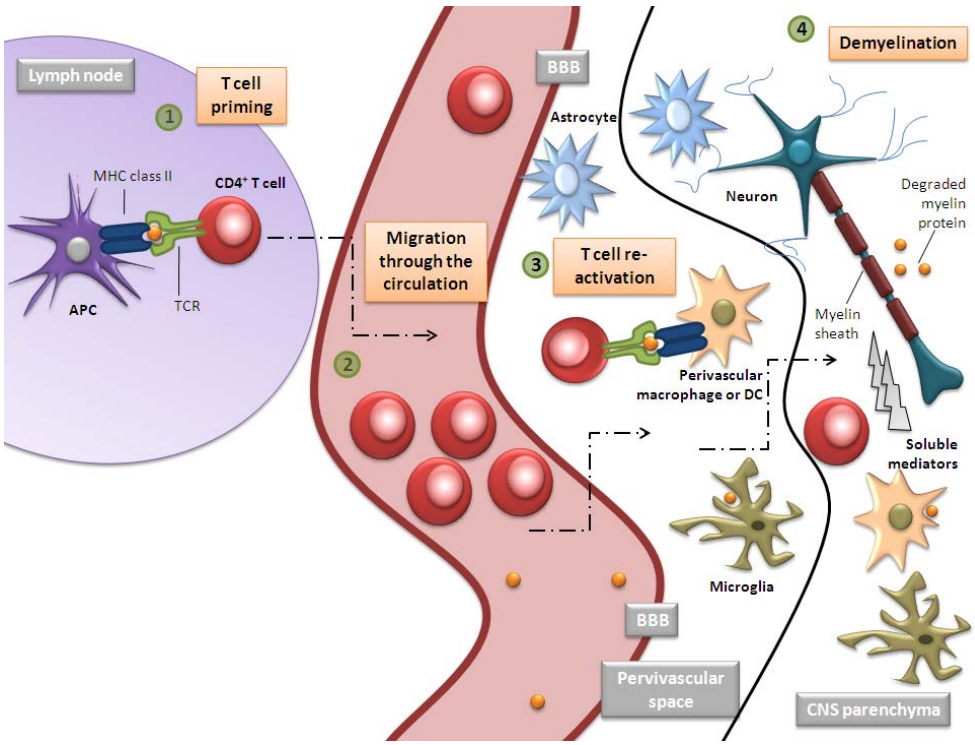


Figure 1.1 – Myelin-specific $CD4^+$ T cells activation in the periphery and CNS [adapted from (Goverman, 2009)].

After breakage of immune cell tolerance, the immune system attack to the CNS can be divided into several stages. First, in order to be able to migrate to the CNS, the auto-reactive T lymphocytes must be activated in peripheral lymphoid organs, such as lymph nodes and the spleen, by antigen-presenting cells (APCs) which include B cells, macrophages and dendritic cells (DCs). Then, these activated cells cross the blood brain barrier (BBB) and infiltrate the CNS perivascular space and parenchyma. Within the CNS, it is believed that the effector T helper cells subsets are re-activated both by CNS antigen presenting resident cells, such as astrocytes and microglia, and infiltrating macrophages and DCs, where they release inflammatory cytokines and attract further cells from the immune system (Dittel, 2008) **(Figure 1.1)**.

A second invasion wave follows, which seems to be independent of antigen recognition or activation status, and inflammation develops contributing to myelin loss and, consequently, axonal degeneration and neuronal death.

1.3.1 CD4$^+$ HELPER T CELLS

Several data clearly demonstrate the importance of CD4$^+$ T cells in EAE (Linington et al., 1984;Schluesener and Wekerle, 1985). The discovery that CNS antigen-specific T cells cultured and activated *in vitro* could induce EAE after transfer to naïve recipients (Ben-Nun et al., 1981) also proved the need for T cell activation, a requirement for migration across the BBB to reach the CNS (Flugel et al., 2001). In fact, one pioneering study showed that mice transgenic in their TCR specific for MBP, found to be pathogenic in EAE experiments, developed spontaneous EAE if kept in conventional, not specific pathogen-free (SPF) conditions (Goverman et al., 1993). This showed that the mere presence of CNS antigen-specific T cells in the immune system is not sufficient to trigger disease but that cells need to be activated first.

Though it is difficult to translate CD4$^+$ T cell-mediated EAE to all MS clinical aspects, CD4$^+$ T cells are generally believed to be central in pathogenesis (Kitze et al., 1988;Pette et al., 1990b;Pette et al., 1990a), and this view is supported by the genetic association

studies between MS susceptibility and the MHC class II region. The development of "humanized mice", in which the animals express transgenes derived from MS patients, has helped better understanding of the effects of individual genetic variations on disease development. For example, mice engineered to express both the HLA-DRB1*1501 allele and a human TCR that recognizes MBP peptide 85-99 in the context of HLA-DR2b were found to spontaneously develop MS-like disease (Madsen et al., 1999).

The importance of $CD4^+$ helper T cells in EAE is the main topic of this thesis and will be addressed in more detail in further sections.

1.3.2 $CD4^+$ REGULATORY T CELLS

Regulatory $CD4^+$ T cells (Tregs) were described for the first time some years ago and were defined as an independent T cell population, derived from a naïve precursor and capable of suppressing the function of other T cells. These cells express the CD25 marker, the alpha chain of the IL-2 receptor as found later, and their development and function is dependent on the transcription factor forkhead box P3 (Foxp3). $Foxp3^+$ Tregs derived from the thymus are denominated as naturally occurring Tregs (nTregs). Alternatively, they can be differentiated from naïve $CD4^+$ T cell precursors, the induced Tregs (iTregs). These cells produce high amounts of TGFβ, an anti-inflammatory cytokine. Mice mutant in Foxp3, termed scurfy mice, develop lethal autoimmune syndrome, resultant from deficiency of CD4+CD25+ regulatory T cells (Fontenot et al., 2003). $Foxp3^+$ Tregs have been shown to play a role in the resolution of inflammatory immune responses, and therefore have been suggested as targets of a potential therapeutic target in the treatment of CNS autoimmune disease (Stephens et al., 2009). Indeed, depletion of $CD4^+$ $CD25^+$ Tregs inhibited EAE recovery and adoptive transfer of these cells provided protection (McGeachy et al., 2005). However, the mechanisms of action of Tregs in autoimmunity are not well understood and there are EAE studies reporting the failure of Tregs to control the auto-antigenic inflammatory responses, which can doubts of the possible inefficacy of treating autoimmune diseases with Tregs (Korn et al., 2007b). One possible explanation might be the close relationship between

the differentiation of induced Tregs and Th17 cells, highly inflammatory CD4$^+$ T cells, where transforming growth-factor beta (TGFβ) plays a common and crucial role.

1.3.3 CD8$^+$ CYTOTOXIC T CELLS

The contribution of auto-reactive CD8$^+$ T cells to the MS pathogenesis is still ambiguous but has been addressed in closer detail in the last years. Arguments for an important role of CD8$^+$ T cells in MS has emerged from therapeutic MS studies, where CD4$^+$ depletion did not improve the patients health status. On the contrary, treatment with alemtuzumab, a monoclonal antibody that targets the CD52 antigen, present in more than 95% of the immune cells including CD4$^+$ and CD8$^+$ T cells, B cells and macrophages, is currently tested in clinical trials for MS and appears to be beneficial (Rommer et al., 2008). Additionally, high frequency of CD8$^+$ populations recognizing myelin proteins can be found in MS lesions (Crawford et al., 2004). Clonal expansion of CD8$^+$ T cells in lesions from brain tissue of MS patients was also reported (Babbe et al., 2000). Recent genetic studies have also related MS susceptibility with the HLA class I alleles, and "humanized" mouse models have supported this idea. For example, mice expressing human HLA class I allele HLA-A3 combined with PLP-specific T cells, develop mild early disease similar with clinical manifestations in MS patients. However, the disease progressed to a more severe state with the contribution of MHC class II-restricted CD4$^+$ T cells, suggesting an interaction between these two populations (Friese et al., 2008). EAE studies also suggest the existence of both regulatory and pathogenic CD8$^+$ T cell populations. While CD8$^+$ T cell deficiency resulted in less mortality but more relapses, adoptive transfer of MBP-specific CD8$^+$ T cells induced a demyelinating disease (Goverman, 2009).

1.3.4 B CELLS

B cells belong to the adaptive immune system through the presence of unique antigen-specific B cell receptors (BCR), membrane-bound immunoglobulins. When B cells are activated, they can differentiate into memory B cells or plasma B cells and the latter produce and release large amounts of antigen-specific antibodies, as well as a panel of cytokines depending on the inflammatory milieu. Another role of B cells concerns antigen presentation as they also express the MHC class II molecule on their surface. This is of particular importance in the B and T cell crosstalk which, as mentioned above, can contribute to the EAE pathogenesis. EAE studies showed that injection of MOG-specific autoantibodies amplified T cell attack by increasing the severity and duration of clinical signs and inducing large scale demyelination (Linington et al., 1988). The presence of elevated amounts of immunoglobulins (Igs) in the CSF of MS patients was demonstrated many years ago (Kabat et al., 1950) and since then, association between the presence of CSF Igs and the MS clinical worsening suggested a role of B cells in the pathogenesis. Clonally expanded memory B cells have also been found in MS CSF and lesions. The increased Igs discovered in the CSF but not in the serum of MS patients suggested antibody production by local B cells. In fact, a recent report compared the transcriptomes and proteomes from CSF of MS patients. The data obtained demonstrated a strong overlap, indicating that CSF B cells produce the oligoclonal Ig bands (Obermeier et al., 2008). B cells are not able to cross intact BBB but, once disrupted B cells along with antibodies and complement molecules enter the CNS. The auto-reactive antibodies can cause demyelination by opsonization of myelin for phagocytosis, or via complement activation leading to antibody dependent complement cytolysis (ADCC), which can be found in MS lesions and EAE CNS (Sospedra and Martin, 2005).

1.3.5 INNATE IMMUNE CELLS

Innate immune responses comprise variable functions that range from non-specific recognition of non-self molecular structures by Toll-like receptors (TLRs) and release of molecules such as nitric oxide, cytokines and chemokines, to the activation of antigen-specific adaptive immune responses. The innate immune cells include DCs,

macrophages, mast cells, NK cells and humoral factors like complement. In the CNS resides an innate immune-derived population, the microglia. Although the main role of innate immune cells is to distinguish self from non-self, activate specific adaptive immune responses and maintain homeostasis, they can also contribute to destructive autoimmunity.

Innate immune cells as APCs are extremely important in the activation of CD4$^+$ T cells, both in periphery and in the CNS. Macrophages, DCs and microglia express constitutively MHC class II molecules and thus are able to present antigen both to naïve and effector T cells and contribute, therefore, to antigenic spread. For example, DCs were demonstrated to bias effector T cell to Th17 differentiation in the CNS from a relapsing EAE model (Bailey et al., 2007). Macrophages, on their side, are also activated in EAE, release inflammatory cytokines and promote T helper responses, further contributing to pathogenesis (Tran et al., 1998;Martiney et al., 1998). However, type II macrophages, recognized as anti-inflammatory cells, were very recently described as suppressors of EAE (Tierney et al., 2009). Macrophages also act as professional phagocytic cells, a key feature in the immune response against pathogens but probably also important in the removal of myelin debris in CNS damaged areas [though it is known that this process is more efficient in the peripheral nervous system (PNS) through optimal crosstalk with myelin-producing Schwann cells]. Removal of myelin debris can enhance re-myelination but also promote antigen presentation and re-activation of encephalitogenic T cells.

A less explored field concerns the status of activation and maturation of the innate immune system during different phases of MS. For example, cytokines such as IL-12, IL-18 and TNFα are found in different levels in blood cell samples from relapsing-remitting and chronic progressive MS patients and are mostly produced by dendritic cells (Weiner, 2008). This emphasizes the importance of innate immune cells in different phases of CNS inflammation, whose markers might help to predict the disease course.

Microglial cells, on their side, reside in the CNS but are of hematopoietic origin. They respond to TLR ligands in pathogen infections, to neuronal dysfunction, pro-

inflammatory cytokines and cellular debris after injury or necrosis. *In vitro* studies have shown that microglia are able to secrete a panel of inflammatory cytokines and chemokines such as IL-6, macrophage inflammatory protein-2 (MIP-2), nitric oxide, adhesion molecules, and neurotrophic factors in response to IL-17. Additionally, these cells can also be an innate source of IL-17 in response to IL-23 or IL-1β, contributing therefore to inflammation in autoimmune diseases such as MS (Kawanokuchi et al., 2008).

1.4- THE PARADIGMS OF HELPER T CELL SUBSETS: MORE COMPLEX THAN INITIALLY THOUGHT

There are several subsets of effector helper $CD4^+$ T cells. Depending on the microenvironment and cytokines released by the APCs, a naïve T cell responds to presentation of the cognate antigen, in the context of the major histocompatibility complex (MHC) class II, by differentiating to different helper T cell subsets. In 1986, helper T cells were classified for the first time into two subsets, type 1 helper T cells (Th1) and type 2 helper T cells (Th2), based in their cytokine production (Mosmann et al., 1986). Th1 cells produce high amounts of interferon gamma (IFNγ) whereas Th2 cells produce interleukin-4 (IL-4), IL-5 and IL-13. The Th1/Th2 paradigm associated these T cell populations with specific immune responses. While Th1 cells are specialized in macrophage activation and defense against intracellular pathogens, including viruses and some bacteria such as mycobateria, Th2 cells, in contrast, are particularly important for defense against large extracellular pathogens such as helminths, for the induction of immunoglobulin E (IgE) by B cells, mast cells activation and eosinophils mobilization. Allergic diseases are characterized mainly by a Th2 response, whilst autoimmune diseases are driven by Th1 cells. However, this paradigm soon proved to be too simplistic as many observations from clinical diseases could not be explained by this simple categorization. More recently, several subsets of Tregs capable of controlling effector T cells responses were described: the thymus derived nTregs, and the iTregs, Tr1 and Th3 regulatory T cells derived from peripheral helper T cell precursors. Furthermore, in 2005 Dan Cua and colleagues described a new pathogenic $CD4^+$ T cell population that is driven by IL-23, produces IL-17, IL-17F, IL-6, and tumor

necrosis factor (TNF), and induces autoimmune inflammation (Langrish et al., 2005). This new CD4$^+$ T cell population was later termed Th17 and identified as a distinct lineage from Th1 and Th2 cells (Harrington et al., 2005;Park et al., 2005). The most recent member of the helper T cell population is the IL-9-producing subset, Th9, (Veldhoen et al., 2008;Dardalhon et al., 2008) but this subject is still not thoroughly explored and needs further maturation as there are indications of IL-9 production also by classical Th2, Th17 and Foxp3$^+$ regulatory T cells (Liu et al., 2006;Nowak et al., 2009;Forbes et al., 2008), suggesting that IL-9 plays a role both in inflammation and immunosuppression.

1.4.1 CD4$^+$ HELPER T CELL DIFFERENTIATION: TH1, TH2, TH17 AND TREGS

A naïve CD4$^+$ T cell can be differentiated into a helper T cell subset - Th1, Th2, Th17 and Treg - that is determined by the cytokine milieu, released by innate immune cells (**Figure 1.2**). IL-12 released by activated APCs is the driving cytokine for the development of Th1 cells. With IL-18 synergizes for the production of IFNγ, the Th1 main cytokine produced. Also, IL-27 potentiates Th1 development by activating the transcription factor signal transducer and activator of transcription 1 (STAT1), which in turn induces T box expressed in T cells (T-bet), a key transcription factor in Th1 differentiation. STAT4, T-bet, HLX and RUNX3 activate IFNγ expression, which reinforces Th1 cell commitment through the activation of STAT1 in a positive-feedback loop.

Th2 cells are driven by IL-4 through the activation of STAT6 and the transcription factor GATA binding protein 3 (GATA-3). STAT6 and GATA-3 together activate the transcription of IL-4, IL-5, IL-10 and IL-13. IFNγ and IL-4, produced by Th1 and Th2 cells, reciprocally suppress the expansion of Th2 and Th1 populations.

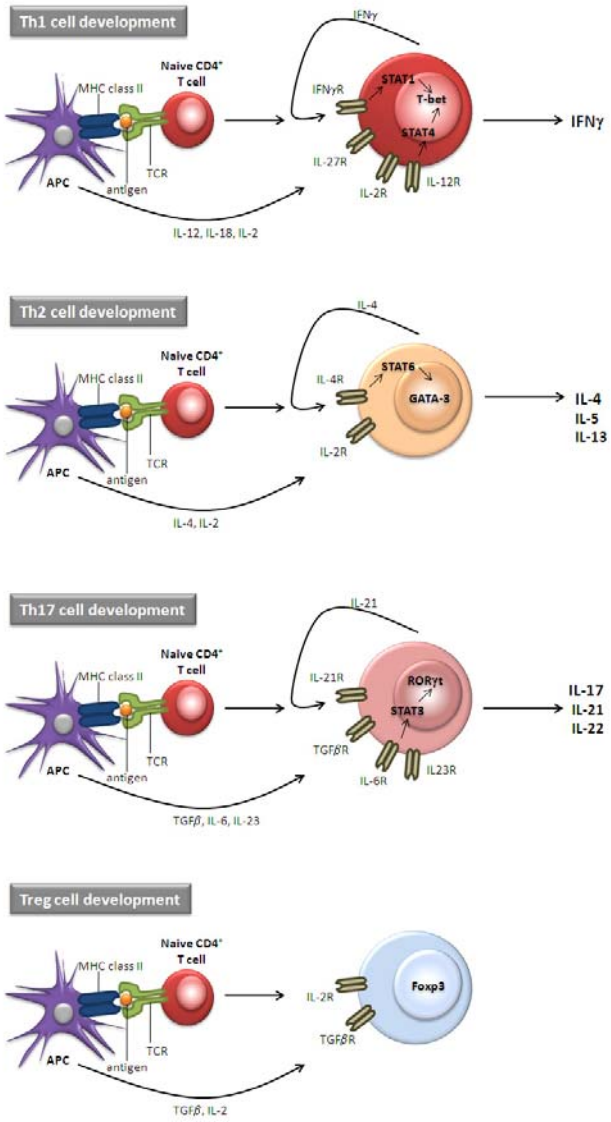


Figure 1.2 – CD4$^+$ helper T cell differentiation [adapted from (Wilson et al., 2009)]

IL-12 is a heterodimeric cytokine formed by a large (p40) and a small (p35) glycosylated and disulfide-linked subunits to form the bioactive IL-12 (p70). IL-23 was not long ago described and is also a heterodimeric cytokine that shares with IL-12 the p40 subunit plus a smaller subunit, p19 (Oppmann et al., 2000). The discovery of IL-23 led to the identification of Th17 cells as another distinct CD4$^+$ T cell lineage, which is induced by IL-23 and inhibited by IFNγ and IL-4 (Harrington et al., 2005). However, later studies shed doubt on IL-23 as the true driving force of the *de novo* differentiation of Th17 cells, and suggested instead that Th17 and Tregs cells are closely linked by TGFβ1. While Tregs,

driven by the transcription factor Foxp3, are induced by TGFβ1 only, the pro-inflammatory cytokine IL-6 blocks the generation of Foxp3$^+$ cells and instead, together with TGFβ, supports the differentiation of Th17 cells, with IL-23 controlling their survival and expansion (Bettelli et al., 2006b;Veldhoen et al., 2006a).Once this new T cell subset was described, a new exploratory era took place in T cell biology in order to understand which molecules determine its development and differentiation. Two retinoic acid receptor-related orphan nuclear receptor gamma t (RORγt) and alpha (RORα) were described as the key transcription factors that orchestrate Th17 differentiation (Yang et al., 2008;Ivanov et al., 2006). Th17 cells produce mainly IL-17A and IL-17F, but also IL-22 (Liang et al., 2006) and IL-21. The latter is induced by IL-6 and acts in an autocrine manner to induce the expression of both IL-21 and IL-17 through STAT3 and RORγt, therefore having a potent role in Th17 differentiation (Zhou et al., 2007;Nurieva et al., 2007;Korn et al., 2007a). Importantly, IL-2 besides promoting Treg generation and Th1 cells expansion was recognized as a negative regulator of Th17 differentiation (Laurence et al., 2007). Finally, further molecules involved in Th17 cells commitment and differentiation were identified, such as the aryl hydrocarbon receptor (Ahr) and Runx1 acting as inducers; and the nuclear zinc-finger orphan receptor subfamily 2, group F, member 6 (NR2F6), acting as a suppressor (Kimura et al., 2008;Hermann-Kleiter et al., 2008;Zhang et al., 2008;Veldhoen et al., 2009) (**Figure 1.3**).

Helper T cell subsets, by their specialized cytokine production, can orchestrate specific immune responses. Th1 cells are known to be important in macrophage activation and fight against intracellular pathogens. Th2 cells on their side respond to chronic eosinophilic inflammation and helminth infection and, finally, Th17 cells act in response to chronic neutrophilic inflammation and extracellular pathogens. The main function of Tregs is the downmodulation of the helper T cell responses in order to limit inflammation and minimize collateral damage to the host. The involvement of these subsets in “self-directed diseases” such as allergy and autoimmunity is less clear. Initially, Th1 cells were thought to be involved in autoimmunity, while Th2 cells were implicated in allergic reaction. At the present time, T cell-mediated reactions are known to be more complex and need a more detailed understanding.

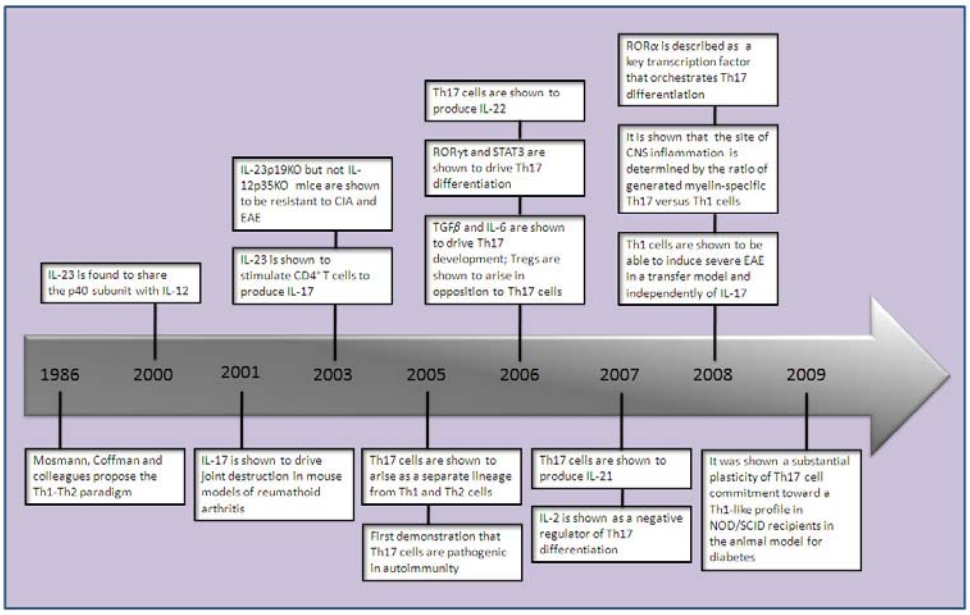


Figure 1.3 - Important events in the history of the IL-17 and T_H17 cell field [adapted from (Gaffen, 2009)]

The discovery of Foxp3$^+$ iTregs and Th17 cells and their close relationship, as well as the identification of molecules that affect more than one T cell subset, described above, has shown that these subsets have more in common than distinctions and, therefore, have revised researcher's concepts of CD4$^+$ T helper cell commitment to a specific phenotype as an endpoint. The concept of plasticity in T helper cells was based on the observation that T cells show great flexibility in their differentiation options both *in vivo* and *in vitro* depending on the milieu. For example, IL-17 and IFNγ double positive population was identified within CD4$^+$ CNS-infiltrating MOG peptide 35-55 specific T cells in the CNS of actively induced EAE sick mice (Suryani and Sutton, 2007). In the context of Th17 and Foxp3$^+$ iTregs cells, TGFβ can orchestrate both T cell differentiation programs in a concentration-dependent manner (Zhou et al., 2008). Also, all-trans retinoic acid (ATRA) induces Foxp3$^+$ cells and decreases the frequency of Th17 cells, mediated by the nuclear retinoic acid receptor-alpha (RARα) (Schambach et al., 2007). Finally, there are Th1 cells which produce simultaneously with IFNγ the anti-inflammatory cytokine IL-10 in certain conditions such as infections in order to regulate their inflammatory reactions and minimize immunopathology (O'Garra and Vieira, 2007).

1.4.2 TH1 AND TH17 CELLS: FRIENDS AND/OR FOES IN AUTOIMMUNITY?

Over the past three years, during the development of this thesis, the scientific community witnessed a quite dramatic change of point-of-views on the pathogenic roles of T cells in autoimmunity. This subject is directly correlated to the discovery of the Th17 subset and, to date continues to be a theme of debate.

As mentioned, traditionally, MS and EAE were thought to be mediated by IFNγ-producing Th1 cells. However many EAE experiments targeting Th1-related molecules led to perplexing results and demonstrated that the pathogenic mechanisms were more complex than initially expected. First evidence came from studies where the absence of IFNγ or IFNγR conferred on mice high susceptibility to EAE, with neutrophil invasion and disseminated demyelination (Krakowski and Owens, 1996;Tran et al., 2000b). Further, CD4$^+$ T cells unable to produce IFNγ were fully able to passively transfer EAE (Chu et al., 2000). Then, while mice devoid of the IL-12p40 subunit (common receptor chain to IL-12 and IL-23) were resistant to EAE, IL-12p35 knock-out mice, only deficient in IL-12 remained susceptible to EAE (Gran et al., 2002). The following observations that IL-12 and IL-23 share the p40 subunit, and that IL-23p19 deficient mice (no IL-23) are resistant to EAE (Cua et al., 2003), explaining the previous observed IL-12 redundancy, led to the conclusion that IL-23 but not IL-12 was the pathogenic cytokine in EAE. IL-12Rβ2-deficient mice were found to develop earlier and more severe EAE, with extensive demyelination and CNS inflammation. These mice exhibited significantly increased autoantigen-induced proliferative response, increased production inflammatory cytokines which included TNFα, GM-CSF and IL-17, and increased expression levels of IL-23p19 mRNA (Zhang et al., 2003). However, mice deficient in the transcription factor T-bet, important in the differentiation of Th1 cells, were completely resistant to active induction of EAE by immunization with MOG (Bettelli et al., 2004).

After the discovery of Th17 cells as a distinct effector T cell lineage, numerous reports involving specific cytokine neutralizations or knock-out mice in EAE studies associated this T cell subset with EAE. Neutralization of IL-17 with IL-17-receptor-Fc-protein or with

a monoclonal antibody was found to be beneficial in acute EAE (Hofstetter et al., 2005) and blocking IL-23 function with anti-IL-23p19-specific antibodies in EAE sick mice inhibited multiple inflammatory pathways functions such as serum levels of IL-17 and CNS expression of IFNγ and IL-17 (Chen et al., 2006). Also, IL-17 deficient mice exhibited delayed EAE onset and adoptive transfer of IL-17 deficient CD4+ T cells, which carried an increased number of IFNγ-producing cells, inefficiently were able to induce EAE in recipient mice (Komiyama et al., 2006). IL-27 is a cytokine that suppresses Th17 cells and IL-27R alpha-deficient mice were also found to be hypersusceptible to EAE (Batten et al., 2006). In the absence of TGFβ signaling, mice were found to be deficient in Th17 cells and resistant to EAE immunization (Veldhoen et al., 2006c). More recently, the restriction of IL-23 receptor deficiency to defined cell populations *in vivo* showed that Th17 cells failed to down-regulate IL-2 and maintain IL-17 production, associated with less proliferation and capacity to migrate to certain tissues (McGeachy et al., 2009), demonstrating the requirement of IL-23 signaling in the development and function of Th17 cells in autoimmunity.

The observations involving the Th17 rather the Th1 cells pathogenicity in EAE might comes might be explained by some interesting studies. First, the study of the role of pertussis toxin as an immune adjuvant in active EAE demonstrated that pertussis toxin inhibits the development of Tregs and induces IL-17-producing CD4 cells (Chen et al., 2007). Additionally, mycobacteria and zymosan, a cell wall component of *Saccharomyces cerevisiae*, when emulsified with MOG and incomplete Freund's adjuvant for immunization potentiate Th17 cell differentiation (Veldhoen et al., 2006c). Therefore, the active EAE model might be already pre-conditioned to a Th17 response, masking the true role of T cells responses in CNS autoimmunity.

Other studies suggested the involvement of cytokines and transcription factors affecting both Th1 and Th17 populations, previously associated with only one T cell subset. MOG-specific T cells from IL23p19KO mice, found to be highly deficient not only in the production of IL-17A but also of IFNγ, induced EAE with delayed onset and much lower severity when transferred to WT recipient mice, indicating a critical role of IL-23 in development of encephalitogenic T cells, towards both Th1 and Th17 pathways (Thakker et al., 2007). Notwithstanding, another work focused on the suppression of T-bet described an amelioration of EAE, not only by limiting the differentiation of auto-

reactive Th1 cells but also by inhibiting pathogenic Th17 cells via regulation of IL-23R (Gocke et al., 2007).

Some studies indicated that distinct EAE clinical outcomes are correlated with the presence/absence of IFNγ and the location of T cell infiltration in the CNS. Depending on the nature of MBP peptide that was used to immunize, IFNγ knock-out mice either developed a conventional disease characterized by ascending weakness and paralysis, or an axial-rotatory disease characterized by uncontrolled axial rotation. These two clinical phenotypes were characterized by inflammation and demyelination primarily in spinal cord and brain, respectively (Abromson-Leeman et al., 2004). Similarly, another report described a spontaneous EAE model (MBP-specific TCR transgenic RAG-1 knock-out) that develops classical EAE but, in the absence of IFNγ develops a non-classical form of EAE characterized by a slight head tilt, progressing to a severe head tilt, spinning, or a rotatory motion. The cellular infiltrate in non-classical EAE is predominantly found also in the brainstem and cerebellum, with very little inflammation in the spinal cord, which is primarily affected in classical disease (Wensky et al., 2005). A later study reported the mentioned evidence by demonstrating that absence of IFNγ promotes T cell infiltration of brain stem and cerebellum rather spinal cord (Lees et al., 2008).

The parallel observations on T helper cell plasticity together with paradoxical EAE data led researchers to the conclusion that, after all, both Th1 and Th17 may participate in the pathogenic processes in EAE, which coincided with the observations described in this thesis. This was initiated by a report demonstrating that Th1 cells are indeed highly pathogenic and, only after these establish CNS inflammation, Th17 cells follow to infiltrate the CNS (O'Connor et al., 2008). Another study proposed that it is the ratio of myelin-specific Th17 versus Th1 cells generated that determines the site of CNS inflammation. This occurs in the brain parenchyma when Th17 outnumber Th1 cells but in the spinal cord when Th1 outnumber Th17 cells (Stromnes et al., 2008). Similarly, adoptive transfer of myelin-reactive T cells driven *in vitro* in the presence of IL-12 or IL-23 was encephalitogenic in both cases and induced indistinguishable clinical features though with distinct immune profiles (Kroenke et al., 2008). Also, in the related

experimental autoimmune uveitis (EAU) animal model for autoimmune uveitis in humans, Th17 cells played a dominant role in the immunization model with the retinal antigen interphotoreceptor retinoid-binding protein. However, Th1 cells were able to induce severe EAE in a transfer model and independently of local IL-17 (Luger et al., 2008) showing that the dominant effector T helper cell phenotype in pathological conditions may be determined by the model. Similarly, the adoptive transfer of either hen egg lysozyme-specific Th1 or Th17 cell lineages into hosts expressing hen egg lysozyme as pseudo autoantigen their eyes induced ocular inflammation but with slight differences in histological pathology and selective cytokine and chemokine expression, indicating that Th1 and Th17 might have distinct activities in mediating inflammation (Cox et al., 2008). More recently, in the experimental model for autoimmune diabetes, adoptive transfer of Th1 or Th17 cells from BDC2.5 transgenic mice into NOD/SCID recipient mice induced diabetes with similar rates of onset. Interestingly, a substantial plasticity of Th17 cell commitment toward a Th1-like profile was observed in the NOD/SCID recipients (Bending et al., 2009). In fact, a recent report emphasized the plasticity of Th17 cells through the use of fluorescent reporter mice for IL-17F by showing that Th17 cells convert to a Th1 phenotype when transferred into lymphopenic hosts but not into normal animals (Nurieva et al., 2009).

In summary, in the past years we have witnessed a real revolution in the understanding of helper T cell subsets biology and differentiation and their role in pathological conditions. This subject was found to be more complex than initially thought and too haste rush was taken in the evaluation of the experimental data, leading to some confusion in the field.

1.5 - CHEMOKINES AND CHEMOKINE RECEPTORS

Chemokines and chemokine receptors are important cell migration regulators during an immune response. Chemokines exert their function via interaction with their cognate receptors in the target cell, which are G protein-coupled receptors (GPCRs), thus activating multiple signaling transduction pathways depending on the cell type.

Many chemokines and chemokine receptors are constitutively expressed in certain tissues, involved in homeostatic functions such as hematopoiesis, homing to lymphoid organs, immunosurveillance, organogenesis and brain development. Nevertheless, most of them are up-regulated in inflammatory conditions to influence T cells, B cells and DCs in differentiation and cell migration. In the context of CNS inflammation, the events of disruption of BBB integrity and inflammatory leukocyte trafficking are regulated not only by adhesion molecules and matrix metalloproteinases, but also by chemokines. These can be produced by the endothelial cells and resident CNS cells to direct the movement of mononuclear cells, expressing chemokine receptors, into the CNS. The full understanding of the role of these molecules is complicated by their redundancy and promiscuity, either by individual chemokines binding to more than one receptor, or single receptors being activated by several chemokines (Cartier et al., 2005;Hamann et al., 2008).

There are an enormous amount of studies addressing the role of chemokines and chemokine receptors in EAE an MS, both from the CC and CXC families of chemokines. Here, only CCR5, CCR6, CCR8 chemokine receptors and their ligands will be briefly discussed by reason of their exclusive expression in Th1 and Th17 cells, which could possibly help to explain the dissimilar migration behaviors of Th1 and Th17 cells in EAE. While CCR5 is for long described to be only expressed in Th1 cells, CCR6 was recently found to be expressed by Th17 cells. CCR8, previously described in Th2 cells, was found in the microarray data described in Chapter 5, to be expressed by Th17 but not Th1 cells.

1.5.1 CCR5 AND ITS LIGANDS

CCR5 is expressed mainly by T lymphocytes (Th1 cells) but also by monocytes/macrophages and immature DCs. Several chemokines bind to CCR5: CCL3 (MIP1-α), CCL4 (MIP1-β), CCL5 (RANTES) and CCL8 (MCP-2), which are produced by neurons, astrocytes, microglia and endothelial cells. MS lesions with high levels of

inflammation show increased levels of CCR5, as well as of CCL3, CCL4 and CCL5 (Balashov et al., 1999). In mice with EAE the same is true, but CCL3 and CCR5 deficient mice are not resistant to EAE immunization nor those treated with the antagonist met-RANTES (Glabinski et al., 1998;Tran et al., 2000a;Matsui et al., 2002).

1.5.2 CCR6 AND ITS LIGANDS

CCR6, preferentially expressed by most B cells, immature DCS and effector memory T cells, was recently described to be expressed also in Th17 cells and regulatory T cells. CCL20 (MIP3-α) but also, with lower affinity, the β-defensin group of antimicrobial peptides bind CCR6. Th17 cells are also able to express CCL20, making them also potential recruiters of both Th17 and Tregs. Absence of CCR6 in Th17 cells was found to slightly delay EAE onset, as in the case of CCR6 knock-out mice (Yamazaki et al., 2008). CCR6 and CCL20 are up-regulated in draining lymph nodes and spinal cord during EAE, and the disease can be attenuated with a neutralizing antibody or antagonist for CCR6 (Liston et al., 2009). CCL20 is expressed either constitutively or following induction in a variety of epithelial cell types, including keratinocytes, pulmonary and intestinal epithelial cells and is highly induced following inflammatory stimuli. CCR6 seems to play a role in Th17 cells migration to certain intestine microenvironments expressing CCL20 where these receptors can regulate the balance between effector and regulatory T cells in the gut (Wang et al., 2009).

1.5.3 CCR8 AND ITS LIGANDS

CCR8 has been detected in Th2 cells, regulatory T cells, macrophages and microglia. CCR8 has only one ligand, CCL1 (I-309). In MS lesions, phagocytic macrophages and activated microglia were found positive for CCR8 and correlated with active demyelination (Trebst et al., 2003). CCR8 is also up-regulated in EAE brain and spinal cord, as well as CCL1 in spinal cord before EAE onset (Godiska et al., 1995). CCR8-CCL1 interaction seems to be essential for the pathogenesis as CCR8 deficient mice develop later onset and milder EAE symptoms (Murphy et al., 2002)

1.6- CNS RESIDENT CELLS: THE PARTICULAR CASE OF ASTROCYTES

The interaction between glial cells such as microglia, oligodendrocytes and astrocytes, and neurons is critical for myelination and remyelination in MS. Glial cells, in particular astrocytes, are highly abnormal in MS. Astrocytes are a very abundant star-shaped glial cell type in the CNS, and through their highly dynamic communication with neurons and other glial cells perform many functions. Those include biochemical support of neurons and endothelial cells from the BBB, maintenance of extracellular ion balance and pH homeostasis, immunity and neuroprotection following traumatic injuries. Astrocytes are able to extend their processes to neuronal synapses, nodes of Ranvier, endothelial cells of the BBB and oligodendrocytes. Upon pathological insults such as in MS or Alzheimer's disease, astrocytes are activated, a process denominated astrogliosis where hypertrophy of processes occurs in parallel with glial fibrillary acidic protein (GFAP) up-regulation and astrocytes proliferation. Moreover, astrocytes change their expression of adhesion molecules, antigen presentation molecules, cytokines, chemokines and receptors (Nair et al., 2008;Williams et al., 2007). For example, astrocytes up-regulate glutamate transporters in order to sequester glutamate excess and protect neurons and oligodendrocytes (Liberto et al., 2004). In pathological conditions such MS, due to their diverse functions astrocytes can be not only neuroprotective cells but also detrimental in the CNS by promoting or inhibiting remyelination.

1.6.1 PROTECTIVE AND DETRIMENTAL ROLES OF ASTROCYTES IN CNS

Astrocytes can play both toxic and protective roles in oligodendrocytes, and thus affect neuronal survival. An example of the first case comes from a study where the astrocyte hypertrophic response was associated with axonal dysfunction before massive T cell infiltration in the CNS and EAE onset (Wang et al., 2005). Additionally, necrotic oligodendrocytes were detected amongst reactive astrocytes in EAE acute lesions (Bannerman et al., 2007). One possible explanation for this might be the decreased capacity of reactive astrocytes to maintain extracellular glutamate homeostasis, which

in turn increases vulnerability of oligodendrocyte to excitotoxic insults (Korn et al., 2005). On the other hand, reactive astrocytes synthesize proteins such as insulin-like growth factor (IGF-1), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), ciliary neurotrophic factor (CNTF) known to enhance neuronal and oligodendrocyte survival (Nair et al., 2008).

1.6.2 THE IMMUNE FUNCTIONS OF ASTROCYTES IN EAE AND MS

There are increasing evidences suggesting the involvement of astrocytes in the innate and adaptive immune responses in the CNS.

The first encounter of astrocytes with infiltrating T lymphocytes happens at the BBB. Astrocytes modulate BBB function through their endfeet surrounding CNS capillaries and perivascular macrophages. Under inflammatory conditions, astrocytes can increase BBB permeability by acting directly on endothelial cells and tight junctions through secretion of inflammatory cytokines such as TNFα, IL-6 and IL-1β (Nair et al., 2008). Expression of TNFα by astrocytes correlates with active demyelination activity and oligodendrocytes apoptosis in MS brain. In order to cross the BBB, T cells express the α4- integrin on their surface, which binds to vascular cell adhesion molecule-1 (VCAM-1). VCAM-1 is expressed not only by endothelial cells but also by astrocytes (Gimenez et al., 2004), therefore contributing to T cell entry in the CNS and to pathogenesis. However, in apparent contradiction, astrocytes are also able to secrete the anti-inflammatory cytokines TGFβ and IL-10, which tightens the BBB.

Inside the CNS, T cells are re-activated by professional APCs such as infiltrating dendritic cells and macrophages, and also CNS resident microglia. In opposition to microglia, of hematopoietic origin and regarded as the resident macrophages of the CNS, astrocytes are of neural origin and considered non-professional APCs as they do not express constitutively the MHC class II molecule as DCs or microglia. However, astrocytes can up-regulate MHC class II after activation and present myelin antigens to auto-reactive T cells (Fontana et al., 1984). There is *in vitro* evidence of MHC class II up-regulation in astrocytes by IFNγ (Fierz et al., 1985) and TNFα. Additionally, astrocytes can present MBP, PLP or MOG epitopes to T cells but are not able to efficiently process MOG

protein, suggesting that astrocytes may participate as secondary APCs after the release of degraded proteins from damaged cells (Kort et al., 2006). A recent study reported the expression of both IL-12 and IL-23 by astrocytes after stimulation, and the subsequent blocking of antigen presentation to T cells after neutralization of the IL-12/IL-23 p40 subunit, reinforcing the idea of their capacity to present antigen to encephalitogenic T cells (Constantinescu et al., 2005). With the identification of Th17 cells, this study suggests a contribution of astrocytes in the modulation of T helper cell Th1 and Th17 responses. Notwithstanding these *in vitro* observations, it is still not clear whether antigen presentation by astrocytes also occurs *in vivo* during demyelinating disease due to contradictory data on MHC class II expression by astrocytes in MS lesions. However, this may be due to the diverse pathologies in MS patients, which may require different astrocytes responses. In a T cell driven pathology, it is possible that the activated astrocytes release cytokines and chemokines in response to T cell-derived factors, contributing to maintenance of inflammation-mediated demyelination.

Another aspect relevant for the contribution of activated astrocytes to inflammation in the CNS concerns the up-regulation of numerous chemokines and cytokines, key molecules in the attraction, guidance and communication of immune cells within the CNS parenchyma. Monocyte chemoattractant protein-1 (MCP-1, also called CCL2) and interferon-inducible protein-10 (IP-10, also called CXCL10) were found to be over-expressed in astrocytes in EAE (Ransohoff et al., 1993), and MCP-1 expression is also found in astrocytes in MS lesions (van, V et al., 1999). Other examples of chemokines found to be expressed in astrocytes include CX3CL1 (fractalkine), CXCL12 (also called SDF-1), CCL20 (MIP3α) and IL-8 (CXCL8), also correlated with EAE and MS severity. The production of these chemokines by astrocytes influences the integrity of the BBB, and therefore the migration of T cells into the CNS.

In summary, the immune functions of astrocytes during CNS inflammation as antigen presenting cells to auto-reactive T cells and as producers of inflammatory cytokines and chemokines contribute to the perpetuation of inflammation by potentiating the activation and further recruitment of inflammatory cells into damaged areas.

1.7- STRATEGIC THERAPIES IN MS: RENDITION FROM EAE STUDIES?

Due to the complexity of CNS autoimmunity, only recently have researchers started to understand MS pathology and EAE studies have been invaluable in the development of each of the strategies described bellow and more. Positive results obtained in such of these studies can be further confirmed with ones in MS and then follow to the clinical trial phases. Naturally, many therapies have demonstrated to be of poor efficacy and others with severe collateral effects. Nevertheless, EAE models are able to mimic the relapse and remission phases of MS and, for that reason, are a precious tool to explore the pathogenic mechanisms in order to design better strategies in MS treatment.

It is believed that immune cell migration, their adhesion to surface molecules of the BBB and subsequent infiltration into the CNS parenchyma are crucial steps in the MS and EAE pathogenesis (Engelhardt, 2008). In fact, the most famous therapeutic strategy aiming at the targeting of the leukocyte trafficking across the BBB is by blocking the alpha 4 subunit of the integrin VLA4 (very late antigen 4), a drug named Natalizumab. This strategy results in the blockade of VLA-4, expressed by T cells, binding to the vascular cell-adhesion molecule (VCAM-1), expressed in the BBB endothelium, and is at present the most potent drug approved so far in the treatment of relapsing-remitting MS. Another drug aiming at the targeting of leukocyte trafficking is the oral immunosuppressant FTY720 (Fingolimod), a sphingosine-1-phosphate (S1P) analogue that sequesters lymphocytes into the lymph nodes and therefore prevents immune cell migration to the CNS. This drug is under investigation as a therapy and has demonstrated a beneficial effect on disease activity in RR-MS patients and in EAE models (Miron et al., 2008).

As the humoral response also plays a role in MS, strategies aiming at B cells targeting have been implemented. Rituximab is a chimeric murine-human antibody against $CD20^{+}$ pre-B cells and mature B cells, therefore interfering with the production of autoreactive antibodies. The mixed cell targeting strategy counts with Alentuzumab as an example. It is a humanized monoclonal antibody against CD52, a cell surface molecule present in T cells, B cells and monocytes, and induces immediate cytolysis of these cells,

intercepting the autoreactive lymphocytes to reach the CNS (Lopez-Diego and Weiner, 2008).

Pro-inflammatory cytokines play a crucial role in the effector phase of MS pathogenesis thus supporting the use of respective blocking antibodies or anti-inflammatory cytokines as a therapeutic option. However, the outcomes are not always predictable and many have shown undesirable/unpredictable side-effects or limited therapeutic efficacy. For example, the blocking of the pro-inflammatory cytokine TNFα, produced by innumerous cell types and known to be involved in CNS damage, was found to both improve and protect against EAE but in clinical trials it increased MS lesions (McCoy and Tansey, 2008).Similarly, the IL-23 cytokine, found recently to be tightly correlated with Th17 cells, was claimed to be highly pathogenic in EAE and mice deficient in this molecule were completely resistant to EAE immunization. The therapeutic targeting of IL-23 was able to inhibit EAE (Chen et al., 2006) but so far neutralization of IL12/IL23 p40, ustekinumab, failed to block inflammation in relapsing-remitting MS phase II study (Segal et al., 2008).

Finally, glatiramer acetate is the generic name for the drug Copaxone or Copolymer 1 and is a synthetic polymer composed of four amino acids that are found in MBP. It is an immunomodulator shown in clinical trials to reduce the frequency of relapses in relapsing-remitting MS. Though glatiramer acetate has been extensively studied and found to be protective in EAE, the mechanisms of action are still not entirely understood (Ruggieri et al., 2007).

1.8- THE ORPHAN NUCLEAR RECEPTOR REV-ERBα: A DIFFERENTIAL MOLECULE EXPRESSED IN TH1 AND TH17 CELLS?

Rev-Erbα, also called NR1D1 and its isoform Rev-Erbβ (NR1D2) belong to the Rev-Erb family of the nuclear hormone receptors (NHRs) and are encoded on the opposite strand of the alpha-thyroid hormone receptor (TR) gene. NHRs are transcription factors

that regulate diverse biological processes such as carbohydrate and lipid metabolism, circadian rhythm and cell differentiation. NHRs share a highly conserved domain structure with a variable amino-terminal AB region that encodes the activation function 1 domain (AF-1), followed by the highly conserved C region that contains the DNA binding domain (DBD), a linker region D and the carboxy-terminal E region that encodes the ligand binding domain (LBD) and AF-2 (**Figure 1.4a**).

Though many NHRs have identified ligands such as steroid hormones, fatty acids and lipophilic vitamin derivatives, which bind to the LBD, many others are orphan receptors with their ligands still unidentified. NHRs exert gene expression regulation function by binding to specific DNA sequences of their target genes in a ligand binding-dependent manner, either as homodimers, heterodimers or, to less extent, as monomers. Rev-Erbα and Rev-Erbβ recognize two classes of DNA response elements, the (A/G)GGTCA half-site with a 5' AT-rich extension and the dimeric site (RevDR2) composed of a direct repeat of the core motif (AGGTCA) spaced by two nucleotides. The Rev-Erbs regulate gene transcription when bound to these response elements within the promoter region of the target gene (Burris, 2008).

1.8.1 REPRESSOR ACTIVITIES AND LIGANDS OF REV-ERBα

Rev-Erbα and Rev-Erbβ are unique NHRs in the sense that both lack the AF-2 in the LBD, required for recognition of co-activators necessary for transcription activation. As a consequence, Rev-Erbs, in opposition to other NHRs, repress transcription by binding to co-repressor molecules. This repressive process requires the recruitment of the co-repressor NCoR that complexes with histone deacetylase 3 (HDAC3), altering local chromatin structure that is not favorable to transcription (Harding and Lazar, 1995;Yin and Lazar, 2005). Rev-Erbα is not, at the present time, a classic orphan receptor as recently heme was identified as the reversible ligand for Rev-Erbα and Rev-Erbβ (Raghuram et al., 2007;Yin et al., 2007). Heme binds do the LBD of these receptors, enhancing the proteins stability, which enhances the recruitment of the co-repressor NCoR and leads to repression of target genes (**Figure 1.4b**).

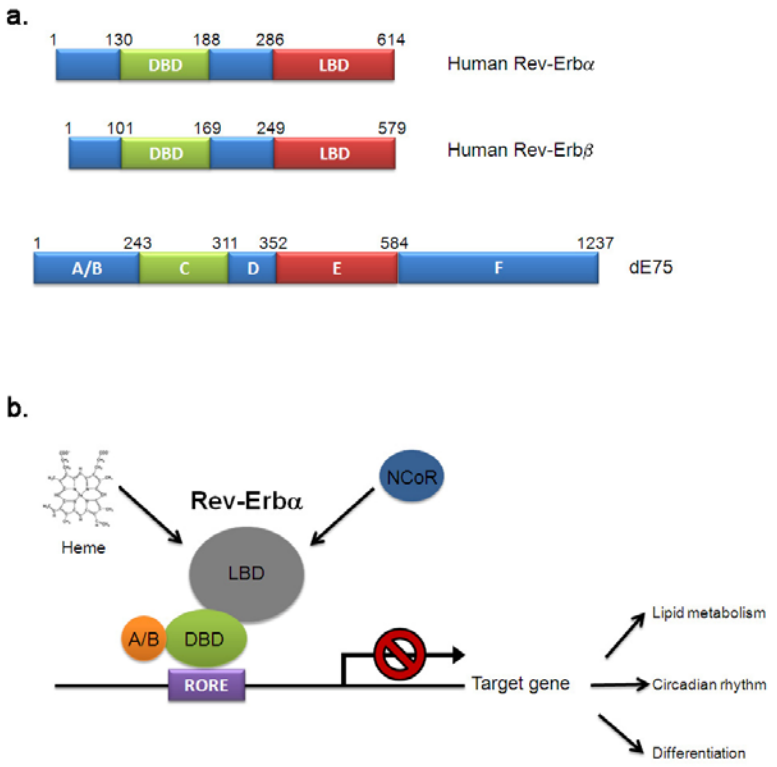


Figure 1.4 – Structure and mechanism of action of Rev-Erbα. [Adapted from (Burris, 2008)]. A. Comparative gene structure of human Rev-Erbα and Rev-Erbβ, with high homology with mouse Rev-Erbα and Rev-Erbβ, and its homolog in *Drosophila* dE75. B. Rev-Erbα-mediated regulation of target genes. Rev-Erbα binds to heme through its ligand binding domain (LBD), which helps the recruitment of co-repressor molecules such as NCoR. The DNA binding domain (DBD) then binds to the responsive DNA elements (ROREs) in the promoter region of target genes, leading to repression of transcription.

1.8.2 ROLES OF REV-ERBα

Rev-Erbα is as regulator of many genes in numerous tissues such as adipocytes, skeletal and smooth muscle cells, liver and vasculature (Ramakrishnan and Muscat, 2006).

Rev-Erbα plays an unequivocal role in lipid metabolism. Rev-Erbα deficient mice are dyslipidemic with increased levels of triglycerides, very-low-density lipoprotein (VLDL) and apoliprotein CIII (apoCIII) in liver and serum. Elovl3, a liver and adipose tissue fatty acid elongase, and plasminogen activator inhibitor type I (PAI-1), a fibrinolysis regulator, were found to be target genes of Rev-Erbα. Additionally, Rev-Erbα is up-regulated during adipogenesis, possibly linked to PPARγ, which is also enhanced.

Rev-Erbα is critically involved in the circadian rhythm, which is generated by feedback loops in gene expression. The genes on the mammalian clock with a circadian expression include the brain and muscle Arnt-like protein 1 (Bmal1), the circadian locomoter output cycles kaput (Clock), cryptochrome (Cry1, Cry2) and period (Per1, Per2). Rev-Erbα is the major regulator (repressor) of the cyclic Bmal1 transcription but is also regulated by the Bmal1/ Clock heterodimer. Rev-Erbα influences the period length and phase shifting and Rev-Erbα deficient mice have attenuated amplitude of mRNA oscillations in Bmal1, Clock and Cry1. Accordingly, Rev-Erbα affects circadian regulation of processes such as blood pressure and hypertensive fluctuations associated with cardiovascular disease.

Finally, there is some evidence of Rev-Erbα involvement in inflammation. For example, over-expression of Rev-Erbα in smooth muscle cells upregulated the expression of IL-6 and cyclooxygenase-2, and increased transactivation by NF-κB (Migita et al., 2004). Additionally, Rev-Erbα was found to be expressed in endothelial cells, macrophages and chondrocytes, cell capable of producing inflammatory molecules.

1.8.3 RELATIONSHIP BETWEEN REV-ERBα AND RORα: A LINK FOR TH17 CELLS?

Rev-Erbα and Rev-Erbβ are both expressed in skeletal muscle and brain but also in liver and heart (Forman et al., 1994). The finding that Rev-Erbα and Rev-Erbβ are co-expressed with RORα in several tissues, suggested that these orphan nuclear receptors are integrated in an overlapping network of responsive genes. In fact, Rev-Erbs and RORα bind to the same target DNA sequence (the ROR responsive element or RORE),

and compete for transcriptional activation, being the former a repressor and the latter an enhancer of transcription. Additionally, Rev-Erbα and RORα are reciprocal target genes. Rev-Erbα expression represses RORα transcription but RORα promotes Rev-Erbα transcription (Forman et al., 1994;Raspe et al., 2002;Delerive et al., 2002). Nevertheless, one cannot exclude the hypothesis that Rev-Erbα might also function as an activator on certain target genes by recruiting co-activators. In fact, RORα was reported to recruit both co-activators and co-repressors (Harding et al., 1997). Thus, the balance of ROR versus Rev-Erb has been suggested to be critical for dynamic regulation of target genes containing the ROREs, such those involved in lipid metabolism, circadian rhythm and inflammation. For example, Rev-Erbα is the major regulator of the cyclic Bmal1 transcription, as Bmal1 contains ROREs in its promoter region for Rev-Erbα binding, but also influences other circadian genes such as Clock and Cry1 (Preitner et al., 2002). Along this line, more recently, RORα was also described to regulate Bmal1 transcription (Akashi and Takumi, 2005), re-enforcing the idea of crosstalk between RORα and Rev-Erbα in gene regulation.

Recently, another orphan nuclear receptor, NR4A2, was described to be involved in the pathogenesis of EAE and MS. It is up-regulated in the peripheral blood T cells of MS patients as well as in CNS infiltrating T cells from EAE sick mice. Moreover, NR4A2 was found to increase IFNγ and IL-17 production, contributing therefore for the pathogenesis (Doi et al., 2008). These facts together with the recent findings connecting orphan nuclear receptors such as RORγt and RORα with Th17 cells (described above) plus our data described in Chapter 5 about Rev-Erbα, suggest that these receptors might play a role in T cell biology besides their importance in lipid metabolism and circadian rhythm.

CHAPTER 2 MATERIALS AND METHODS

2.1 – MATERIALS

2.1.1 BUFFERS AND REAGENTS

Cell culture medium

RPMI-1640 or DMEM medium with sodium bicarbonate (Gibco and Sigma) supplemented with 1% MEM non-essencial aminoacids (100X, Gibco), 1% sodium piruvate (100 mM, Gibco), 1% Pen-Strep (penicillin G 10,000 units/ml; streptomycin 10,000 μg/ml; Gibco), 1% glutamine solution (200mM, Gibco), 400μl/L β-mercaptoethanol (10μl/10ml media, pre-prepared), 10% heat-inactivated fetal calf serum (FCS).

Tail digestion buffer

100 mM Tris HCl pH 8.5

5mM EDTA

0.5% Tween 20

1mg/ml Proteinase K (added before digestion)

FACS buffers

Staining buffer: PBS + 1% bovine serum albumin (BSA) + 0.1% sodium azide

Saponin buffer: 0.1% saponin in staining buffer

PFA/saponin buffer: 4% paraformaldehyde (PFA) + 0.1% saponin in PBS

Erythrocyte lysis buffer

0.83% ammonium chloride in PBS

Antibody purification buffers

Neutralization buffer: 1M Tris HCl pH 9.0

Elution buffer: 0.1M glycine HCl pH 2.7

Binding buffer: 20 mM sodium phosphate pH 7.0

LB medium

10g peptone, 5g yeast extract, 5g NaCl, distilled water up to 1000 ml.

MOG purification buffers

Elution buffer: 6M guanidium chloride + 0.5M imidazole

Equilibration buffer: 6M guanidium chloride

Wash buffer: 6M guanidium chloride + 40 mM imidazole

Solubilization buffer: 6M guanidium chloride + 20 mM β-mercaptoethanol

Sonication buffer: 2X PBS

Lysis buffer: sonication buffer + 0.5% LDAO + lysozyme

Ni-EDTA column regeneration buffer: 1% EDTA + 0.05% Tween-20

ELISA buffers

Wash buffer: PBS + 0.05% Tween-20

Assay diluents: PBS + 10% FCS or PBS + 1% BSA

2.1.2 MICE GENOTYPING BY CONVENTIONAL AND REAL-TIME PCR

The following primers and probes were used for mice genotyping by conventional and real-time PCR:

Genotyping	Gene	Primer name	Oligo sequence (5' -> 3')
Th mouse	Anti-MOG IgH (8.18C5)	8.18C5 sense	TGAGGACTCTGCCGTCTATTACTGT
		8.18C5 anti-sense	GGAGACTGTGAGAGTGGTGCCT
		8.18C5 probe	CCAGTATGGCATGTTTACCATCGTATTACCAGTT
	IgG heavy chain	mIgH sense	ATTGGTCCCTGACTCAAGAGATG
		mIgH anti-sense	TGGTGCTCCGCTTAGTCAAA
		mIgH probe	CCTTGCACCAGTCAGAGACCACAGGG
2D2 mouse	Valpha 3.2 TCR	Va3.2–2D2-M	CCCGGGCAAGGCTCAGCCATGCTCCTG
		Ja18-2D2-M	GCGGCCGCAATTCCCAGAGACATCCCTCC
Rag2KO mouse	RAG2	mRAG-2 sense	AGTCAGGAGTCTCCATCTCACTGA
		mRAG-2 anti-sense	GAATAGTGTAGCTGACTGCCTACC
		Rag2-intron sense	AAAGACCTATTCACAATCAAAAATGTCC
	Neomycin resistant gene	Neo-2 – anti-sense	GGATTGCACGCAGGTTCTCCG
IL-12p35KO mouse	IL-12p35	IL-12p35 sense	CAAATACCAGCACTGGGCTT
		IL-12p35 anti-sense	AAGTCACAGCTTGATGGCTG
	PGK promoter	PGK promoter anti-sense	CACCAAAGAACGGAGCCGGT
IL-12p40KO mouse	IL-12p35 sense	IL-12p40 sense	CACTTGCCAAACTCCTGTGAGCTATGA
		IL-12p40 anti-sense	TTCTTGTGGAGCAGCAGATGTGAGTGG
	IL-12p40 KO	Neo-p40 sense	TGATATTGCTGAAGAGCTTGGCGG
		IL-12p40 anti-sense	TCCTTCTTGTGGAGCAGCAGATGT

2.1.3 GENE EXPRESSION ANALYSIS BY REAL-TIME PCR

The following primers and probes were used for gene expression analysis by real-time PCR:

Gene	Primer name	Oligo sequence (5' -> 3')	References
Bmal1	Bmal1 sense	CCAAGAAAGTATGGACACAGACAAA	(Preitner et al., 2002)
	Bmal1 anti-sense	GCATTCTTGATCCTTCCTTGGT	
	Bmal1 probe	TGACCCTCATGGAAGGTTAGAATATGCAGAA	
CCL1	CCL1 sense	CCGTGTGGATACAGGATGTTG	(Nakamura et al., 2006)
	CCL1 anti-sense	TCAGGACAGGAGGAGCCC	
CCL20	CCL20 sense	ATGGCCTGCGGTGGCAAGCGTCTG	(Yamazaki et al., 2008)
	CCL20 anti-sense	TAGGCTGAGGAGGTTCACAGCCCT	
CCL3	CCL3 sense	AAGTCTTCTCAGCGCCATATG	(Auray et al., 2007)
	CCL3 anti-sense	AAGACTCTCAGGCATTCAGTTCCAGGTC	
CCL4	CCL4 sense	CCAGGGTTCTCAGCACCAATGGGC	(Paranavitana et al., 2005)
	CCL4-reverse	AGGAAATCTGAACGTGAGGAGCAAGG	
CCL5	CCL5 sense	TCTCTGCAGCTGCCCTCACC	(Auray et al., 2007)
	CCL5 anti- sense	TCTTGAACCCACTTCTTCTC	
CCL8	CCL8 sense	TAAGGCTCCAGTCACCTGCT	(McColl et al., 2006)
	CCL8 anti- sense	TCTGGAAAACCACAGCTTCC	
CCR5	CCR5 sense	GACATCCGTTCCCCCTACAAG	(Lacroix-Lamande et al., 2008)
	CCR5 anti- sense	TCACGCTCTTCAGCTTTTTGCAG	
CCR6	CCR6 sense	CCTCACATTCTTAGGACTGGAGC	(Yamazaki et al., 2008)
	CCR6 anti- sense	GGCAATCAGAGCTCTCGGA	
CCR8	CCR8 sense	CAGATAATTGGTCTTCCTGCCTC	(Liu et al., 2007)
	CCR8 anti-sense	TGAGGAGGAACTCTGCGTCACA	
Fas-L	Fas-L sense	GAAGGAACTGGCAGAACTCCG	(Halloran et al., 2004)
	Fas-L anti-sense	CCCTGTTAAATGGGCCACACT	
	Fas-L probe	AAAGCAAATAGCCAACCCCAGCACACC	
GAPDH (for SYBR Green)	GAPDH sense	GTGTCCGTCGTGGATCTGA	(Lau et al., 2004)
	GAPDH anti-sense	CCTGCTTCACCACCTTCTTG	
GAPDH	GAPDH sense	TCACCACCATGGAGAAGGC	Designed by Primer 3 software
	GAPDH anti-sense	GCTAAGCAGTTGGTGGTGCA	
	GAPDH probe	ATGCCCCCATGTTTGTGATGGGTGT	
GATA-3	GATA3 sense	CTACCGGGTTCGGATGTAAGTC	Designed by Primer 3 software
	GATA3 anti-sense	GTTCACACACTCCCTGCCTTCT	

	GATA3 probe	AGGCCCAAGGCACGATCCAGC	
GM-CSF	GM-CSF sense	GCCATCAAAGAAGCCCTGAA	(Overbergh et al., 2003)
	GM-CSF anti-sense	GCGGGTCTGCACACATGTTA	
	GM-CSF probe	ACATGCCTGTCACATTGAATGAAGAGGTAGAAG	
Granzyme A	GzmA sense	ATCTGTGCTGGCGCTTTGA	(Halloran et al., 2004)
	GzmA anti-sense	ACTTAGATCTCTTTCCCACGTTACAGT	
	GzmA probe	TGAAAAGAACTGGGTGTTGACTGCTGCC	
Granzyme B	GzmB sense	CGATCAAGGATCAGCAGCCT	(Halloran et al., 2004)
	GzmB anti-sense	CTTGCTGGGTCTTCTCCTGTTCT	
	GzmB probe	TGCTGCTCACTGTGAAGGAAGTATAATAAATGTCACT	
IFNγ	IFNg sense	TCAAGTGGCATAGATGTGGAAGAA	(Giulietti et al., 2001)
	IFNg anti-sense	TGGCTCTGCAGGATTTTCATG	
	IFNg probe	TCACCATCCTTTTGCCAGTTCCTCCAG	
IL-10	IL-10 sense	CAGAGAAGCATGGCCCAGAA	Designed by Primer 3 software
	IL-10 anti-sense	TGCTCCACTGCCTTGCTCTT	
	IL-10 probe	TGAGGCGCTTGTCATCGATTTCTCCC	
IL-17A	IL-17 sense	AACTCCCTTGGCGCAAAAGT	Designed by Primer 3 software
	IL-17 anti-sense	GGCACTGAGCTTCCCAGATC	
	IL-17 probe	CCACGTCACCCTGGACTCTCCACC	
IL-22	IL-22 sense	TCCGAGGAGTCAGTGCTAAA	(Zheng et al., 2007)
	IL-22 anti-sense	AGAACGTCTTCCAGGGTGAA	
	IL-22 probe	TGAGCACCTGCTTCATCAGGTAGCA	
IL-4	IL-4 sense	ACAGGAGAAGGGACGCCAT	(Giulietti et al., 2001)
	IL-4 anti-sense	GAAGCCCTACAGACGAGCTCA	
	IL-4 probe	TCCTCACAGCAACGAAGAACACCACA	
IL-5	IL-5 sense	CCG CTC ACC GAG CTC TGT T	Designed by Primer 3 software
	IL-5 anti-sense	AGA TTT CTC CAA TGC ATA GCT GG	
	IL-5 probe	CAG GAA GCC TCA TCG TCT CAT TGC TTG T	
IP-10	IP-10 sense	GCCGTCATTTTCTGCCTCAT	(Giulietti et al., 2001)
	IP-10 anti-sense	GCTTCCCTATGGCCCTCATT	
	IP-10 probe	TCTCGCAAGGACGGTCCGCTG	
Perforin	Perforin sense	GAAGACCTATCAGGACCAGTACAACTT	(Halloran et al., 2004)
	Perforin anti-sense	CAAGGTGGAGTGGAGGTTTTTG	
	Perforin probe	ACCAGGCGAAAACTGTACATGCGACACT	

Rev-Erbα	Rev-erba forward	GGCAAGGCAACACCAAGAA	(Panda et al., 2002)
	Rev-erba reverse	GGCCGCTGCGTCCAT	
	Rev-erba probe	GTTCTGCTGGCATGTCCCATGAACATG	
RORα	Rora sense	CAATGCCACCTACTCCTGTCC	(Lau et al., 2004)
	Rora anti-sense	GCCAGGCATTTCTGCAGC	
RORC (human)	RORC Fw	TTTTCCGAGGATGAGATTGC	(Manel et al., 2008)
	RORC Rev	CTTTCCACATGCTGGCTACA	
RORγt	RORgt sense	CCGCTGAGAGGGCTTCAC	(Ivanov et al., 2006)
	RORgt anti-sense	TGCAGGAGTAGGCCACATTACA	
	RORgt probe	AAGGGCTTCTTCCGCCGCAGCCAGCAG	
T-bet	T-bet sense	GCCAGGGAACCGCTTATATG	(Lighvani et al., 2001)
	T-bet anti-sense	GACGATCATCTGGGTCACATTGT	
	T-bet probe	ACCCAGACTCCCCCAACACCGG	

2.1.4 ANTIBODIES FOR ELISA

The following antibody pairs were used for cytokine quantification by ELISA:

Antibodies	Anti-mouse specificity	Clone	Antibody class	Company
Purified capture antibodies	IFNγ	AN-18	Rat IgG1, k	BD Pharmingen
	IL-17	eBio17CK15A5	Rat IgG2a, κ	eBioscience
	GM-CSF	kit	Kit	Peprotech
	IL-10	kit	Kit	R&D systems
	IL-4	BVD4-1D11	Rat IgG2b	BD Pharmingen
	IL-5	TRFK5	Rat IgG1	BD Pharmingen
Biotinylated detection antibodies	IFNγ	XMG1.2	Rat IgG1, k	eBioscience
	IL-17	eBio17B7	Rat IgG2a, κ	eBioscience
	GM-CSF	kit	Kit	Peprotech
	IL-10	kit	kit	R&D systems
	IL-4	BVD6-24G2	Rat IgG1	BD Pharmingen
	IL-5	TRFK4	Rat IgG2a	BD Pharmingen

2.1.5 ANTIBODIES FOR FLOW CYTOMETRY

The following antibodies against extracellular markers and intracellular cytokines were used for flow cytometric analysis:

Specificity	Label	Clone	Antibody class	Company
CD25	PE	PC61	Rat IgG1, κ	BD Pharmingen
CD3e	FITC	145-2C11	Armenian Hamster IgG1, l	BD Pharmingen
CD3e	APC	145-2C11	Armenian Hamster IgG1, l	BD Pharmingen
CD4	PercP	RM4-5	Rat IgG2a, k	BD Pharmingen
CD62L	FITC	MEL-14	Rat IgG2a	Immuno Tools
CD69	PE	H1.2F3	Armenian Hamster Ham IgG1, l3	BD Pharmingen
CD8	APC	53-6.7	Rat IgG2a, k	BD Pharmingen
IFNγ	FITC	XMG1.2	Rat IgG1	BD Pharmingen
IFNγ	APC	XMG1.2	Rat IgG1	BD Pharmingen
IL-17	PE	TC11-18H10	Rat IgG1	BD Pharmingen
IL-4	FITC	BVD6-24G2	Rat IgG1, κ	eBioscience
IgG1 isotype control	FITC	eBRG1	Rat IgG1	eBioscience
IgG1 isotype control	PE	eBRG1	Rat IgG1	eBioscience
IgG2a isotype control	PercP	R35-95	Rat IgG2a,k	BD Pharmingen
IgG2a isotype control	APC	eBR2a	Rat IgG2a	eBioscience
MHC class II (I-A/I-E^b)	Biotin	2G9	Rat IgG2a, k	BD Pharmingen
Streptavidin	APC	-	-	eBioscience
TNFα	FITC	MP6-XT22	Rat IgG1	BD Pharmingen
Vα3.2 TCR	FITC	RR3-16	Rat IgG2b, k	BD Pharmingen
Vβ11 TCR	PE	RR3-15	Rat IgG2b, k	BD Pharmingen

2.2– METHODS

2.2.1 MICE

All animals used in this study were from C57BL/6 background and bred in the animal facilities of the Max Planck Institute of Biochemistry and Neurobiology. The animal procedures were in accordance with guidelines of the committee on animals of the Max Planck Institute for Neurobiology and with the license of the Regierung von Oberbayern (Munich, Germany). Optico-Spinal Encephalomyelitis (OSE) mice, a spontaneous EAE model (Krishnamoorthy et al., 2006), were obtained by crossing TCR^{MOG} x IgH^{MOG} single transgenic animals. TCR^{MOG} mice, also known as 2D2, express a transgenic TCR recognizing autoantigen MOG aa 35–55 peptide in the context of $I\text{-}A^{b}$ on most $CD4^{+}$ T cells (Bettelli et al., 2003); and IgH^{MOG} mice, also known as Th, express a demyelinating MOG-specific Ig heavy-chain in the antibodies-producing B lymphocytes (Litzenburger et al., 1998). The beta-actin TNXXL transgenic mouse (not published) bears a genetically encoded FRET-based (CFP/YFP) calcium indicator under the control of the actin promoter (Mank et al., 2008). Other transgenic animals were also used: the RAG2KO, the IL-12p35KO, the IL-12p40KO and the beta-actin GFP transgenic mice.

2.2.2 MICE GENOTYPING

The fluorescent transgenic mice, actin-GFP and TNXXL, were screened with an ultraviolet lamp. The transgenic animals 2D2, Th, Rag2KO, IL-12p35KO and IL-12p40KO were genotyped by either conventional PCR or real-time PCR of genomic DNA extracted from tail. A small piece of tail was clipped and incubated overnight in tail digestion buffer containing proteinase K (Genaxon, Martinsried, Germany) at 55°C. DNA was isolated with addition and mixture of 500 µl of phenol/chloroform/isoamyl alcohol (Roth). After centrifugation at 14000 rpm for 5 min, an aqueous layer was transferred into a new tube. DNA was precipitated with 1 ml of ethanol for 5 min at 14000 rpm and

washed with 70% ethanol. Precipitated DNA was dissolved in 400 µl of 10 mM Tris buffer at pH 8.0 and stored at -20°C. The typical PCR reactions consist of the following:

Typical PCR reaction		Typical real-time PCR reaction	
Tail genomic DNA	1 µl	**Tail genomic DNA**	0.5 µl
10X Buffer	2.5 µl	**ROX 1:50**	0.5 µl
10 µM sense primer	0.5 µl	**10 µM sense primer**	0.5 µl
10 µM anti-sense primer	0.5 µl	**10 µM anti-sense primer**	0.5 µl
10 mM dNTPs	0.5 µl	**10 µM probe**	0.5 µl
***Taq polymerase* (5 units/ml)**	0.1 µl	**Thermomix**	10 µl
H_2O	Up to 25 µl	**H_2O**	Up to 20 µl

Taq polymerase was purchased either from Roche or Invitrogen, and real-time PCR mix was from ABGene.

2.2.3 PREPARATION OF SPLENOCYTES FOR CELL CULTURE

Spleens were removed and a cell suspension was prepared in RPMI by using a 40 µM cell strainer (BD Biosciences). Cells were centrifuged for 10 min at 1200 rpm and the cell pellet was re-suspended in 0.83% NH_4Cl for erythrocyte lysis and incubated in ice for 2 minutes. Cells were washed with medium, centrifuged and re-suspended in complete RPMI for further cell culture.

2.2.4 ANTIGENS

MOG peptide 35-55 (MEVGWYRSPFSRVVHLYRNGK) was synthesized at BioTrend, Germany. Recombinant MOG protein (MOG 1-125) was purified from bacterial inclusion bodies (Amor et al., 1994). Expression plasmid pQE-12 containing rat MOG 1-125 was grown in LB medium containing 100 mg/ml ampicillin (Sigma) and 25 mg/ml kanamycin (Sigma). The bacterial cultures were induced with isopropyl thiogalactoside (IPTG) and

then pelleted. The pellet was re-suspended in lysis buffer with lysozyme and sonicated. Lysed samples were washed, suspended in solubilization buffer and loaded in a Ni-NTA column prepared with chelating sepharose and 1% NiCl2 (Amersham biosciences). The column was washed with wash buffer and eluted with elution buffer. Eluted MOG was dialyzed against 20 mM sodium acetate at pH 3.0.

2.2.5 ANTIBODY PURIFICATION

α-IL-4 and α-IFNγ producing hybridomas were grown in cell stack (Corning) with supplemented DMEM. Culture supernatants were harvested and filtered to remove cell debris. Supernatants were applied to a protein G column (Amersham Biosciences) and washed extensively with binding buffer to remove non-binding proteins. Antibody was eluted from the column with the elution buffer and neutralized with the neutralization buffer. The antibody-containing fractions (measured by OD 280 nm) were pooled and dialyzed against PBS for 2 days with buffer exchange after 24 hours.

2.2.6 *IN VITRO* $CD4^+$ HELPER T CELL POLARIZATION

Th1 and Th17 polarized cells were obtained after optimization of protocols described previously (Bettelli et al., 2006b;Veldhoen et al., 2006a). Briefly, 20 x 10^6 OSE or 2D2 total erythrocyte-lysed spleen cells per well were cultured for 6 days in the presence of 20 μg/ml recombinant MOG in 6 well-plates with the total volume of 3 ml. For Th0, Th2, Th1, Th17 and Treg polarization, the following cytokines and antibodies were further added in the culture. Th0: α-IFNγ (10μg/ml) and α-IL-4 (10μg/ml); Th1: IL-4 (50ng/ml) and α-IFNγ (10μg/ml); Th1: IL-12 (10ng/ml), IL-18 (25ng/ml) and α-IL-4 (10μg/ml); Th17: human TGF-β1 (5ng/ml), IL-6 (20ng/ml), IL-23 (10 ng/ml), α-IL-4 (10μg/ml) and α-IFNγ (10μg/ml); Treg: human TGF-β1 (20ng/ml) and IL-2 (10ng/ml). All cytokines were purchased from Peprotech except IL-23 (R&D) and IL-18 (MBL). The neutralizing antibodies were produced *in house* as previously described. Th0, Th1 and Th2 cells were supplemented with IL-2 (10ng/ml, Peprotech) and Th17 cells with IL-23 (10 ng/ml) at day 3 of culture. At day 6, living cells were purified by Nycoprep (Axis-Shield) gradient (density=1,077) by overlaying total cells in the same volume of Nycoprep, spinning at

600g for 20 min. Living cells were recovered from the interface and washed in RPMI. Subsequently, CD4$^+$ T cells were magnetically isolated by negative selection (R&D Systems) according to manufacturer's instructions, yielding > 95% purity. Purified CD4$^+$ T cells (approximately 5 x 10^6 cells /well) were polarized for a second time in the same conditions and in the presence of irradiated (300 Rad) splenic antigen presenting cells for additional 3 days. By day 9, activated living cells were again purified by Nycoprep, yielding a > 99% pure CD4$^+$ population. Th2 All cultures were performed in complete RPMI-1640 media.

2.2.7 T CELL PROLIFERATION ASSAY

MOG-specific CD4$^+$ T cells activated in Th1 and Th17 polarizing conditions were stimulated in triplicates (4 x 10^4 T cells/well) in the presence of irradiated splenic APCs (2×10^5 cells/well) for 72h. Antigen specific T cell proliferation was measured by adding 20μg/ml of recombinant MOG or MOG 35-55 peptide, or 1μg/ml of α-CD3 in 96-well, round-bottomed plates in a total volume of 200 μl. 1 μCi of [^{3}H]-thymidine was added during the last 18h and incorporated radioactivity was measured in the Beta counter.

2.2.8 ADOPTIVE TRANSFER EAE

At day 9 of polarization, activated MOG-specific CD4$^+$ Th1 and Th17 cells were suspended PBS, counted and injected intravenously into Rag2KO recipient mice. Each animal received 300 μl of total 5 to 10 x 10^6 of Th1, Th17 or Th1 plus Th17 cells (proportion of 1:2) and animals were evaluated every 1-2 days for clinical symptoms. The classical EAE scores were given as below: score 0 – no disease; score 0,5 – reduced tail tonus; score 1: limp tail; score 1,5 – limp tail and ataxia; score 2 – limp tail, ataxia and hind limb weakness; score 2,5 – at least one hind limb paralysed/weakness; score 3 – both hind limbs paralyzed/weakness; score 3,5 –complete paralysis of hind limbs; score 4 – paralysis until hip; score 5 – moribund or dead. The non-classical (atypical)

EAE scores were given as follows: score 0 – no disease; score 1 – head turned slightly (ataxia, no tail paralysis); score 2 – head turned more pronounced; score 3 – inability to walk on a straight line; score 4 – laying on side; score 4,5 – rolling continuously unless supported; score 5 – moribund or dead.

2.2.9 ACTIVE IMMUNIZATION EAE

Mice were subcutaneously injected at the tail base with 200-500 μl of an emulsion containing equal volumes of CFA (Difco) and 200 μg MOG 35-55 in PBS. CFA was supplemented with 5 mg/ml *Mycobacterium tuberculosis* (strain H37Ra; Difco). 400 ng of pertussis toxin were injected intraperitoneally on days 0 and 2 following immunization. Clinical symptoms were evaluated as previously described.

2.2.10 MONONUCLEAR CELLS ISOLATION FROM CNS TISSUE AND LYMPHOID ORGANS

2-4 days after mice reached the peak of disease, typically scores between 3 and 4, brain, spinal cord, spleen and axilliary plus inguinal lymph nodes were removed for mononuclear cells isolation. Briefly, mice were anesthetized, perfused transcardially through left ventricle with cold PBS and the organs were removed and homogeneized in RPMI-1640 media with no FCS. The suspension was passed through a 40 μm nylon mesh (BD Biosciences). Brain and spinal cord suspensions were centrifuged, re-suspended in 30% Percoll (GE Healthcare), overlaid on 70% Percoll and centrifuged for 20 min at 1200g at room temperature. After centrifugation, the interface, containing the mononuclear cells, was removed, washed with RPMI and resuspended in 10% FCS RPMI. The mononuclear cells from lymphoid organs were isolated as previously described for splenocytes, with exception of erythrocyte lysis in lymph nodes.

2.2.11 FLOW CYTOMETRY (FACS)

For intracellular cytokine staining, cells were stimulated for 4 hours with PMA (50ng/ml), ionomycin (0.5μg/ml) and brefeldin A (5μg/ml) (Sigma Aldrich). Cells were

first stained extracellularly in FACS buffer for 20 minutes on ice, whashed, then fixed and permeabilized with 2% PFA and saponin buffer for 10 minutes, washed and finally stained intracellularly for cytokines in saponin buffer for 30 minutes on ice. After washing and re-suspension in FACS buffer, samples were acquired on a FACSCalibur (BD Bioscience) and data were analysed with CellQuest (BD Bioscience) and Flow Jo version 7.2.5 (Tree Star) softwares.

2.2.12 ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA)

Cytokine production was determined by ELISA using matching antibody pairs. Culture supernatants were collected and frozen at -20°C until quantification. ELISA plates (Nunc) were coated with 100 μl of capture antibodies (1μg/ml in 0.1M $NaHCO_3$ pH 9.0) for overnight at 4°C. Plates were washed with wash buffer and blocked with assay diluents for 1 hour. Plates were again washed and incubated with 100 μl of sample supernatants and serially diluted cytokine standards (BD Biosciences) for 2 hours at room temperature. Subsequently, plates were again washed and incubated for 1 hour with the biotinylated detection antibody. After extensive washing, streptavidin-horseradish peroxidase (HRP) diluted 1:2000 (BD Pharmingen) was added and incubated at room temperature for 30 min. Finally, after the final washing, 100 μl of 2,2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS; Sigma-Aldrich)) activated with H_2O_2 was used as a substrate for colorimetric cytokine determination, and plates were read at 405 nm in an ELISA reader (Victor2 1420 multilabel counter, Perkin Elmer).

2.2.13 QUANTITATIVE REAL-TIME PCR ANALYSIS

Total cell RNA was isolated with TRI Reagent extraction (Sigma-Aldrich). Cells were suspended in 1 ml of TRI Reagent, 200 μl of chloroform was added, the mixture was intensively vortexed and centrifuged at 14000 rpm for 15 min. The aqueous phase was removed and RNA was precipitated with 500 μl of isopropanol. RNA was washed in 1 ml

of 75% ethanol and finally dissolved in RNase-free water. RNA, 1-4 μg, was treated with DNase I and then reverse transcribed cDNA using oligo-dT primers and SuperScript II Reverse Transcriptase (Invitrogen), all according with manufacturer´s instructions. Sense and antisense primers in combination with SYBR Green or FAM/TAMRA TaqMan fluorescent probes (all from Metabion, Martinsried, Germany) were used for PCR analysis. Where possible, the primer/probe sequence combinations spanned contact sequences of subsequent exons. For amplification, the Absolute QPCR mix was used (ABgene). Each reaction was run in triplicate on an ABI 7900 machine (Applied Biosystems) and was normalized to housekeeping gene GAPDH transcripts. Primary data was analyzed with Gene-Amp SDS version 2.3 software (Applied Biosystems).

2.2.14 HISTOLOGICAL ANALYSIS

Animals were anesthetized with ether and perfused through left ventricle with cold PBS and then with 4% paraformaldehyde in PBS, stored in the same fixative for 24 hours at 4°C, washed twice with PBS, and finally kept at 4°C until used. For confocal microscopy, fixed organs were stored for some days at 4°C in 25% sacarose solution. Brain and spinal cord tissue was dissected and in part embedded in paraffin, or snap frozen in Tissue Tek OCT compound on dry ice for immunohistochemistry. Adjacent serial sections were stained with haematoxylin (H&E), luxol fast blue (LFB), or Bielschowsky silver impregnation (Biel). All histological analysis was performed by Prof. Hans Lassmann, University of Vienna, Austria.

2.2.15 FLUORESCENT IMMUNOHISTOCHEMISTRY AND CONFOCAL MICROSCOPY

Frozen tissue sections (20 μm) were washed with PBS, blocked with 4% BSA + 4% goat serum in PBS for 2h and incubated overnight at 4°C with 1μg/ml rat anti-CD4 (RM4-5, BD Biosciences — Pharmingen), 1μg/ml rabbit anti-Iba-1 (Wako) or 2μg/ml rabbit anti-GFAP (Sigma) primary antibodies in 4% BSA + 1% goat serum + 0,1% Triton X-100 in PBS. Slides were washed in 0,3% Triton X-100 in PBS and incubated in the dark for 90 minutes with secondary rabbit anti-rat Alexa 488 and goat-anti-rabbit Alexa 594 (1:200)

antibodies (Molecular Probes), which allowed the cell type specific detection of T cells, microglia and astrocytes. After new washing, slides were counterstained for 5 minutes with 1μg/ml nuclear DAPI dye and mounted with Gel Mount Aqueous Mounting Medium (Sigma) prior to microscopic analysis. Images were taken using an inverted Leica TCS SP2-laser scanning confocal microscope with a 40× /1.25 oil objective. Individual sections represent the mean of 4 scans of approximately 1μm axial resolution and images consist of 8-16 optical sections combined to form a through-focus image. Images were grouped into a single canvas using Image J and Adobe Photoshop software.

2.2.16 ASTROCYTES PRIMARY CULTURE

Primary astrocyte cell cultures were obtained from 2-days-old beta-actin GFP or TNXXL transgenic C57BL/6 mouse pups. Briefly, pups were decapitated and heads removed and placed in a Petri dish. Skin and scull were cut midline with scissors and the brain was removed and placed into new Petri dishes with 15 mM of Hepes in Hanks Balanced Salt Solution (HBSS) (Gibco). Meninges were removed from both cortex and cerebellum. Brains were homogenized in 15 mM of Hepes in HBSS with 1 ml tip and a 27G syringe and then dissociated with incubation at 37 °C for 10 minutes in 2mg/ml of trypsin solution. After washing, cells were suspended in supplemented DMEM (Gibco), passed through a 70 μm cell strainer, and plated in a T75 flask. Cells were allowed to grow for 8 to 10 days and media was changed every 3 days. Cells were shaken overnight at 90rpm to remove contaminating oligodendrocytes, microglia and neurons. Adherent cells contain a majority of astrocytes whose purity was increased with trypsinization and further passages. Experiments were done with astrocytes with a minimum of 2-3 passages.

2.2.17 FT7.1 CELL LINE CULTURE

The fibroblast cell transfectants FT7.1, which overexpress the mouse I-A^b MHC class II molecule on their surface, were cultured in RPMI media as described above plus the selective reagents: mycophenolic acid (2.5mg/100ml), xanthine (25mg/100ml) and hypoxanthine (2.5mg/100ml) (Sigma). For the use in time-lapse fluorescent microscopy experiments, FT7.1 cells were retrovirally transduced with GFP (pMSCVneo-IRES2-eGFP). Briefly, Phoenix ecotropic cells were transfected with 10 μg of pMSCVneoIRES2-eGFP DNA plasmid plus 3,75 μg pCL-Eco using FuGENE (Roche). 48h later, virus containing Phoenix supernatant was filtered and spinned overnight at 6000g. In the following day, plated FT7.1 cells were transduced with concentrated virus supernatant for 1 hour at 2000rpm in the presence of 8mg/ml polybrene. GFP-tranduced cells were selected by neomycin resistance by culture in the presence of 1 μg/ml G418 (Sigma). GFP^{high} FT7.1 cells were further selected by FACS sorting.

2.2.18 T CELLS AND ASTROCYTES OR FT7.1 CELLS CO-CULTURE EXPERIMENTS

2 days before the co-culture, astrocytes were trypsinized, irradiated with 30 Gy and plated $4x10^4$ cells per well in 96-well plate. On the following day, adherent astrocytes were stimulated with IFNγ and TNFα, 10ng/ml each (Peprotech), and GFP-FT7.1 cells were plated, $2.5x10^4$ cells per well in 96-well plate. On day 9 of T cell polarization, T cells were added to overnight stimulated astrocytes or FT7.1 cells in a ratio of 1:10, in the presence or absence of 20 μg/ml MOG 35-55 peptide. The following blocking antibodies and inhibitors were used: 10μg/ml α-MHC class II (α-I-A/I-E^b), 10μg/ml α-IFNγ (clone R4-6A2), 10μg/ml α-Fas-L, 10μg/ml isotype control α-IgG2a (all from BD Pharmingen), 25 μM Granzyme B inhibitor II (Calbiochem) and 25 μM caspase inhibitor Z-VAD (Calbiochem). Cytotoxicity was evaluated by Giemsa staining and fluorescent time-lapse microscopy for 24-48h using the MetaMorph and MetaFluor softwares (Molecular Devices), respectively. The quantification was done based on the measurement of the fluorescent cell covered area. The background area was corrected manually for every image (method found to manipulate less the original data) and a

threshold was applied in order to quantify the threshold area. The data obtained were normalized to the control (no T cells co-cultured) for each time point, every 6 hours.

2.2.19 MICROARRAYS AND PATHWAY ANALYSIS

RNA was prepared from CD4$^+$ T cells, either naive or polarized in Th1 and Th17 conditions at day 9 (activated state) and day 12 (resting state), using TRI Reagent as previously described. RNA was amplified with Illumina® TotalPrep RNA Amplification Kit according with manufacturer's instructions (Ambion) and the resulting labeled cRNAs were hybridized with Illumina's direct hybridization array kits (Mouse WG-6-V1_1 BeadChip, Ilumina). RNA integrity was analyzed on the Agilent Bioanalyzer (RNA 6000 Nano Chip Kit) before and after amplification. The scanning and image analysis were done in an Illumina Array reader and Bead Scan, and data quality control was validated with the Illumina Bead Studio. Data analysis and annotation was performed with the R based custom analysis and pathway analysis was done in Pathway Studio 5 software (Ariadne Genomics).

2.2.20 PLASMIDS AND GENE CLONING

The pcDNA3 plasmids for human RORC and mouse Rev-Erbα and Rev-Erbβ were a kind gift from Toru Takumi, Japan. Mouse RORα gene, obtained by PCR, was subcloned in the pcDNA3.1 plasmid (Invitrogen). The retroviral plasmid vector pMSCVneoIRES2-eGFP containing a neomycin resistant cassette, an IRES2 sequence and the enhanced green fluorescent protein (eGFP) gene [kind gift from Naoto Kawakami neighbor group that subcloned the IRES-eGFP from the plasmid pIRES2-eGFP (Clontech) into the pMSCVneo vector (Clontech)] was used to subclone human RORC and mouse RORα, Rev-Erbα and Rev-Erbβ from the pcDNA3 vectors in the multiple cloning site (MCS) by the use of specific restriction enzymes (New England Biolabs) with the help of the software Vector NTI Advance™ 11 (Invitrogen). The dominant negative (DN) forms of Rev-Erbα and Rev-

Erbβ were obtained by PCR on the respective genes (from the pcDNA3 vectors), where the primers were specifically designed to exclude their LBD (present in the C-terminal) and include specific enzyme restriction sites. The GFP gene was used as a reporter to provide evidence of successful transfection, transduction and expression of the foreign genes. The retroviral plasmid vector pMSCVneoIRES2-eGFP and the genes DNA were all digested with appropriated enzymes (New England Biolabs) and the resulting products were ligated with ligase (New England Biolabs). In order to isolate plasmid DNA, *E. coli* DH5a competent cells (Invitrogen) were heat-shock transformed with plasmid DNA according to manufacturer's instructions, a single colony was used to grow in LB medium plus amplicilin and DNA was isolated with a Mini or Midiprep (Qiagen) according to manufacturer's instructions. The recombinant retroviral plasmid vector was identified by restriction endonuclease analysis and DNA sequence analysis.

2.2.21 EL-4 CELLS TRANSFECTION

EL-4 cells, a mouse T cell lymphoma cell line derived from ascetic fluid of C57BL/6N, were cultured in complete RPMI medium and splited frequently to avoid overgrowth. EL-4 cells were stably transfected with FuGENE HD reagent (Roche) with the pcDNA3 plasmids for human RORC and mouse Rev-Erbα and Rev-Erbβ, RFP or empty as a control. 2 μg of DNA plasmid were complexed with 5 μl of FuGENE HD reagent and added to the cultures as described by the product instructions. The positively transfected cells were selected with 1 μg/ml of G418 antibiotic for several days and used for gene expression and cytokine production analysis.

2.2.22 LUCIFERASE ASSAY IN EL-4 CELLS

EL-4 cells were transfected by nucleofection (Amaxa) according to manufacturer's instructions with 2 μg of pGL4 reporter vector containing 2kb fragment of the IL-17 promoter and the *Renilla* luciferase gene, 0.5 μg of pRL-TK (firefly luciferase) and 2 μg of the pcDNA3 plasmids for human RORC, mouse Rev-Erbα or empty control. After overnight incubation, transfected cells were stimulated for 6h with 20 nM PMA (Sigma)

and 2 μM ionomycin (Sigma). Cells were lysed and luciferase activity was measured according with the dual-luciferase assay system (Promega) instructions.

2.2.23 PHOENIX ECOTROPIC CELLS TRANSFECTION AND CD4$^+$ T CELL TRANSDUCTION

The Phoenix ecotropic packaging cell line (ΦNX) was grown in complete DMEM medium and splitted everyday to avoid overgrowth. On the first day, Phoenix cells were seeded in a 10 cm dish; on day 2, the same cells were transferred to a 15 cm dish and on day 3 were splitted into 10x 10 cm dishes. By day 4, Phoenix cells were transfected with FuGENE as previously described, with the retroviral pMSCV vectors: 10 μg of pMSCV-cDNA, 3.75 μg of pcL-ECO and 25 μl FuGENE HD per 10 cm dish and incubated at 32°C. Phoenix cells confluency for transfection was approximately 50%. On the following day, CD4 cells were isolated from spleen by negative selection magnetic isolation (R&D Systems), and 2 x 10^6 cells per well in a 6-well-plate were stimulated with plate-coated 0.5 μg/ml α-CD3 and 1 μg/ml α-CD28. Also, Phoenix cells were fed with fresh medium. 48 hours post-transfection, the virus-containing supernatant was recovered, spun to remove cell debris, filtrated through a 0.45 μm filter and centrifuged overnight at 6000g, room temperature. Fresh medium was again added to Phoenix cells. On the following day, 48 hours post-T-cell stimulation, new supernatant from Phoenix cells was recovered with same procedure and was used to re-suspend the virus pellet. Cells were transduced by adding the concentrated virus containing supernatant in the presence of 8 μg/ml of polybrene and centrifuged for 1 hour at 2000 rpm, room temperature. Cells were allowed to rest at 37°C for 2-4 hours and then the virus-containing medium was replaced by RPMI medium containing APCs and Th17 polarizing cytokines. Intracellular cytokine staining was done 2 to 3 days post-polarization.

2.2.24 STATISTICS

Descriptive statistical analysis was performed using Prism version 5 software (GraphPad). Differential EAE incidence was analyzed by log-rank (Mantel-Cox) test by an in-built survival curve analysis. One- and two-way ANOVA statistical analysis were used in the other studies. *P* values less than 0.05 were considered to be significant.

CHAPTER 3 *IN VIVO* ANALYSIS OF TH1 AND TH17 CELLS IN ADOPTIVE TRANSFER EAE

3.1 – SUMMARY

MS is an autoimmune disease that affects the CNS and is characterized by inflammation, demyelination and axon destruction. The processes that are involved in triggering the onset and driving inflammation, cellular composition and distribution of autoimmune lesions during the course of MS are not completely understood (Sospedra and Martin, 2005). Yet, the EAE animal model representing human MS has proved a central role for myelin-autoimmune CD4$^+$ helper T cells playing in the initiation of inflammatory demyelination of the CNS. Traditionally, EAE was considered to be an autoimmune disease exclusively mediated by Th1 cells, which secrete IFNγ as a signature cytokine. However, studies using IL-12 and IL-23 subunits-deficient mice identified a new CD4$^+$ T cell subset, Th17 cells, as the encephalitogenic effector helper T cells in autoimmunity (Kastelein et al., 2007;Weaver et al., 2007). Nevertheless, most EAE induction protocols used require complete Freund adjuvant (CFA) together with the auto-antigen, which distorts the immune response. Additionally, the study of two transgenic mouse strains from different genetic backgrounds developed in this lab (Krishnamoorthy et al., 2006;Pollinger et al., 2009) that spontaneously develop EAE revealed the presence of inflammatory CNS lesions, comprising both Th1 and Th17 cells. This raised the possibility that both T cell lineages, Th1 and Th17, may participate in the autoimmune pathogenesis perhaps with different effector functions.

The present study was aimed at evaluating the pathogenic potential and the individual functions of myelin-specific Th1 and Th17 CD4$^+$ T cells, both *in vitro* and *in vivo* in the adoptive transfer EAE model. To achieve this, we established polarized Th1 and Th17 populations, derived from MOG-specific TCR transgenic mice with C57BL/6 genetic background. These cells were adoptively transferred, individually or as combination, to Rag2 deficient animals, deficient in B and T cells, to test their encephalitogenic potential.

Our experiments make several points. First, they indicate that both Th1 and Th17 cells alone are capable of inducing EAE. Second, the clinical disease mediated by either of these $CD4^+$ T cell lineages differs remarkably. While Th1 cell mediated disease affects the spinal cord, resulting in hind limb paralysis, Th17 cells transfer a disease with prominent ataxic gait disturbance. Thirdly, combinations of Th1 and Th17 cells display a higher encephalitogenic capacity than the two lineages transferred separately. Finally, we found strong evidence for T cell plasticity *in vivo* by conversion of transferred Th17 cells into a Th1 phenotype in the CNS, suggesting a pathogenic role for Th1 cells in CNS autoimmunity.

3.2– RESULTS

3.2.1 DEVELOPMENT OF A METHODOLOGY FOR *IN VITRO* TH1 AND TH17 POLARIZATION

In contrast to culture conditions driving Th1 polarization, which are very well defined, those for Th17 cells have been described only recently (Bettelli et al., 2006b). However, in order to obtain sufficient numbers of T cells for the adoptive transfer EAE, these conditions had to be optimized to a large scale. Using the protocol described in Methods, we were able to generate pure Th1 and Th17 populations by stimulating MOG-specific spleen cells from TCR transgenic 2D2 mice with recombinant MOG, without contaminating IL-17 and IFNγ producing cells in Th1 and Th17 polarizations, respectively. While we obtained a major population, constantly more than 50%, of IFNγ-producing Th1 cells by stimulation in the presence of IL-12 and IL-18, Th17 polarization was less efficient. T cell stimulation in the presence of TGFβ, IL-6 and IL-23 induced 20-50% of IL-17 single positive cells. The 2D2 MOG-specific T cells co-expressed the transgenic TCR Vα and Vβ chains in more than 90% (**Figure 3.1a**). ELISA results confirmed that Th1 cells produced IFNγ and Th17 cells produced large amounts of IL-17 in a mutually exclusive manner (**Figure 3.1b**). Also, the specific transcription factors for

Th1 and Th17 differentiation, T-bet and RORγt respectively, together with IFNγ and IL-17 were expressed only in their corresponding T cell subset (**Figure 3.1c**).

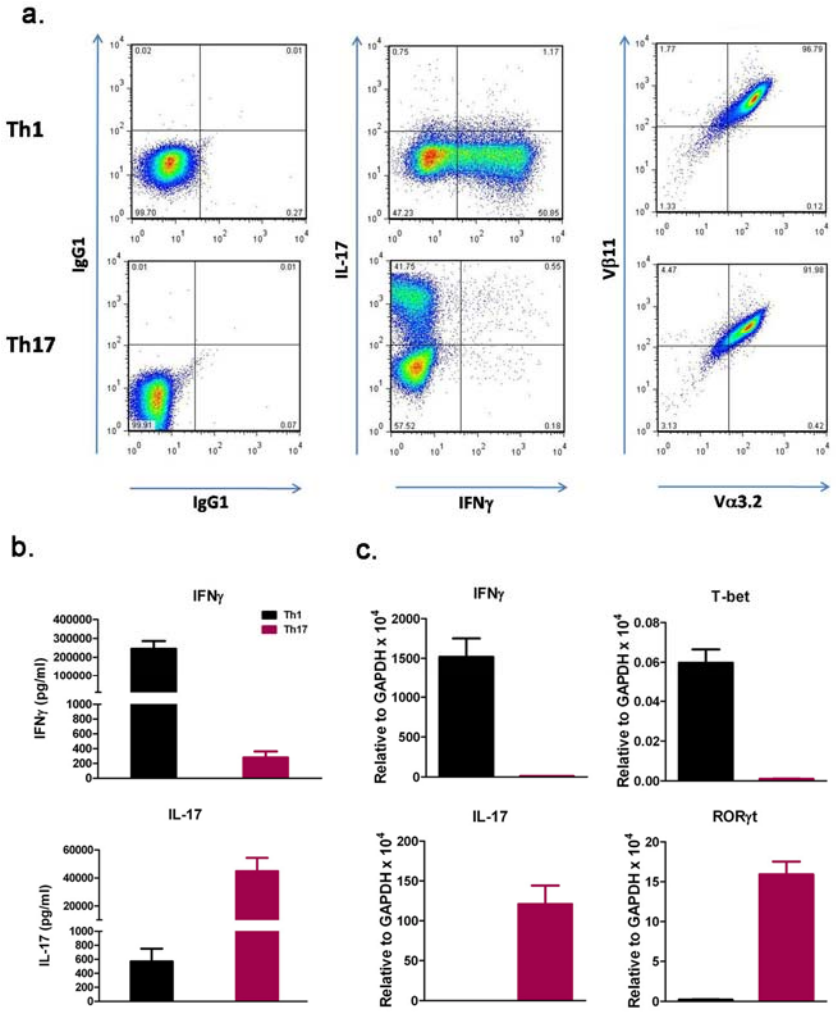


Figure 3.1 – MOG-specific Th1 and Th17 cell differentiation. A. Naive T cells from 2D2 mice were activated under Th1 and Th17 polarizing conditions for 9 days. Vα3.2 and Vβ11 transgenic TCR chains, as well as IL-17 and IFNγ cytokine expression was assessed by FACS extracellular and intracellular staining, respectively. Data shown are gated in the CD4$^+$ population. B. IFNγ and IL-17 cytokines from culture supernatants of Th1 and Th17 polarized cells at day 9 were quantified by ELISA. C. IFNγ, Tbet, IL-17 and RORγt gene expression was quantified by real-time PCR of Th1 and Th17 polarized cells by day 9. Data shown are representative or a mean of a minimum of 5 experiments.

Additionally, IL-4 and IL-5 were not produced by Th1 or Th17 but only by Th2 cells. However, IL-10 was found to be produced in both Th1 and Th17 cells of levels comparable to Th2 cells (**Figure 3.2a**). Gene expression analysis of IL-4 and GATA-3, the transcription factor important for Th2 differentiation, showed higher expression of these molecules in Th17 than in Th1 cells, though there are no comparative values from Th2 cells (**Figure 3.2b**).

Together, these results show the development of stable protocols for obtaining pure MOG-specific Th1 and Th17 populations for the adoptive transfer EAE.

3.2.2 *IN VITRO* POLARIZED TH1 AND TH17 CELLS DISPLAY DIFFERENT CHARACTERISTICS

The expression of GM-CSF, an important inflammatory cytokine found to be important in EAE pathogenesis and expressed in Th17 cells was compared in Th1 and Th17 cells. Contrary to our expectations, GM-CSF was expressed and produced exclusively in Th1 but not in Th17 cells (**Figure 3.2c**).

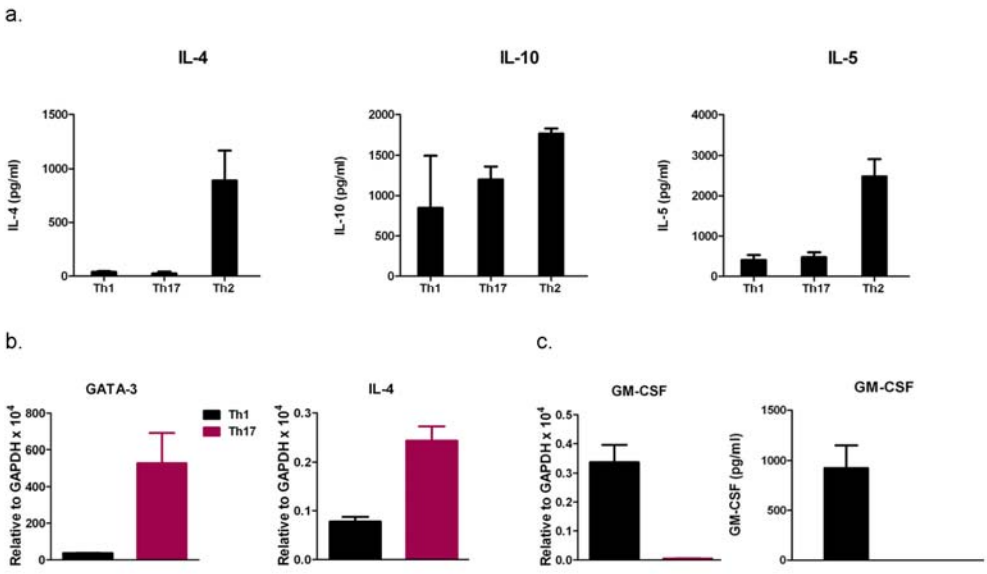


Figure 3.2 – Th2-related cytokines and GM-CSF produced by Th1 and Th17 polarized cells. A. IL-4, IL-5 and IL-10 from culture supernatants from Th1, Th17 and Th2 polarized cells at day 9, as described in Figure 3.1, were quantified by ELISA. B. IL-4 and GATA-3 gene expression was

quantified by real-time PCR of Th1 and Th17 polarized cells by day 9. C. GM-CSF gene expression and production quantification in Th1 and Th17 polarized cells at day 9, as described in Figure 3.1. Data shown are representative or a mean of a minimum of 3 independent experiments.

Moreover, the activation status of Th1 and Th17 cells was analysed by flow cytometry. We found that Th1 and Th17 cells exhibit opposite expression of characteristic cell surface activation markers such as CD62L and CD25. All Th17 cells were CD62L^{low} but only around 50% of Th1 cells down-regulated this receptor. On the other hand, Th17 cells did not express CD25 (IL-2 receptor) as did Th1 cells (**Figure 3.3**).

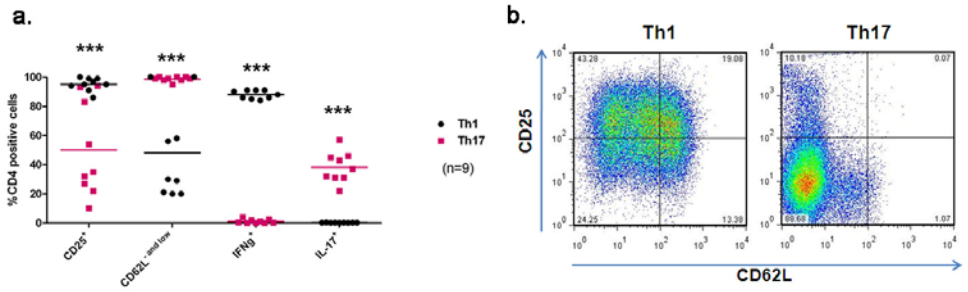


Figure 3.3 – CD25 and CD62L expression by Th1 and Th17 polarized cells. CD25 and CD62L, as well as IL-17 and IFNγ cytokine expression of polarized Th1 and Th17 cells at day 9, as described in Figure 3.1, was assessed by FACS extracellular and intracellular staining. Data shown are gated in the CD4$^+$ population. A. Mean percentage of CD25, CD62L^{low}, IL-17 and IFNγ positive cells in Th1 and Th17 differentiated cells for a total of nine independent experiments analyzed. B. Representative FACS plot of CD25 and CD62L expression in Th1 and Th17 polarized cells. Statistical analysis of data shows a p value < 0.001.

Finally, the antigen reactivity of MOG-specific polarized Th1 and Th17 cells was measured by T cell proliferation assay. Interestingly, we observed that Th17 cells exhibited a higher proliferative response than Th1 cells in response to MOG (**Figures**

3.4a, b). In addition, we consistently obtained much more Th17 cells than Th1 cells, per total number of initial splenocytes, at the end of the polarization period (data not shown). However, in the presence of their corresponding polarizing cytokines, we observe the opposite, Th1 cells proliferating more than Th17 cells (**Figure 3.4c**).

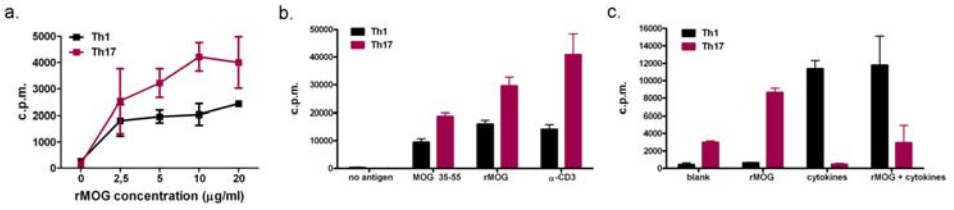


Figure 3.4 – Antigen-specific proliferation capacity of Th1 and Th17 polarized cells. Polarized Th1 and Th17 cells, 6 days post-first stimulation, were re-stimulated in the presence of irradiated antigen-presenting cells and different antigens for 3 days. Antigen-specific proliferation was measured by radioactive ^{3}H-thymidine uptake. A. Proliferation assay of Th1 and Th17 cells with titrated concentrations of recombinant rat MOG. B. Proliferation assay of Th1 and Th17 cells in the presence of 20 μg/ml MOG35-55 peptide, 20 μg/ml recombinant rat MOG or 1 μg/ml α-CD3. C. Proliferation assay of Th1 and Th17 cells in the presence of 20 μg/ml recombinant rat MOG and respective polarizing cytokines (10 ng/ml IL-12 and 25ng/ml IL-18 in Th1 polarization and 10 ng/ml IL-23, 5ng/ml TGFβ and 20 ng/ml IL-6 in Th17 polarization) either alone or in combination. Data shown are representative of a minimum of 3 independent experiments.

3.2.3 BOTH TH1 AND TH17 CELLS ARE ABLE TO INDUCE EAE

In order to evaluate the encephalitogenic potential of CD4^{+} helper T cell subsets in EAE, *in vitro* polarized Th1 and Th17 cells in the activated were transferred either individually or in combination to Rag2KO recipient mice, which are deficient both in B and T cells. By using lymphopenic hosts we could, therefore, evaluate the effector role of these T cell subsets. All recipient mice started to develop clinical EAE symptoms between 11 and 18 days post-transfer. In this model, we always observed 100% EAE incidence with similar severity and day of onset in Th1 and Th17 cells recipients. Interestingly, co-transfer of Th1 and Th17 cells, in a proportion of 1:2 respectively, induced an earlier EAE onset, between 10 and 13 days post-transfer and more severe disease (**Figures 3.5a, b**).

However, the most intriguing observation came from the clinical features produced by Th1 and Th17 cell single adoptive transfers. In contrast to Th1 cell adoptive transfer, where recipients developed in almost all cases the classical EAE phenotype characterized by an ascending paralysis from tail to head, approximately 50% of Th17 cells transferred animals came down with a different neurological picture, which could not be classified by the classical EAE scores. These animals exhibited an ataxic phenotype, with an unbalanced/ataxic gait, and few with a severe axial and barrel rotatory EAE (**Figure 3.5c, Videos 3.1 and 3.2 in CD**). Mice that recovered from such an ataxia eventually developed classical EAE symptoms such as paralysis. However, we rarely observed this type of atypical EAE either in Th1 cells recipient mice or in the co-transfer case.

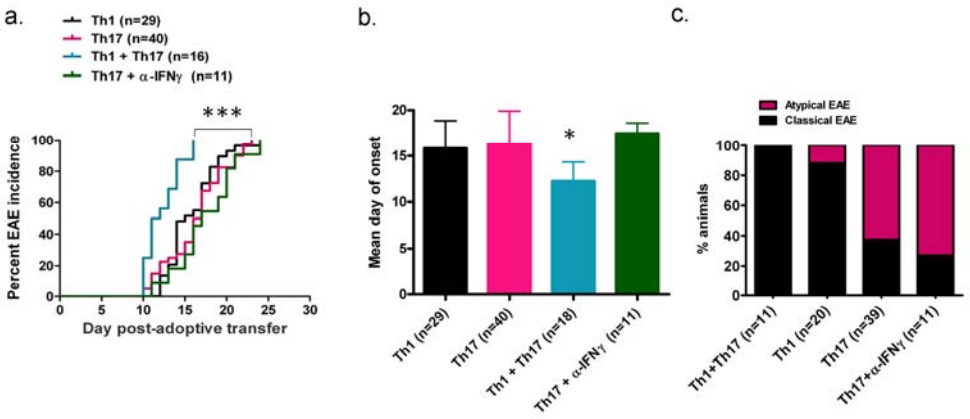


Figure 3.5 – Adoptive transfer EAE with polarized Th1 and Th17 cells. Th1 and Th17 polarized cells at day 9 were adoptively transferred to Rag2KO recipient mice (approximately 5 x 10^6 cells per mouse, intravenously in the tail), either alone or in combination or with α-IFNγ treatment in the Th17 transfer. Recipient mice were daily scored for EAE disease. A. Evaluation of percentage of EAE incidence in the different recipient mice. P value < 0.001 in Th1+Th17 vs. all other conditions. B. Quantification of mean day of EAE onset. P value < 0.05 in Th1+Th17 vs. all other conditions. C. Analysis of the percentage of animals developing classical or atypical EAE phenotype. The number of animals used in each adoptive transfer case is stated in each figure.

The CNS infiltrating mononuclear cells as well as the peripheral lymphoid organs of sick mice with EAE at the peak of disease with minimum score 3 were analysed. Major CNS infiltrates were found in the brain and spinal cord. Characterization of these infiltrating cells yielded surprising results. In contrast to Th1 transferred animals, where only IFNγ-producing $CD4^+$ T cells were found in brain and spleen, both IFNγ and IL-17 producing $CD4^+$ T cells were present in the Th17 cells transferred mice (**Figure 3.6**). In addition, a double positive population, $CD4^+$ $IFN\gamma^+$ $IL\text{-}17^+$ was also found in the CNS of Th17 transferred mice, but not in the periphery. Spinal cord and lymph nodes (axilliary and inguinal) were also analysed and demonstrated similar results (data not shown).

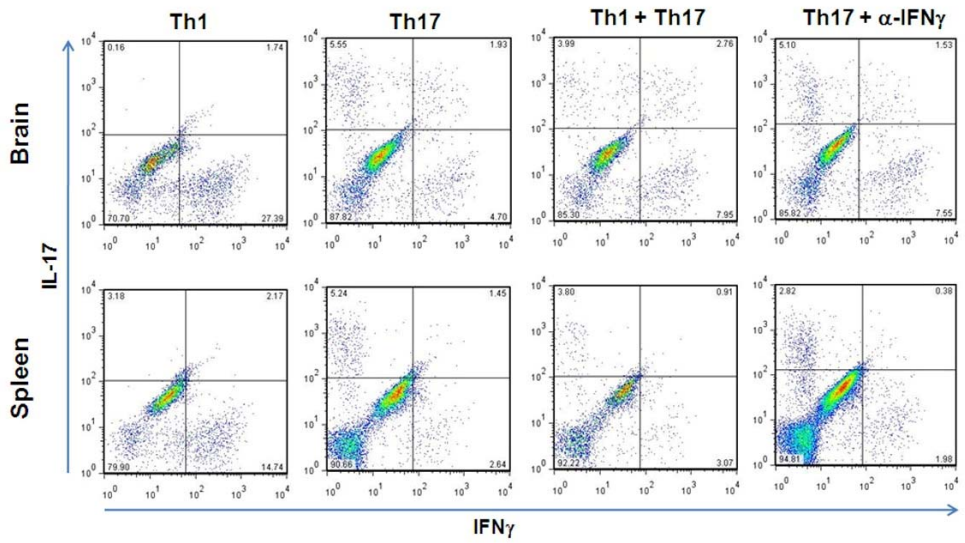


Figure 3.6 – Characterization of CNS infiltrating T cells in adoptive transfer EAE. EAE sick mice from all the adoptive transfer cases (Th1, Th17, Th1+Th17 and Th17+α-IFNγ) with classical EAE, minimum score of 3 were sacrificed and brain, spinal cord, spleen and lymph nodes were removed. Immune cells were isolated and characterized for IFNγ and IL-17 expression by cytokine intracellular FACS. Data shown is gated in the $CD4^+$ population and is representative of a minimum of 5 animals analyzed.

The existence of $IFN\gamma^+$ and $IFN\gamma^+$ $IL\text{-}17^+$ double positive cells in Th17 transferred mice suggested a possible conversion *in vivo* of Th17 cells to the Th1 phenotype. Since IFNγ is a negative regulator of Th17 polarization we evaluated whether IFNγ produced by other

cells in the host could be the responsible for such T cell conversion of phenotype. For that purpose, Th17 cell recipient mice were treated with a blocking antibody for IFNγ. However, we observed no differences in the EAE onset or severity, and the majority of the treated mice developed an ataxic phenotype. Additionally, T cell conversion from Th17 to a Th1 phenotype could not be avoided by blocking IFNγ (**Figures 3.5 and 3.6**). To clarify whether Th1 and Th17 polarizing cytokines, such as IL-12 and IL-23 respectively, released by innate immune cells could have a role in this phenomenon, Th17 cells were transferred into Rag2KO x IL-12p35KO (devoid of IL-12) and Rag2KO x IL-12p40KO recipient mice (deficient both in IL-12 and IL-23). We hypothesized that in both cases, IFNγ production by the host would be compromised, making difficult this change of phenotype to occur. Nevertheless, all mice developed EAE independently of host derived IL-12 of IL-23, although the absence of these cytokines led to delayed EAE onset (**Figure 3.7**). Again, Th17 cells were able to convert to IFNγ producing cells (data not shown).

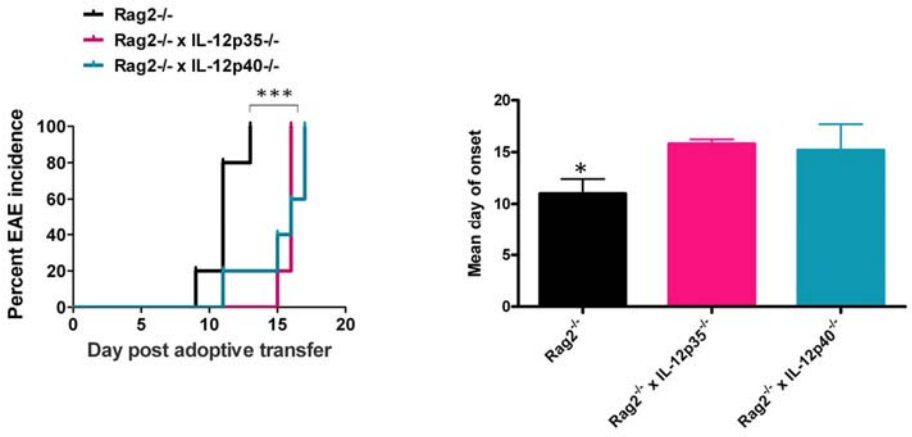


Figure 3.7 – Adoptive transfer EAE of polarized Th17 cells in mice deficient in IL-12 or IL-12 plus IL-23 together. Th17 polarized cells at day 9 were adoptively transferred to Rag2KO, Rag2KO x IL-12p35KO and Rag2KO x IL-12p40KO recipient mice (approximately 5 x 10^6 cells per mouse, intravenously in the tail). Recipient mice were daily scored for EAE disease. A. Evaluation of percentage of EAE incidence in the different recipient mice. P value < 0.001 in Rag2-/- vs. other conditions. B. Quantification of mean day of EAE onset. P value < 0.05 in Rag2-/- vs. other conditions. 5 animals per group were used.

Histology and immunohistochemistry from spinal cord of sick mice from all the adoptive transfers with classical EAE, score 4, was performed. All cases exhibited severe immune cells infiltration and no major differences could be established in terms of demyelination and axonal damage between Th1 and Th17 single transfers, as seen by Haematoxylin & Eosin, Luxol Fast Blue and Bielschowsky's silver stainings. Curiously, the damaged areas in the Th1+Th17 co-transfer comprised all those found in the cases of Th1 and Th17 alone and the extent of demyelination and axonal damage was more severe, which is in agreement with the EAE incidence data. Additionally, infiltrating CD3 and MAC3 positive cells, comprising T cells and phagocytes respectively, were co-localized with the demyelinated areas (**Figure 3.8**).

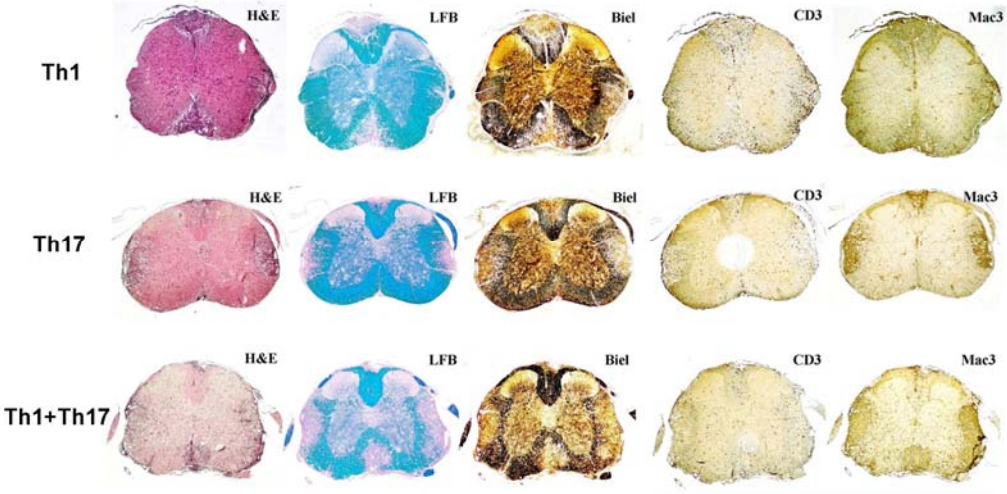


Figure 3.8 – Histological analysis of Th1 and Th17 cell recipient mice. Spinal cord sections of recipient mice transferred with Th1 and Th17 cells, either alone or in combination, with classical EAE with score 4, were characterized histologically and by conventional immunohistochemistry. H&E – haematoxylin & eosin, LFB – Luxol Fast Blue, Biel – Bielschowsky, CD3 stains $CD4^+$ and $CD8^+$ T cells, and MAC3 is a marker for phagocytes. Magnification: 25x. Histology performed by Professor Hans Lassmann, Vienna.

The infiltration and demyelination in sections of spinal cord, brain and PNS of all the animals analysed, including the ones shown in **Figure 3.8**, are described in **Table 3.1** in a qualitative and semi-quantitative manner.

Table 3.1 – Quantification of inflammation and demyelination in spinal cord, brain and PNS based in histological sections. This quantification is according with the classification developed by (Storch et al., 1998). SC Inf – inflammatory infiltrates per spinal cord section; SC DM – spinal cord extent of demyelination determined semi-quantitatively: 1-perivenous, 2-confluent, 3-profound (half of spinal cord section), 4-complete (entire spinal cord section); Brain Inf and Brain DM – Areas of the brain with inflammation and demyelination (with score as in SC): Cer-cerebellum, Obl-medulla oblongata, ON-optic nerves, Trig-central portion of the trigeminal root; PNS – inflammation in the peripheral nervous system: TG-trigeminal root, RO-spinal roots. Samples in bold correspond to the representative figures shown in **Figure 3.8**.

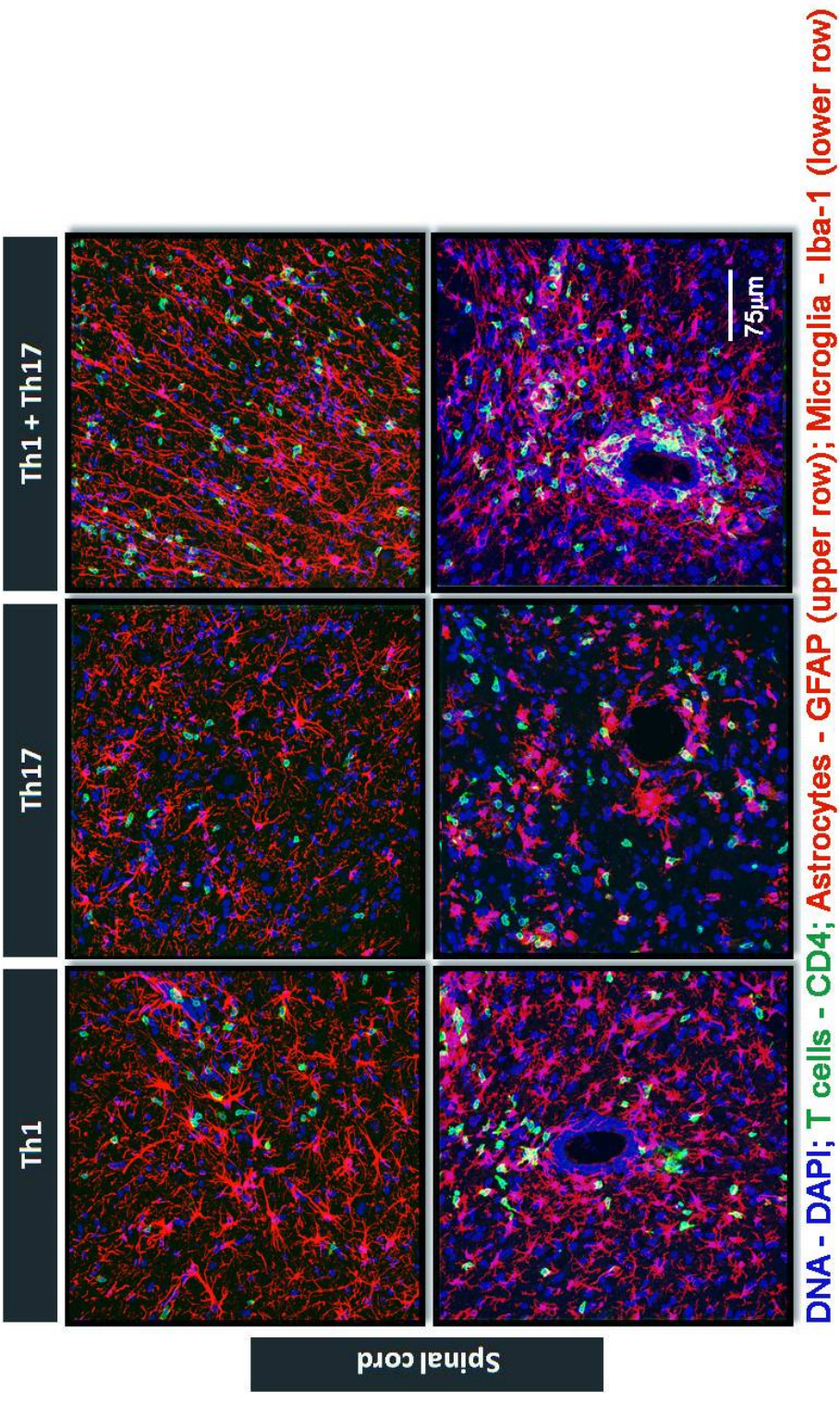
Adoptive transfer EAE	EAE score	SC Inf	SC DM	Brain Inf	Brain DM	PNS
Th1	3,5	0,7	1	Cer, Obl, ON	Cer:1, Obl: 1, ON:0	TG
	3,5	0,5	1	Cer, Obl,	Cer 3; Obl 1,	TG
	3,5	1,9	2	Cer, Obl, ON	Cer 2, Obl 1, ON 1	TG +/-,
	4	2,1	1	ON	ON 1	TG, RO
	4	**3,5**	**2**	**Cer, ON**	**Cer 1, ON 1**	**TG, RO**
	4	2,8	2	Cer, Obl, Trig, ON	Cer 1, Obl 1, ON 1	TG, RO
Th17	4	2	2	Cer, (obl)	Cer 2	TG
	4	1,7	2	Cer, Obl, ON,	ON 1	TG, RO
	4	**2,4**	**2**	**Cer, Obl, Trig**	**Trig 2**	**TG, RO**
	ataxic	4,5	2	Cer, Obl, Trig, ON	Cer 2, Obl 1, Trig 3, ON 1	TG
	ataxic	1,3	1	Cer, Obl, Trig, ON	Cer 1, Obl 1, ON 2	TG, RO
Th1+Th17	4	2,2	3	Cer, Obl, ON,	ON 1	(TG, RO)
	4	**2,2**	**2**	**Obl, Trig, ON**	**ON 1, Trig 2**	**TG, RO**
	4	2	3	Cer, Obl, ON, Trig	ON 2, Trig 2	TG, RO

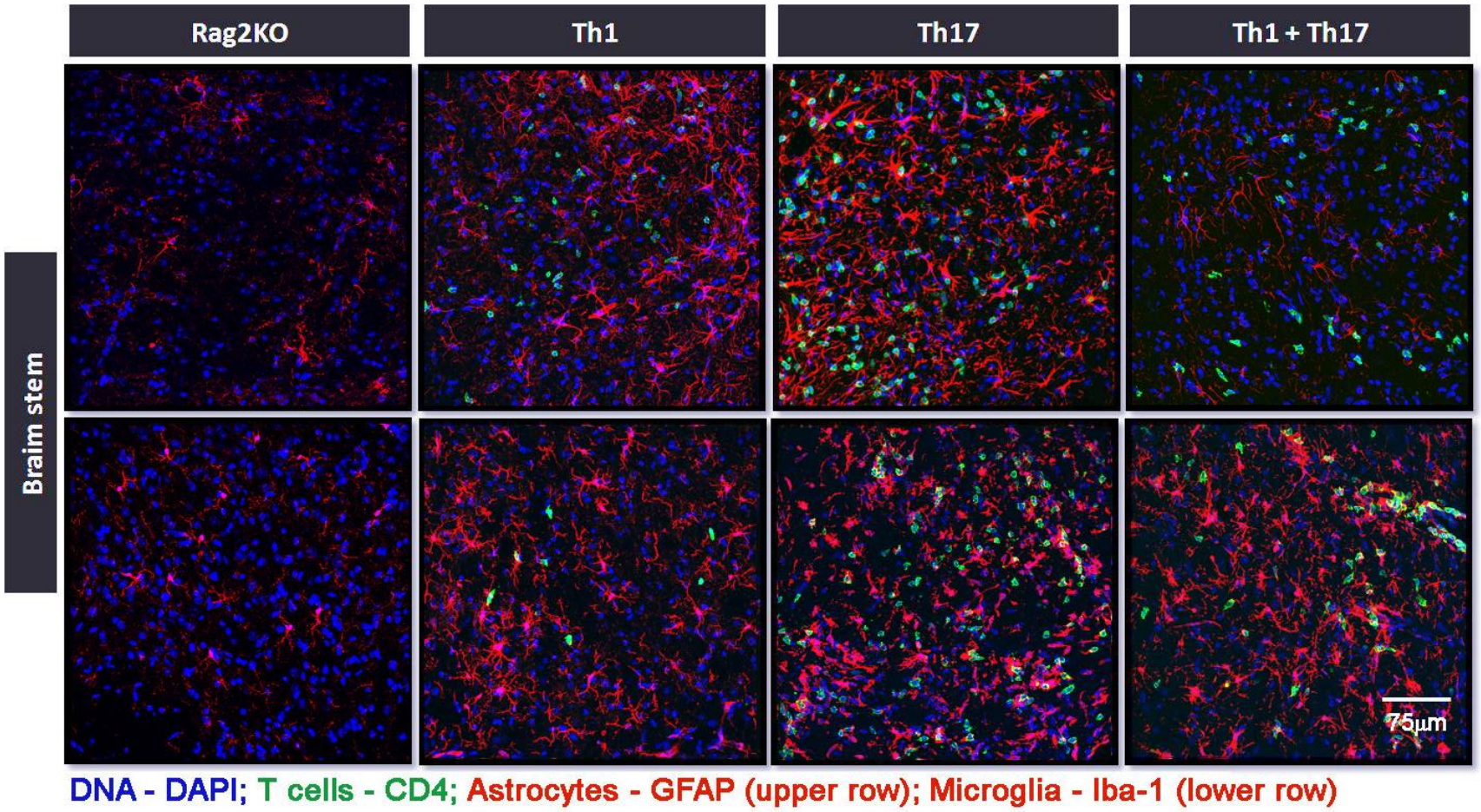
In general, no major differences in terms of demyelination and infiltration extension were found between Th1 and Th17 cells single transfers though spinal cord demyelination was more pronounced in the co-transfer case. Intriguingly, we found no significant histological differences between the Th17 cells transferred animals with classical and ataxic EAE phenotype. Additionally, T cell infiltration and demyelination did not extensively correlate with the clinical EAE symptoms, since a more pronounced affection of the spinal cord is usually correlated with classical paralytic EAE symptoms

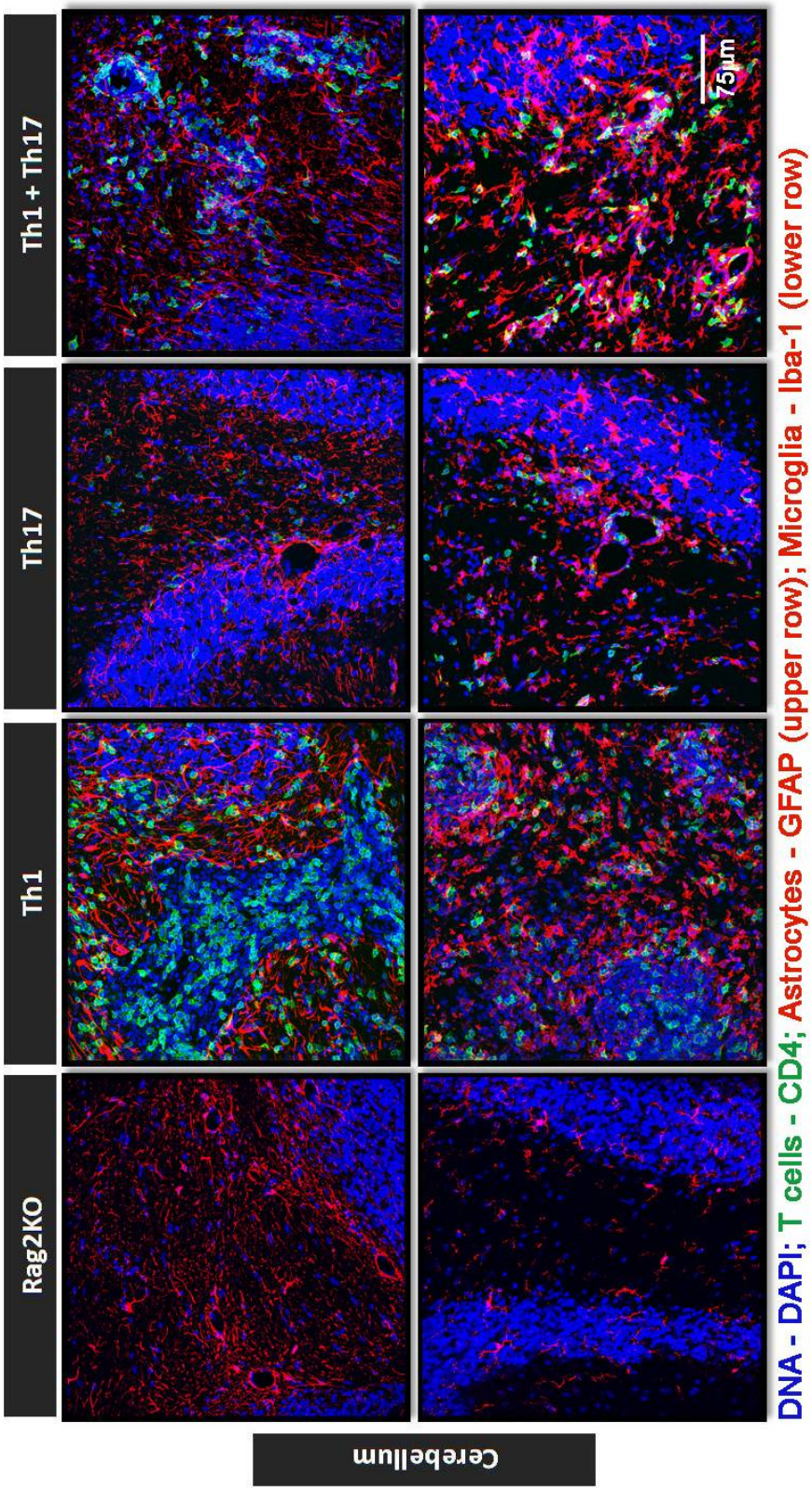
and brain stem and cerebellum more correlated with an ataxic non-classical EAE phenotype. Though the majority of the animals analysed developed classical EAE and had severe spinal cord infiltration, Th1 transferred animals showed also infiltration and demyelination in the cerebellum and in some cases to higher degree than ataxic Th17 transferred mice. Another exciting aspect concerns the observation of immune infiltration and demyelination in the PNS, in particular the trigeminal root and the spinal roots. This is in agreement with the parallel observation that described for the first time the recognition by 2D2 TCR transgenic mice of another auto-antigen besides MOG, the neurofilament (NF-m), which is also expressed in the PNS (Krishnamoorthy et al., 2009).

Brain, spinal cord and cerebellum were also analysed by fluorescent immunohistochemistry. $CD4^+$ T cell infiltration largely correlated with astrogliosis and microglia activation (GFAP and Iba-1 staining, respectively). In the case of Th1+Th17 cells co-transferred mice this was evident in practically all regions, especially in spinal cord and cerebellum. On the other hand, Th1 cells recipient mice showed more T cell infiltration (CD4 staining) of spinal cord, perivascular spaces and cerebellum, while Th17 cells recipient mice with classical EAE presented major T cell infiltration both in spinal cord and brain stem (**Figure 3.9**).

Figure 3.9 – Immunohistochemistry analysis of Th1 and Th17 cell recipient mice. Recipient mice transferred with Th1 and Th17 cells, either alone or in combination, were sacrificed at the peak of disease (classical EAE, minimum score 3). After perfusion with phosphate buffer, spinal cord, brain stem and cerebellum were isolated. Sequential frozen tissue sections were stained for CD4, Iba-1, GFAP and DAPI by fluorescent immunohistochemistry. DAPI stains DNA of all cells, Alexa 488-CD4: helper T cells; Alexa 594-GFAP: astrocytes (upper rows); Alexa 594-Iba-1: microglia (upper rows). Pictures were taken with 40x magnification. Scale bar: 75μm. The figures shown are representative of 4 animals per group. *On the following pages.*


Th1
Th17
Th1 + Th17
Spinal cord
75µm
DNA - DAPI; T cells - CD4; Astrocytes - GFAP (upper row); Microglia - Iba-1 (lower row)


Rag2KO
Th1
Th17
Th1 + Th17
Braim stem
75µm
DNA - DAPI; T cells - CD4; Astrocytes - GFAP (upper row); Microglia - Iba-1 (lower row)


Th1 + Th17
Th17
Th1
Rag2KO
75 µm
DNA - DAPI; T cells - CD4; Astrocytes - GFAP (upper row); Microglia - Iba-1 (lower row)
Cerebellum

We assessed whether chemokines and chemokine receptors could explain the results observed in the immunohistochemistry data, that is, why Th1 cells recipient mice exhibited higher infiltration in the cerebellum and Th17 cells recipient mice exhibited higher infiltration in the brain stem. First, we analyzed the expression of the chemokine receptors CCR5, CCR6 and CCR8, known to be differently expressed by T cell subsets. We confirmed that CCR5 is only expressed by Th1 cells. In addition, CCR6 is expressed by Th17 and iTregs but not Th1 or Th2 cells, as recently described. Surprisingly, we described for the first time that Th17 but not Th1 cells also express CCR8 (found in the microarray data described in Chapter 5), though to lower extent than Th2 cells (**Figure 3.10**).

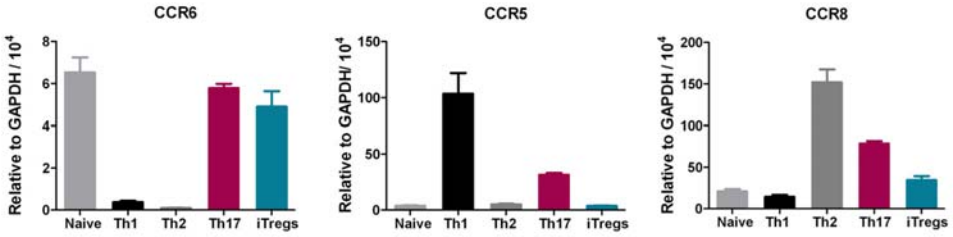


Figure 3.10 – Chemokine receptors CCR5, CCR6 and CCR8 gene expression analysis in polarized CD4$^+$ T cell subsets. Naïve T cells (n=4) and polarized Th1 (n=7), Th2 (n=4), Th17 (n=7) and induced regulatory T cells (iTregs) (n=4) were analyzed for the expression of the chemokine receptors CCR5, CCR6 and CCR8.

Subsequently, by using the adoptive transfer EAE model, we analyzed the expression of these chemokine receptors and their respective ligands in different regions of the CNS: spinal cord, cerebellum, brain stem and cortex (**Figure 3.11**). CCR5 and its ligands were more expressed in the spinal cord and in the Th1 and Th1+Th17 cells co-transfer than in the Th17 cells transfer. However, the expression behavior of CCR6 and CCR8 and its ligands was also very similar, meaning that these molecules could not explain the differential migration in the different CNS regions.

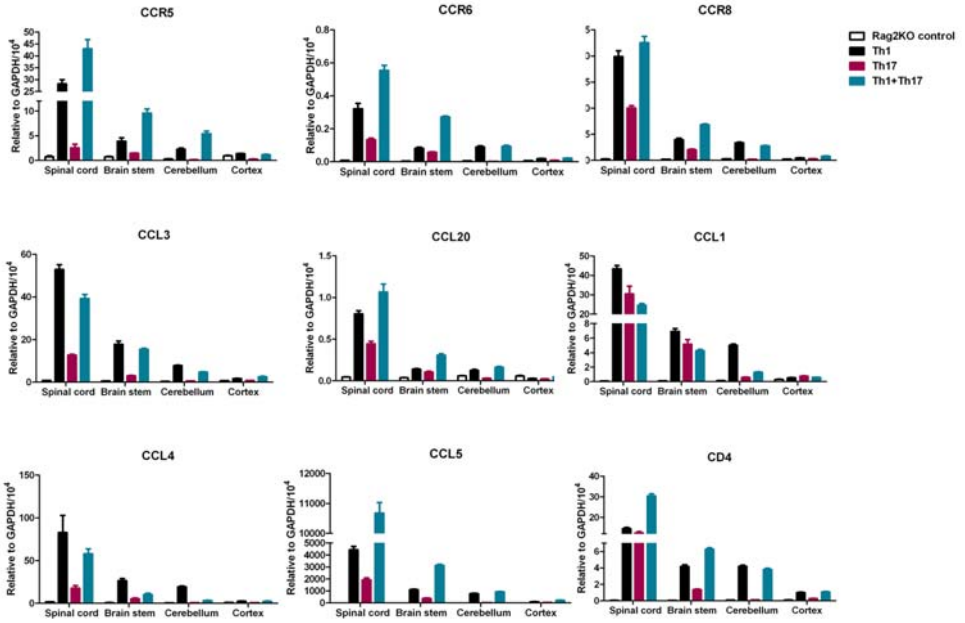


Figure 3.11 – Chemokines and chemokine receptors gene expression in different regions of the CNS in the adoptive transfer EAE model. Rag2KO mice were transferred with Th1 and Th17 cells, either alone or in combination as explained above. At the peak of EAE disease, the recipient animals (3 animals per group) were sacrificed and perfused with phosphate buffer. Spinal cord and brain were removed, cerebellum and brain stem were separated from the cortex and all organs were prepared for RNA isolation and gene expression analysis of CCR5, CCR6, CCR8, CCL3, CCL4, CCL5, CCL8, CCL20 and CCL1.

The same analysis was also performed in the active immunization EAE model (**Figure 3.12**). In general, CCR5 and its ligands, CCL3, CCL4 and CCL5, were mainly expressed in the spinal cord. On the other hand, CCR6 and its ligand CCL20 expression did not correlate so much, though in proportion to spinal cord their expression was higher in cerebellum and cortex in relation to CCR5 and relative ligands. Finally, CCR8 expression did not correlated with its ligand CCL1. While CCR8 was mainly expressed in the cortex, which included the brain stem, the one of CCL1 was higher in the spinal cord and almost absent in the cortex.

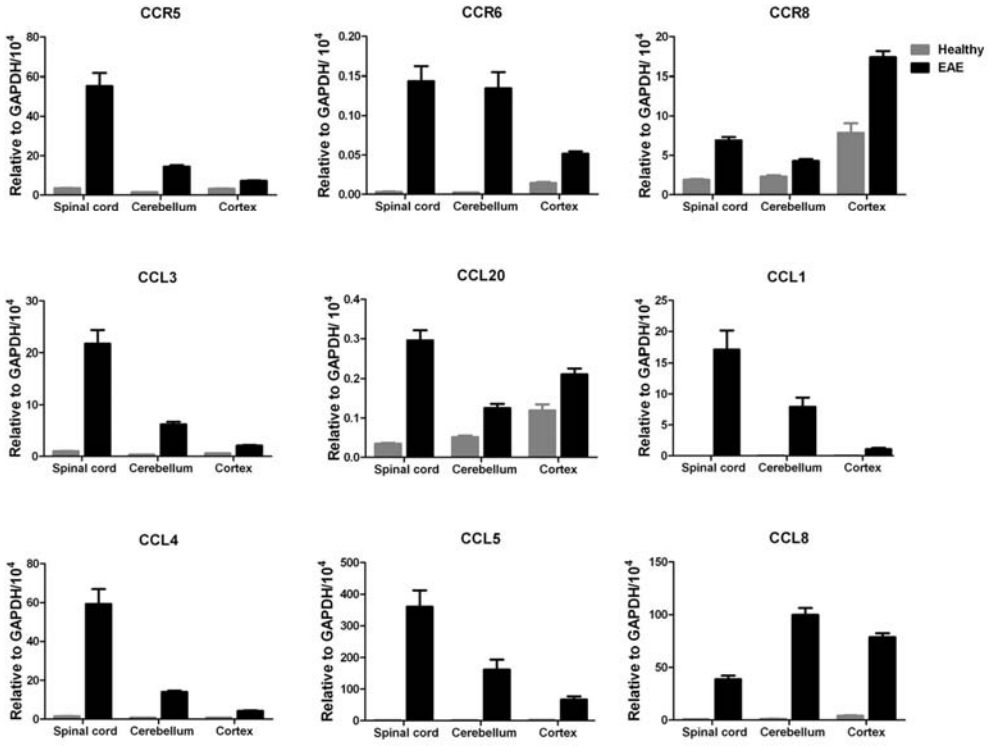


Figure 3.12 – Chemokines and chemokine receptors gene expression in different regions of the CNS of MOG immunized EAE sick mice. Mice were immunized with recombinant rat MOG in CFA and pertussis toxin. At the peak of disease, animals were sacrificed (3 animals per group) and perfused with phosphate buffer. Spinal cord and brain were removed; cerebellum was separated and RNA was isolated from all organs for gene expression analysis of CCR5, CCR6, CCR8, CCL3, CCL4, CCL5, CCL8, CCL20 and CCL1.

Finally, we tested whether IFNγ and IL-17 positive cells in the adoptive transfer EAE model could be different in their capacity to infiltrate different regions of the CNS of adoptively transferred animals with Th1 and Th17 cells. However, the isolation of infiltrating cell from the different regions of the CNS showed no differences between the spinal cord, brain stem and cerebellum in terms of percentages of infiltrating IFNγ and IL-17 positive cells in all adoptive transfers (**Figure 3.13**).

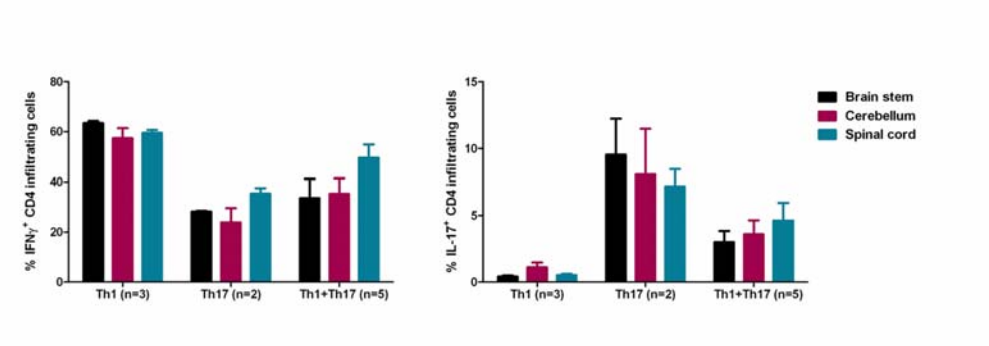


Figure 3.13 – Analysis of IFNγ and IL-17 positive T cells in different regions of the CNS. Recipient animals transferred with Th1 and Th17 cells, both alone or in combination, were sacrificed at the peak of EAE and perfused with phosphate buffer solution. Brain and spinal cord were isolated, cerebellum and brain stem were separated and the infiltrating cells were isolated from the CNS to evaluate IL-17 and IFNγ expression of CD4 T cells by intracellular FACS.

Finally, the expression of several genes known to be involved in the EAE pathogenesis was also evaluated in the brains of Th1, Th17, Th1+Th17 and Th17+α-IFNγ cells Rag2KO recipient animals. Th17-associated genes, IL-17, IL-22 and RORγt, were highly expressed in Th17 recipient animals whereas in Th1 cells transfer case the levels were very close to the control. These observations are in agreement with those made by flow cytometric analysis where IL-17 production was not observed in the brain of Th1 cells transferred mice. The levels of these cytokines were even higher in the cases when Th17 cells were co-transferred with Th1 cells or with the blocking antibody for IFNγ. Th1-related molecules such as IFNγ and IP-10 but, strangely not T-bet, were more expressed in Th1 than in Th17 recipient animals. The fact that IFNγ was also expressed in Th17 transferred mice reinforced the idea of T cell phenotype conversion in the CNS observed in brain FACS analysis. Inversely to the Th17-related molecules, the levels of IFNγ and Interferon-inducible protein-10 (IP-10) were even lower in the cases when Th17 cells were co-transferred with Th1 cells or with the blocking antibody for IFNγ. The cytokines IL-4 and IL-10 and the transcription factor GATA-3, were found in all adoptive transfers but with different gene expression profiles **(Figure 3.14)**.

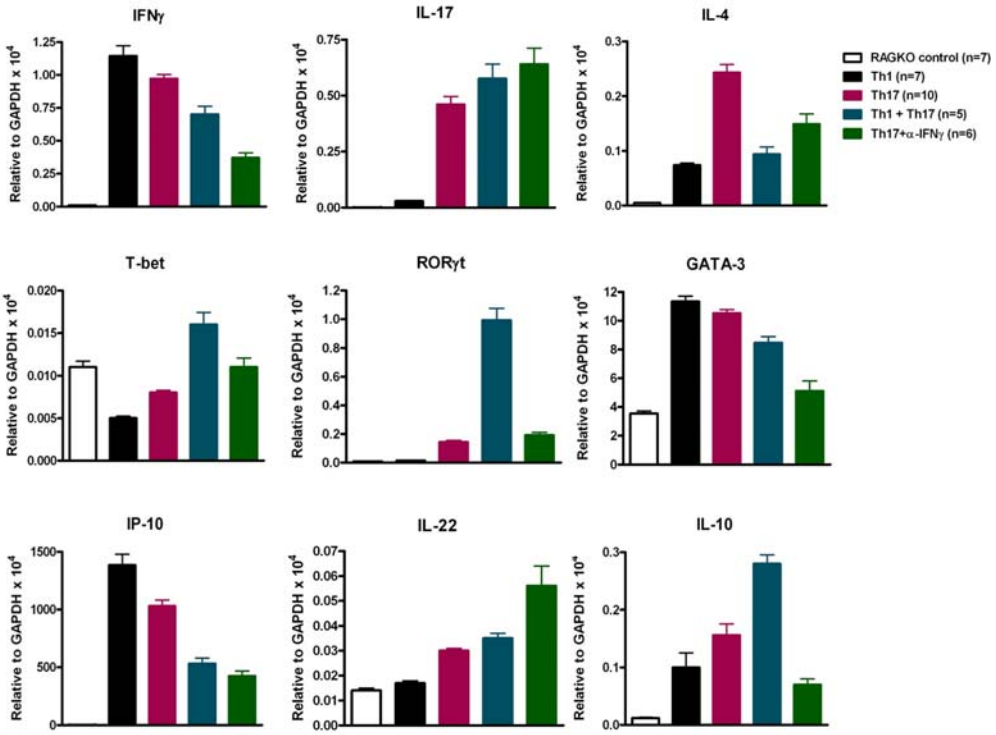


Figure 3.14 – Inflammatory genes expression in the brain of EAE sick mice. Recipient Rag2KO were adoptively transferred with Th1, Th17, Th1+Th17 and Th17+α-IFNγ cells. EAE sick mice with several scores developing both classical and atypical EAE were sacrificed and perfused with phosphate buffer. Brain was removed and RNA was isolated for gene expression analysis of IFNγ, Tbet, IP-10, IL-17, RORγt, IL-22, IL-4, GATA-3 and IL-10. The number of animals analysed in each case is shown in the figure.

3.3 – DISCUSSION AND CONCLUSIONS

The clarification of the effector roles of auto-reactive helper $CD4^+$ T cell subsets in autoimmunity has been, in the last years, a central theme in the immunology and in neuroimmunology in particular. IFNγ-producing Th1 cells were for a long time considered the mediators of disease in MS and EAE. Nevertheless, with the discovery in 2005 of the new Th17 helper T cell subset (Harrington et al., 2005) many studies mainly

based in the active EAE model led the researchers to claim that Th17, rather than Th1 cells, were the potential pathogenic helper CD4$^+$ T cell subset in CNS autoimmunity (Chen et al., 2006;Komiyama et al., 2006).

Spontaneous EAE mouse models are precious tools for the understanding of CNS autoimmunity since they mimic more naturally the immune processes that take place in MS. Our lab has contributed significantly to the development of such models in two different genetic backgrounds (Krishnamoorthy et al., 2006;Pollinger et al., 2009) and the analysis of CNS infiltrating cells of EAE sick mice exhibited the presence of both IFNγ and IL-17-procucing T cells. This fact suggested that both Th1 and Th17 cells may participate in the disease process.

Therefore, we developed an adoptive transfer EAE model by transferring MOG35-55 peptide-specific polarized Th1 and Th17 cells from TCR transgenic 2D2 mice into Rag2KO lymphopenic hosts. In this model, it is possible to evaluate in a more direct way, in comparison with the active EAE model, the effector role of the transferred cells since use of adjuvant is not required, avoiding therefore possible masking of the true immune responses. Importantly, this is the first description of the development of pure Th1 and Th17 subsets and their use in the adoptive transfer EAE model.

Data shown in Figures 3.1 and 3.2 confirmed the successful development of polarized MOG-specific Th1 and Th17 lineages where their signature molecules such as IFNγ, IL-17, T-bet and RORγt are expressed and produced in a mutually exclusive manner. In addition, Th2-related molecules such IL-4 and IL-5 were not found in Th1 and Th17 cells, though IL-10 was produced in significant amounts. A possible explanation for this finding might come from the fact that in highly inflammatory conditions, such as those used *in vitro* to differentiate these two populations, T cells might find necessary to produce an anti-inflammatory cytokine to control excess of inflammation. Indeed, it was already observed *in vivo* IL-10 production by Th1 cells in an infection model in order to dampen inflammation (O'Garra and Vieira, 2007). Also, McGeachy and colleagues described the production of IL-10 by Th17 cells but only when differentiated in the presence of IL-6 and TGFβ, while further addition of IL-23 abolished IL-10

production (McGeachy et al., 2007). However, our Th17 differentiation culture conditions also contained IL-23 besides IL-6 and TGFβ, which is in disagreement with the described findings.

Polarized Th1 and Th17 cells were characterized for surface activation markers expression (Figure 3.3) and their capacity to proliferate (Figure 3.4) and interesting observations were made. We found that Th17 but not Th1 cells completely down-regulated CD62L. This selectin is known to be important in cell activation and migration. Naive T cells are $CD62L^{high}$ and down-regulate this receptor when activated in order to be able to migrate to the sites of inflammation. This data may be indicative of higher migratory capacity of Th17 than of Th1 cells. On the other hand, CD25, the IL-2 receptor, is a marker associated with activation and proliferation of activated T cells but only Th1 cells expressed this receptor. IL-2 was recently described as a negative regulator of Th17 differentiation (Laurence et al., 2007), suggesting that Th17 cells may proliferate through an IL-2 independent mechanism, probably through IL-23. In fact, IL-23 has been considered an important factor for the expansion and survival of Th17 cells (Veldhoen et al., 2006b). While developing Th1 and Th17 differentiation, we constantly observed that from the same initial number total splenocytes we always obtained four to eight times more Th17 than Th1 cells (data not shown). Additionally, antigen-specific proliferation assays showed that Th17 cells proliferated more than Th1 cells to MOG. Interestingly however, in the presence of their respective polarizing cytokines, the opposite was observed. Indeed, it is known that IL-12 is a proliferative cytokine and that TGFβ dampens proliferation. One possible explanation might come from the fact that the highly proliferative Th1 cells produce high amounts of IFNγ, which is an apoptotic cytokine. This induces Th1 cell death and what we obtain in the end is more Th17 than Th1 cells.

After two rounds of stimulation, activated Th1 and Th17 cells were adoptively transferred, either alone or in combination to lymphopenic Rag2KO mice to induce EAE (Figure 3.5). We observed that Th1 and Th17 cells alone were both able to induce EAE with no differences in the EAE incidence and mean day of EAE onset. However, in combination, in a proportion of 1 to 2 of Th1 to Th17 cells, both T cell subsets together induced more severe EAE with earlier onset. This data shows, for the first time, a synergism and cooperation of these two cell lineages to trigger and modulate

autoimmune inflammation in the CNS. Some time after these data were obtained, reports in other models of EAE and EAU claimed similar data stating a similar pathogenic role for Th1 and Th17 cells (Cox et al., 2008;Kroenke et al., 2008;Luger et al., 2008;O'Connor et al., 2008;Stromnes et al., 2008). This re-enforced the veracity of our data. However, we made other interesting observations regarding the EAE clinical pictures of the different recipient mice. While Th1 cells alone and co-transfer of Th1 and Th17 cells induced basically only a classical EAE phenotype, characterized by an ascending paralysis from tail to head, approximately half of the Th17 transferred mice developed atypical EAE characterized by an ataxic gait phenotype. These mice lose their balance and are unable to walk properly on a straight line, leading to hypothesize that the non-classical EAE symptoms might be correlated with infiltrating Th17 cells in the CNS.

While severe paralysis is suggestive of spinal cord demyelination, ataxia is related with affection of certain regions of the brain such as brain stem and cerebellum. Examination of spinal cord sections showed, however, similar infiltration and demyelination in Th1 and Th17 single transfers, though happening in different areas. A possible explanation for this might come from the fact that the animals were analysed at the peak of disease with classical EAE, making it difficult to observe differences in the spinal cord. In contrast, in the co-transfer case demyelination and axonal damage was more severe, which is in agreement with EAE data. Interestingly, no major differences were found between Th17 cells transferred animals that developed classical and atypical EAE. In fact, mice that developed atypical EAE, associated with higher brain stem and cerebellum immune cells infiltration, showed less brain demyelination in the cerebellum than some Th1 recipient cases developing classical EAE.

Immunohistochemistry data confirmed similar T cell infiltration in the spinal cord of Th1 and Th17 single transfers and demonstrated higher T cell infiltration in the brain stem of Th17 recipient mice though cerebellum was more affected in the Th1 case (Figures 3.8 and 3.9, Table 3.1). These data demonstrated some regional differential T cell migration in the CNS but it does not totally correlate with the EAE phenotype and previous reports (Lees et al., 2008;Wensky et al., 2005). A possible explanation might

come from the evidences demonstrated in Figure 3.6. Upon analysis of IFNγ and IL-17 expression by infiltrating $CD4^+$ T cells in the CNS, it was observed a conversion of phenotype of transferred Th17 cells to IFNγ producing Th1 cells. While in Th1 cells recipient mice only IFNγ positive cells were found, infiltrating T cells from Th17 transferred animals expressed both IL-17 and IFNγ, including a double positive population. This phenomenon has also been recently reported in the adoptive transfer diabetes model (Bending et al., 2009).

Therefore, admitting the hypothesis that Th1 and Th17 cells have a different capacity to infiltrate different regions of the CNS, the extent of T cell conversion of phenotype could determine the different regions being infiltrated, that is, the more Th17 cells convert to IFNγ-producing cells the more similar pathology would be observed. However, these different intrinsic properties of Th1 and Th17 cells to migrate to different CNS regions did not correlate with their production of IFNγ and IL-17 as no differences in the percentages of infiltrating $CD4^+$ $IFN\gamma^+$ or $CD4^+$ $IL\text{-}17^+$ cells could be found between spinal cord, cerebellum and brain stem (Figure 3.13). Th1 and Th17 cells express different chemokine receptors. While CCR5 is expressed in Th1 cells, CCR6 and CCR8 are expressed in Th17 cells, the latter being demonstrated for the first time (Figure 3.10). Therefore, we hypothesized whether different chemokines could be differentially expressed in different CNS regions such as spinal cord, cerebellum, brain stem and cortex, and for that reason could determine the place of T cell infiltration. However, CCR5, CCR6, CCR8 and the majority of their ligands were mainly expressed in the spinal cord and no significant differences could be found between cerebellum and cortex, both in the active and adoptive EAE models (Figures 3.11 and 3.12) showing no role for chemokines in differential CNS regions T cell migration. However, this data nicely show that all the CCRs analyzed and their respective ligands are mostly expressed in the spinal cord of all the adoptive transfers, therefore supporting the similar spinal cord infiltration and demyelination evidenced by the histology data.

Additionally, in order to understand which mechanism could explain the Th17 cell conversion to a Th1 phenotype in the CNS in the Th17 cells adoptive transfer, many possibilities could be envisioned. Either IFNγ-positive cells came from the transferred double negative population in response to IFNγ producing cells from recipient mice; or negative regulation of the IL-17 positive population occurred by the IFNγ producing cells

from recipient mice; or both first and second hypothesis occurred. It is also possible that the CD4$^+$ IFNγ$^+$ IL-17$^+$ population found may be an intermediate population during T cell phenotype conversion. Since it is known that IFNγ down-modulates Th17 responses (Cruz et al., 2006), we tested whether local IFNγ produced by the host could contribute to such behaviour by treating Th17 transferred animals with blocking IFNγ antibody. However, we observed IFNγ positive cells in similar proportions as in non-treated Th17 cells transfer case (Figure 3.6). Other possibility could come from the polarizing cytokines IL-12 and IL-23 since it is believed that T cells are re-activated in the CNS by the contact with infiltrating macrophages, DCs and CNS resident cells, such as microglia. The innate cytokines could therefore contribute to the modulation of the T cell phenotype. For example, in the absence of IL-12, IFNγ production could be compromised and it would be speculated that Th17 cells would not have conditions to convert to Th1. To test this hypothesis, we transferred Th17 cells into IL-12p35KO (deficient in IL-12) or IL-12p40KO (deficient in both IL-12 and IL-23) Rag2KO mice (Figure 3.7). Surprisingly, all the animals developed EAE, though with later EAE onset in comparison with the controls and, again IFNγ-producing cells were found (data not shown). These data suggest that, in our EAE adoptive transfer model the *in vitro* activated Th1 and Th17 transferred cells by themselves can induce disease independently of innate immune cells or that other unknown cytokines, besides IL-12 and IL-23, can help Th1 and Th17 cells to trigger the disease. Finally, gene expression of Th1 and Th17-related molecules was analysed in the brain of EAE sick mice of all adoptive transfer cases. The fact that Th1-related molecules such as IFNγ and IP-10 are found not only in the Th1 but also in the Th17 transferred animals emphasizes the data discussed above about T cell conversion of Th17 to Th1 phenotype.

In conclusion, our data clearly demonstrate a clear pathogenic role for both Th1 and Th17 cells in EAE. In the adoptive transfer EAE model, we were able to dissect the individual roles of Th1 and Th17 CD4$^+$ helper subsets. We found that both lineages are able to induce disease with similar histopathological features but, importantly, together synergize to trigger a more severe autoimmune response in the CNS. Nevertheless, the clinical EAE features differ between Th1 and Th17 transferred animals. While Th1

induces a classical EAE phenotype, more than half of Th17 transferred mice develop an ataxic EAE phenotype. In this model we also observed a phenotype conversion of transferred Th17 cells to IFNγ producing Th1 cells, which might explain why some Th17 cells recipient mice developed classical EAE and others ataxic EAE. Though we found T cell regional migration differences in cerebellum and brain stem of Th1 and Th17 cell recipient mice, this did not correlate with the clinical picture.

CHAPTER 4 EVALUATION OF CYTOTOXIC POTENTIAL OF HELPER $CD4^+$ T CELL SUBSETS ON BRAIN RESIDENT ASTROCYTES

4.1 – SUMMARY

Astrocytes are glial CNS resident cells that among other functions support neurons and endothelial cells from the BBB and maintain the extracellular ion balance and pH homeostasis in the CNS.

In pathological conditions, astrocytes are activated, a process denominated astrogliosis, and change their expression patterns of adhesion and antigen presentation molecules, cytokines, chemokines and receptors. Several evidence on immune functions of astrocytes during CNS inflammation emphasize the contribution of astrocytes to the perpetuation of inflammation by potentiating the activation and further recruitment of inflammatory cells into damaged areas. On the other hand, the fact that astrocytes also play neuroprotective roles shows that their cell death as a consequence of direct or indirect immune cell attack can be detrimental to neuroprotection and re-myelination.

The functional complementation of Th1 and Th17 cells in CNS autoimmunity, described in Chapter 3, and their vastly distinct gene profiles, further described in Chapter 5, point out distinct effector mechanisms of Th1 and Th17 lineages in CNS inflammation. This led us to reinvestigate the early finding reported by Sun & Wekerle in 1986 that in the Lewis rat encephalitogenic CD4$^+$ T cells that mediate EAE differ from non-encephalitogenic counterparts by their high cytotoxic activity against antigen presenting glia cells, in particular the case of astrocytes (Sun and Wekerle, 1986). However, by that time Th17 cells subset were not yet described. Consequently, we examined MOG-specific Th1 and Th17 cells for their potential to lyse syngeneic primary astrocytes, CNS resident glial cells, in co-cultures. Our data clearly show that Th1 cells are capable of inducing astrocyte death whereas Th17 are not. In addition, since astrocytes are not excellent antigen presenting cells, we performed parallel experiments with a fibroblast cell line stably transfected with the MHC class II molecule in the context of I-A(b) of the C57BL/6 genetic background, called FT7.1 cells. We

characterized the mechanisms of Th1 induced astrocyte death and the inability by Th17 cells to do so, namely whether cytotoxicity is dependent on antigen presentation, MHC class II binding, cell death related molecules or IFNγ release.

We found that Th1 cells cytotoxicity is dependent on T cell activation and antigen presentation. Additionally, this cytotoxicity can be prevented, at least partially, by blocking MHC class II and IFNγ molecules. *In vivo* data show a severe destruction of the dendate gyrus of the hippocampus in the CNS in Th1 adoptive transfer, with a decreased presence of astrocytes in the surrounding areas. These data emphasize a pathogenic role for Th1 cells, in agreement with the data described in Chapter 3.

4.2 – RESULTS

4.2.1 *IN VITRO* CO-CULTURE OF TH1 AND TH17 CELLS WITH ASTROCYTES

Astrocytes are highly activated in MS lesions and correlate with demyelinated lesions. Though there are *in vitro* evidences of their capacity to present antigen to T cells, the *in vivo* data is controversial. Nevertheless, though astrocytes might behave as perpetuators of inflammation by helping CNS immune infiltrating cells re-activation, they are neuroprotective cells in the CNS and their death can be unfavourable to mechanisms of re-myelination. Therefore, we decided to re-investigate an old finding of $CD4^+$ T cell cytotoxicity on cultured brain-derived astrocytes (Sun and Wekerle, 1986). This was described before the discovery of Th17 cells, so we questioned which $CD4^+$ T cell subset, Th1 or Th17 could be cytotoxic to astrocytes. For that, MOG-specific Th1 and Th17 cells from 2D2 TCR transgenic mice were polarized as described in Chapter 3 and prepared for the co-culture with astrocytes either in the pre-activated and resting state (**Figure 4.1a**). Given that astrocytes are not professional APCs, it was necessary first to irradiate and pre-treat with IFNγ and TNFα to up-regulate the MHC class II molecules (**Figure 4.1b, left**). T cells were co-cultured with astrocytes for 24 or 48 hours

and cytotoxicity was determined by examining the surviving cells that remained attached to the wells.

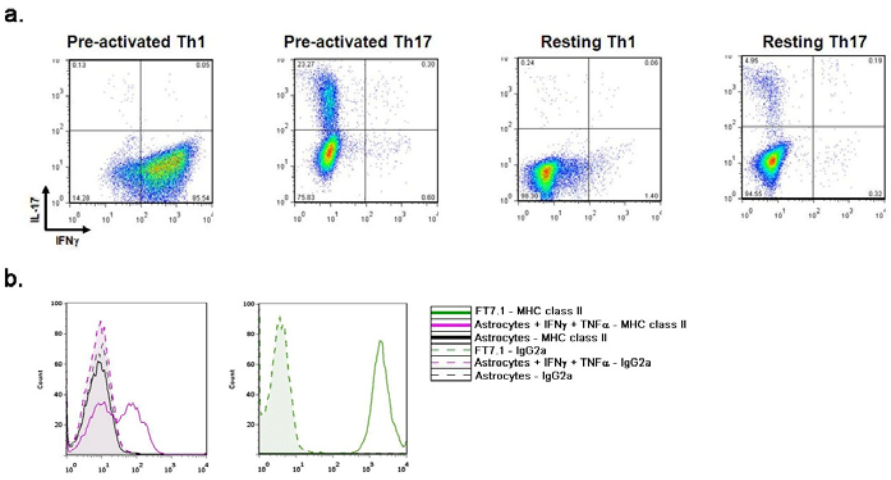


Figure 4.1 – Characterization of polarized Th1 and Th17 cells, astrocytes and FT7.1 cells. A. 2D2 MOG-specific T cells were polarized in Th1 and Th17 cells and prepared in the pre-activated (day 3 of second polarization) and resting state (day 9 of first polarization maintained in the presence of growth factors) to co-culture with activated astrocytes and FT7.1 cells. IL-17 and IFNγ expression was analyzed by ICCS FACS. B. MHC class II (I-A^b) expression analysis by extracellular FACS in GFP positive astrocytes with or without previous stimulation (10 ng/ml IFNγ and TNFα each) (left), and GFP positive MHC class II transfected FT7.1 cells (right). Isotype control staining, IgG2a, was also performed

In a first approach of co-culture of the two T cell subsets with astrocytes, cytotoxicity was evaluated with Giemsa staining, which stained all the living astrocyte monolayer. **Figure 4.2** shows that pre-activated Th1 but not Th17 cells were able to almost completely kill the astrocytic monolayer in 24 hours.

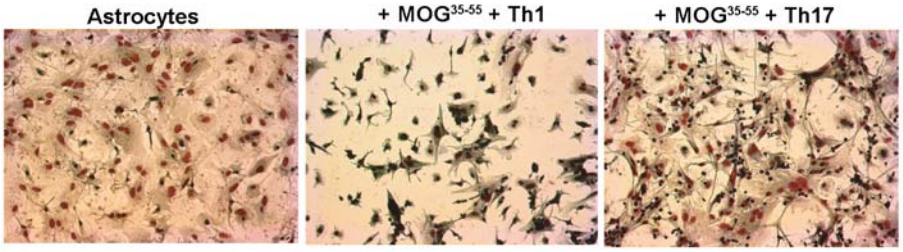
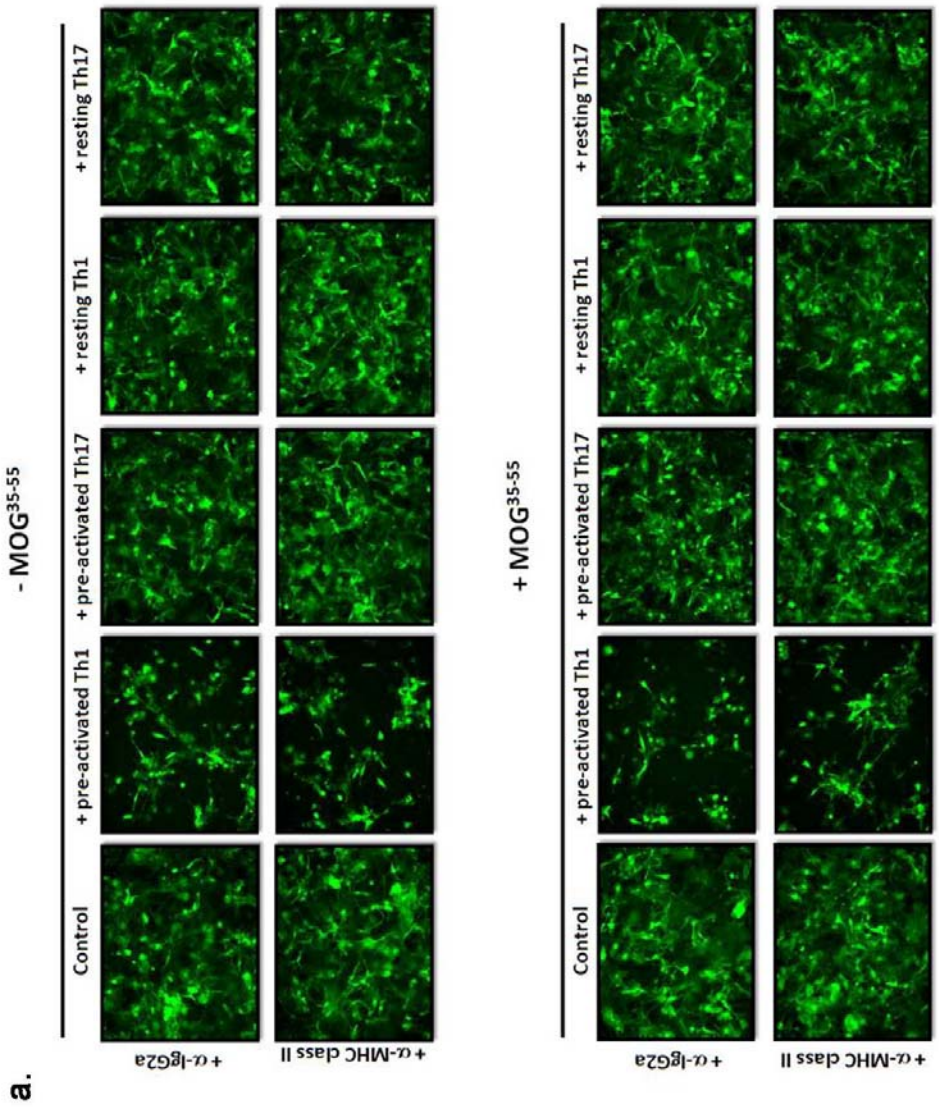


Figure 4.2 – Th1 but not Th17 cells are cytotoxic to astrocytes. Pre-activated polarized 2D2 MOG-specific Th1 and Th17 cells (day 3 of second polarization)) were prepared as described in Chapter 3 and were co-cultured for 24 hours with an astrocytic monolayer (ratio 10:1, respectively) in the presence of MOG 35-55 peptide. The surviving astrocytic cells were washed and visualized with Giemsa stain.

Nevertheless, in order to obtain a quantification method and to clearly prove that the results were not an artefact of the staining method, which includes several washing steps, we developed a non-invasive strategy by co-culturing the T cell subsets with primary astrocyte cultures from neonatal brains of actin-GFP mice. By using fluorescent astrocytes we were able to track the cytotoxicity by time-lapse fluorescent microscopy. Pictures were taken every 20 or 30 minutes for 48h (**Videos 4.1, 4.2 and 4.3 in CD**). **Figure 4.3a** represents the pictures at 48h time point of co-culture of pre-activated and resting Th1 and Th17 cells with GFP-astrocytes either in the presence or absence of MOG35-55 peptide. Only pre-activated Th1 cells were able to kill astrocytes and MHC class II blocking did not prevent cell death. The presence of antigen only to some extent intensified astrocyte cell death as demonstrated by the quantification of living cells (**Figure 4.3b**). ELISA data demonstrated that only pre-activated Th1 cells produced high amounts of IFNγ, which diminished with MHC class II blocking. The same was true for the IL-17, only produced by Th17 cells (**Figure 4.3c**).

Th1 but not Th17 cytotoxicity on astrocytes was emphasized with the results of co-culture with TNXXL CFP/YFP fluorescent astrocytes. These cells bear a genetically encoded FRET-based calcium indicator under the control of the actin promoter. Calcium influx triggers a program of mitochondrial dysfunction leading to cell death. Therefore, astrocytic cell death could be monitored by the calcium influx translated by the change

of CFP/YFP ratio values (**Videos 4.4 and 4.5**). Here, we observed astrocyte calcium influx in Th1 but not Th17 co-cultures.



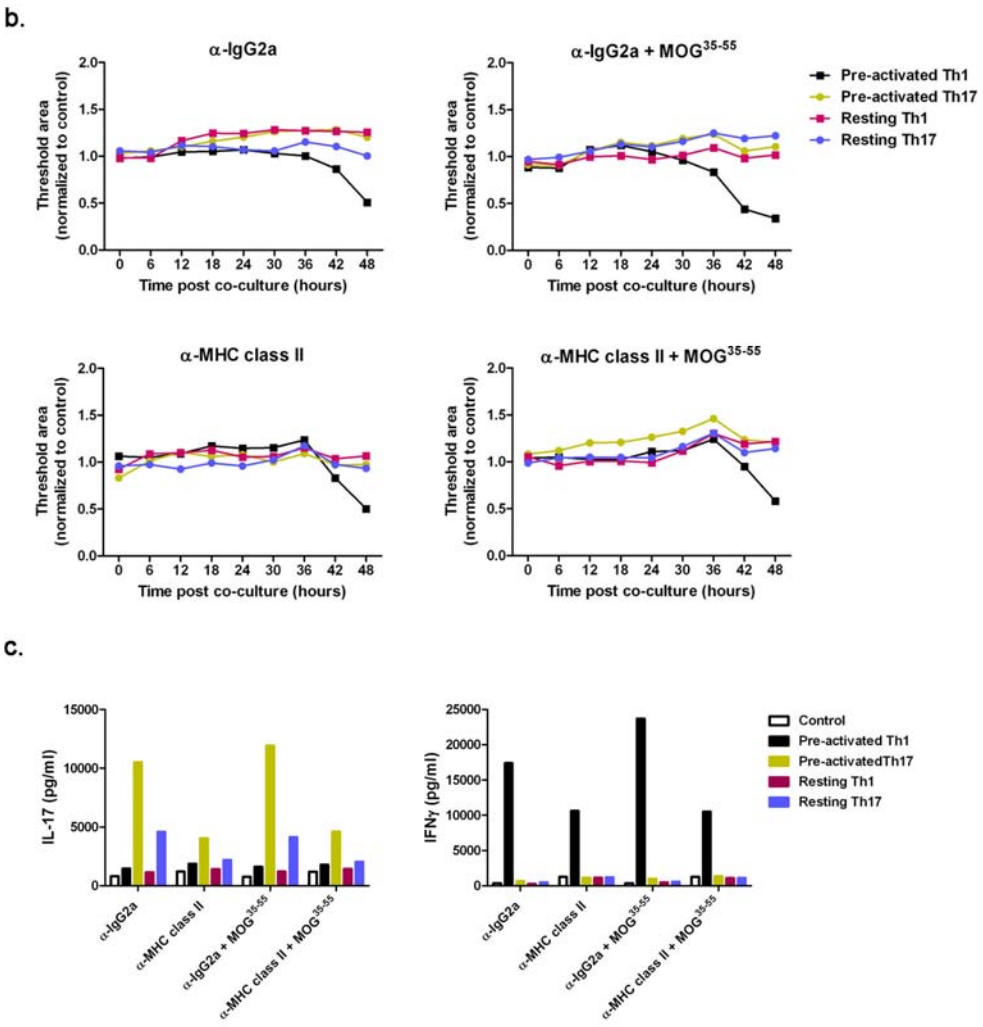
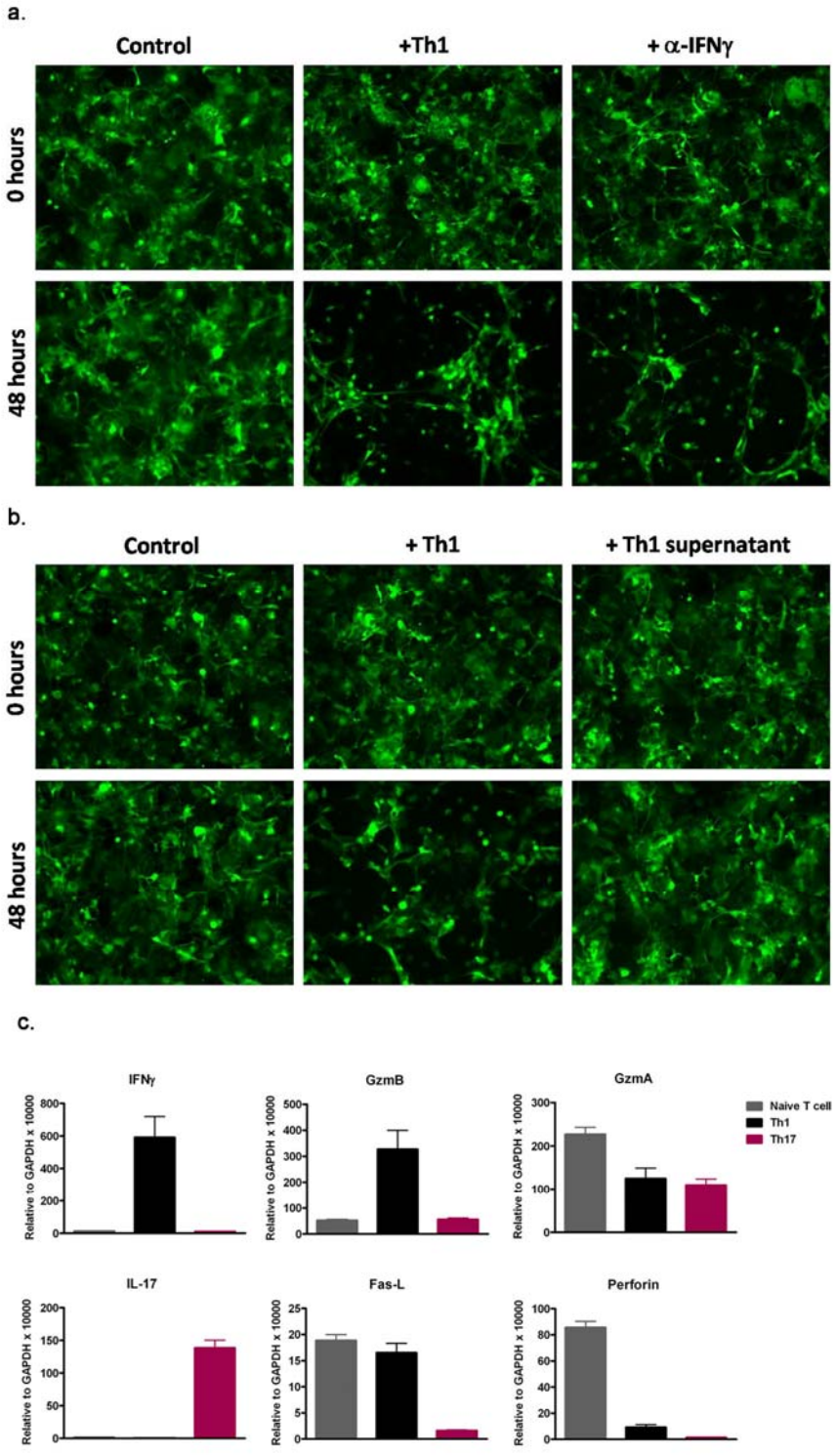


Figure 4.3 – Only pre-activated Th1 cells are cytotoxic to astrocytes. 2D2 MOG-specific T cells were polarized in Th1 and Th17 cells and prepared in the pre-activated (day 3 of second polarization) and resting state (day 9 of first polarization maintained in the presence of growth factors). These cells were co-cultured for 48 hours with astrocytes in the presence or absence of MOG 35-55 peptide. Antigen presentation was tested by blocking MHC class II. Cells were tracked every 30 minutes by fluorescent time-lapse microscopy. Magnification: 10x. A. Final image at 48 hours time-point. B. Quantification of fluorescent area (surviving cells) every 6 hours and values normalized to the ones obtained in the control. C. Quantification of IL-17 and IFNγ production in the co-culture supernatants at 48h time-point by ELISA.

We further addressed which cytotoxic molecules could mediate Th1 cytotoxicity. Blocking IFNγ did not prevent astrocyte cell death demonstrating that IFNγ is not the cytotoxic molecule in Th1 cells (**Figure 4.4a**). Also, the addition of Th1 culture-derived supernatant to astrocytes monolayer was not cytotoxic, meaning that this process is cell-to-cell contact dependent. Therefore, we analyzed the expression of cytotoxic molecules such as granzymes A and B, Fas-L and perforin in Th1 and Th17 cells, which mediate their action in a cell contact manner. Only Granzyme B and Fas-L were up-regulated in Th1 cells in relation to Th17 cells, though Fas-L expression of Th1 cells was similar to the one in naive T cells (**Figure 4.4c**). However, blocking Fas-L, and the use of Granzyme B and pan-caspases (ZVAD) inhibitors, as well, did not prevent Th1-mediated astrocyte cell death (**Figure 4.4c**).

4.2.2 *IN VITRO* CO-CULTURE OF TH1 AND TH17 CELLS WITH FT7.1 CELLS

The data obtained from co-culture of T cells with astrocytes revealed a poor capacity os astrocytes to present antigen as only pre-activated but not resting Th1 cells were cytotoxic. As well, blocking the MHC class II molecule in the presence of MOG peptide had a very mild effect. To evaluate the antigen presentation in Th1 mediated cytotoxicity, we preformed parallel experiments with FT7.1 cells, a fibroblast cell line stably transfected with the MHC class II molecule (**Figure 4.1b, right**). When co-culturing FT7.1 cells with Th1 and Th17 cells, both in the pre-activated and resting state, for 48h we made interesting observations. All T cell subsets were able to kill FT7.1 cells but only in the presence of MOG 35-55. Moreover, cell death was prevented by blocking IFNγ and the MHC class II molecule (**Figures 4.5a, b and videos**). The surprising fact that, in the case of FT7.1 cells, Th17 cells were cytotoxic was explained by the ELISA data. These demonstrated that in the presence of antigen, both pre-activated and resting Th17 cells dramatically increased their production of IFNγ, which suggested a conversion to a Th1 phenotype (**Figure 4.5c**), in agreement with the *in vivo* Th17 conversion to Th1 phenotype data described in Chapter 3.


a.
Control
+Th1
+ α-IFNγ
0 hours
48 hours
b.
Control
+ Th1
+ Th1 supernatant
0 hours
48 hours
c.
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Th1
Th17
Relative to GAPDH x 10000
IL-17
Fas-L
Perforin

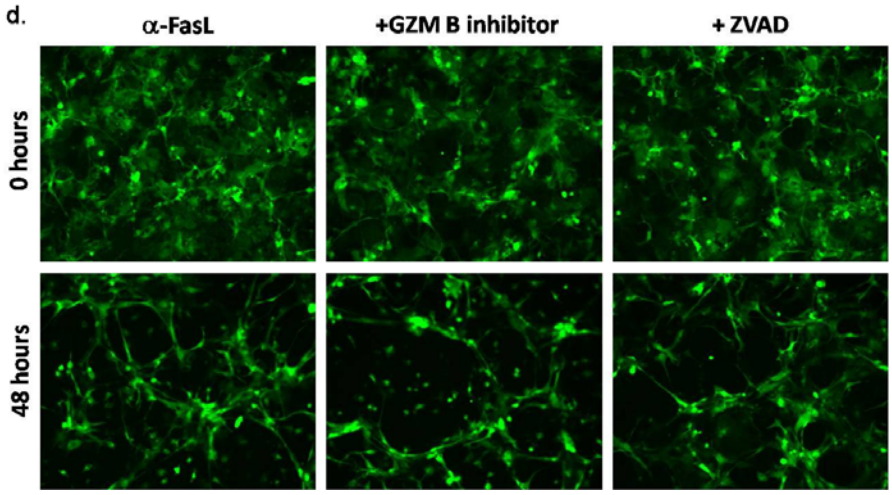
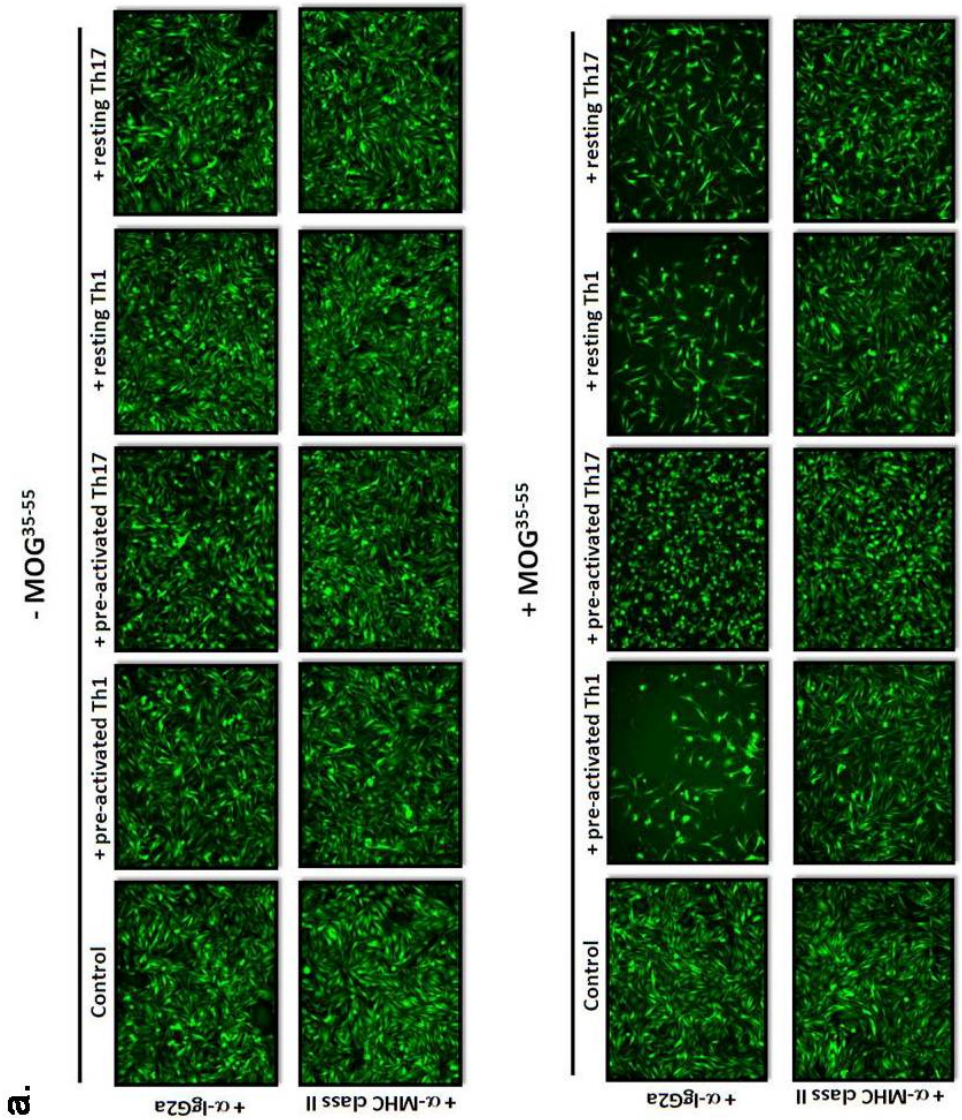


Figure 4.4 – Evaluation of cytotoxic molecules produced by Th1 cells to mediate astrocytes cell death. 2D2 MOG-specific T cells were polarized in Th1 conditions and prepared in the pre-activated (day 3 of second polarization) to be co-cultured for 48 hours with astrocytes in the presence or absence of MOG 35-55 peptide. Cells were tracked every 30 minutes by fluorescent time-lapse microscopy. Cytotoxicity evaluation on astrocytes by co-culture with: Th1 cells in the presence of blocking IFNγ antibody (A), Th1 culture-derived supernatant (B), and Th1 cells in the presence of blocking Fas-L antibody, Granzyme B (GZMB) inhibitor and pan-caspases inhibitor (D). C. Gene expression analysis of IFNγ, IL-17, granzyme A, granzyme B, Fas-ligand and perforin in naive T cells (n=4) and polarized Th1 (n=6) and Th17 cells (n=7) at day 9 of polarization.

4.2.3 *IN VIVO* EVIDENCE OF TH1 CYTOTOXICITY IN THE HIPPOCAMPUS

The *in vitro* data of co-culture with both astrocytes and the MHC class II transfected FT7.1 cells emphasized a cytotoxic role to Th1 but not to Th17 cells. In the context of the adoptive transfer EAE model, immunohistochemistry data revealed an extensive neuronal cell death in the hippocampus, especially in the CA3 region and the dendate gyrus. In the dendate gyrus, we observed a much weaker GFAP staining in the Th1 transferred animals when compared with the healthy Rag2KO controls (**Figure 4.6**). The same was true in the Th17 transferred animals but it is important to take into consideration the fact of Th17 conversion to Th1 phenotype in the brain (**Figure 3.6**).

Also, of interest is the observation that $CD4^+$ T cells were not found in the destroyed area but just in the surroundings of the hippocampus (not shown). Additionally, other areas of the CNS were not thoroughly explored for cell death derived of Th1 cytotoxicity but will surely be addressed in the future.



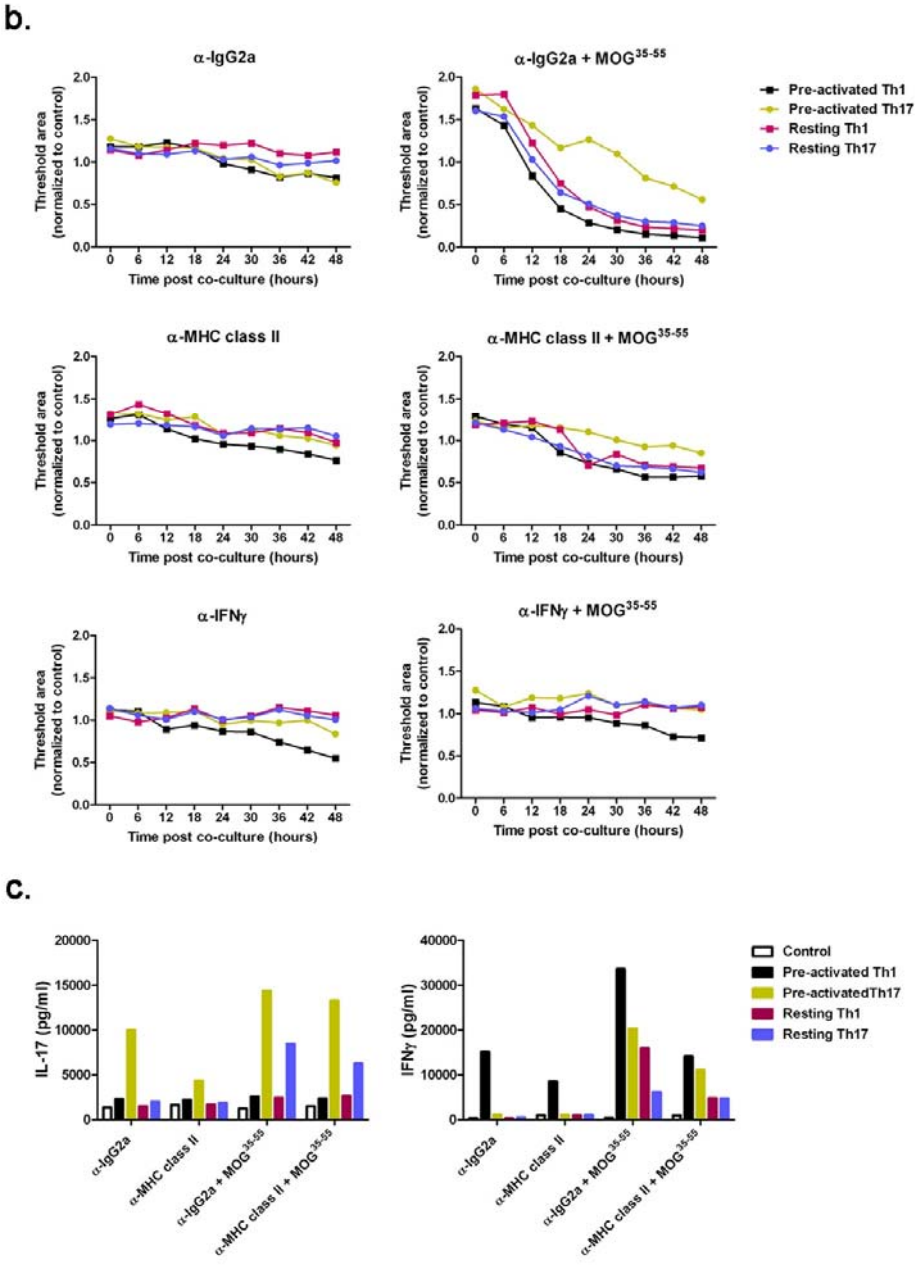


Figure 4.5 –Th1 cells are cytotoxic to FT7.1 cells only in the presence of antigen. Th17 cells are cytotoxic if converted to a Th1 phenotype. 2D2 MOG-specific T cells were polarized in Th1 and Th17 cells and prepared in the pre-activated (day 3 of second polarization) and resting state (day 9 of first polarization maintained in the presence of growth factors) to be co-cultured for 48

hours with FT7.1 cells in the presence or absence of MOG 35-55 peptide. Antigen presentation was tested by blocking MHC class II and Th1 cytotoxicity was evaluated by blocking IFNγ. Cells were tracked every 30 minutes by fluorescent time-lapse microscopy. Magnification: 10x. A. Final image at 48 hours time-point. B. Quantification of fluorescent area (surviving cells) every 6 hours and values normalized to the ones obtained in the control. C. Quantification of IL-17 and IFNγ production in the co-culture supernatants at 48h time-point by ELISA.

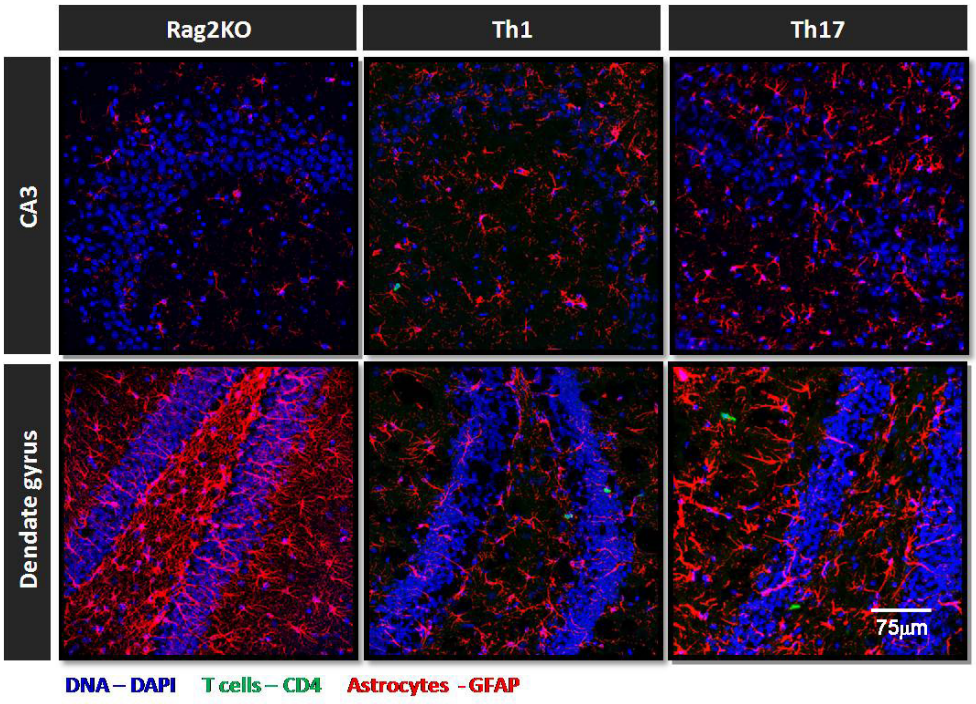


Figure 4.6 – Immunohistochemistry analysis of astrocytes in the hippocampus of Th1 and Th17 cell recipient mice. Brains were isolated from recipient RAG2KO EAE sick mice at the peak of disease (classical EAE, minimum score 3), transferred with Th1 or Th17 cells. Sequential frozen tissue sections of the hippocampus were stained for CD4, GFAP and DAPI by fluorescent immunohistochemistry. DAPI stains DNA of all cells, Alexa 488-CD4: helper T cells; Alexa 594-GFAP: astrocytes. Pictures were taken with 40x magnification. Scale bar: 75µm. The pictures shown are representative of 4 animals per group.

4.3 – DISCUSSION AND CONCLUSIONS

Astrocytes are important CNS resident cells that support neuronal survival and endothelial cells at the BBB but are greatly activated in inflammatory conditions, which can be detrimental in the process of neuroprotection. In fact, there are some *in vitro* evidences of antigen presentation by astrocytes (Kort et al., 2006). Astrocytes can up-regulate MHC class II after activation and present myelin antigens to auto-reactive T cells (Fontana et al., 1984;Fierz et al., 1985). However, astrocytes are not professional APCs and there are suggestions that astrocytes do not efficiently process the antigen bu rather present degraded antigen released by other cells. Additionally, astrocytes express IL-12 and IL-23 after stimulation and block antigen presentation to T cells after neutralization of IL-12/IL-23 p40 subunit (Constantinescu et al., 2005). This reinforces the idea of astrocytes capacity to present antigen to encephalitogenic T cells. However, there is not much literature related with this topic. This study aimed at the identification of the auto-reactive CD4$^+$ helper T cell subset, Th1 or Th17, which was previously found to lyse syngeneic astrocytes (Sun and Wekerle, 1986). By that time, Th17 cells were still not described, so we established *in vitro* astrocyte co-cultures with Th1 or Th17 cells to identify the cytotoxic T cell subset.

A first approach demonstrated that Th1 but not Th17 cells were cytotoxic to astrocytes (Figure 4.2). Later, using fluorescent time-lapse microscopy we were able visualize in real-time astrocytic cell death when co-cultured with pre-activated Th1 cells (Videos 4.2 and 4.4). Astrocytes cell death happened either in presence or absence of MOG 35-55 peptide and could not be prevented by blocking MHC class II. In addition, only pre-activated but not resting Th1 cells were able to kill astrocytes, suggesting a poor capacity of astrocytes to present the antigen to T cells (Figure 4.3). Th1 cytotoxicity could be correlated with the high amounts of produced IFNγ, however blocking IFNγ did not prevent astrocytes cell death. Interestingly, IFNγ-containing supernatant from Th1 differentiation cultures was not cytotoxic to astrocytes. This suggests a cell-to-cell contact mediated cytotoxicity of Th1 cells through other molecules than IFNγ. In fact, we analyzed the expression of cell death molecules in Th1 and Th17 cells, such as

granzymes A and B, FasL and perforin, known to be expressed by cytotoxic $CD8^+$ cells and mediate their action in a cell-to-cell contact manner. We observed that Th1 expressed higher levels of granzyme B and FasL than Th17 cells though the latter was not higher in Th1 than in naive T cells (Figure 4.4). Again, blocking FasL and granzyme B did not prevent Th1-mediated killing of astrocytes. It will be necessary in the future to identify further molecules that could be responsible for Th1-mediated cytotoxicity.

Later, we performed parallel experiments by using a MHC class II transfected fibroblast cell line, the FT7.1 cells, to evaluate antigen presentation as these cells express higher amounts of MHC class II than astrocytes (Figure 4.1). Our data again emphasized the pathogenic role of Th1 but not Th17 cells. Only in the presence of MOG35-55 peptide Th1 cells were able to kill FT7.1 cells, either in the resting and pre-activated state, which could be prevented by blocking the MHC class II and IFNγ as well. Th17 cells here were also cytotoxic but ELISA data from co-culture supernatants confirmed that the antigen presentation to Th17 cell led to conversion to a Th1 phenotype as they dramatically increased the IFNγ production (Figure 4.5).

Finally, *in vivo* analysis of hippocampal sections of EAE sick mice transferred with Th1 and Th17 cells showed a decreased presence of astrocytes, especially in the dendate gyrus region (Figure 4.6). More infiltrating $CD4^+$ cells could be observed in the hippocampus surroundings rather than in the damaged areas (data not shown). This suggests that after T cell mediated cytotoxicity took place towards astrocytes and neurons, the formers also died or migrated to other areas where activation conditions could perpetuate their survival. Interestingly, there is recent evidence of astrocyte death in neuromyelitis optica (NMO), an MS-related disease (Sabater et al., 2009). Therefore, it is relevant in the future to address *in vivo* a deeper analysis of astrocyte cell death in CNS related autoimmune diseases and corresponding animal models and evaluate whether there Th1 cells play a role.

Altogether, these data show for the first time *in vitro* and *in vivo* evidence that demonstrate a true pathogenic role of differentiated Th1 helper T cells to those presenting their cognate antigen. However, other Th1-related molecules, which we were not able to identify, contribute to the astrocyte cell death. The microarray data presented in Chapter 5 verifies the presence of many death related molecules in activated Th1 cells. A future exploitation of these molecules could be addressed and

validated for a deeper understanding of possible mechanisms of Th1-mediated cytotoxicity. A consideration for the future will be to evaluate cell death in brain and spinal cord tissue sections so as to reinforce the hypothesis of *in vivo* neuronal and astrocytic cell death when Th1 cells are transferred to recipient mice.

CHAPTER 5 MICROARRAY ANALYSIS OF T CELLS: TH1 *VERSUS* TH17

5.1 - SUMMARY

The observations described in Chapter 3 demonstrated that Th1 and Th17 cell populations differ not only in phenotypic and functional aspects but also in their effector mechanisms to induce EAE. Therefore, we were interested in identifying the differential gene expression profiles of these two T cell lineages. To this goal, we performed gene microarray analysis of *in vitro* polarized Th1 and Th17 cells, both in the activated and resting state.

A pathway analysis showed the confirmation of known signature genes associated to Th1 and Th17 cells. Nevertheless, new genes either still not described or with unknown functions in T cells were found and were considered for validation and exploitation. In particular, we focused our attention in a molecule exclusively up-regulated in Th17 cells: Rev-Erbα. It is an orphan nuclear receptor of the nuclear hormone receptors family, which has been described to be integrated in an overlapping network of responsive genes with RORα, a well known transcription factor in Th17 differentiation. Rev-Erbα plays critical roles in the regulation of the circadian rhythm and lipid metabolism. Rev-Erbα and RORα compete to bind to the same responsive DNA elements (ROREs) in the promoter region of target genes. While RORα is a transcription activator, Rev-Erbα behaves as a repressor since it lacks a specific region in the ligand binding domain, thus binding to co-repressor molecules. Thus, the balance of RORα versus Rev-Erbα has been suggested to be critical for dynamic regulation of target genes containing the ROREs.

In this chapter the validation of the significance of Rev-Erbα in Th17 differentiation is described, in particular its effect on IL-17 production. A close kinetics of Rev-Erbα expression in the different T cell subsets during T cell polarization showed that Rev-Erbα is down-regulated after T cell activation but later on increases during Th17 but not Th1 or Th0 differentiation. However, ectopic expression of Rev-Erbα in the thymoma cell line, EL-4, and mouse CD4$^+$ T cells revealed that Rev-Erbα does not influence IL-17

production. Additionally, there were no clear evidences that Rev-Erbα binds to the IL-17 promoter.

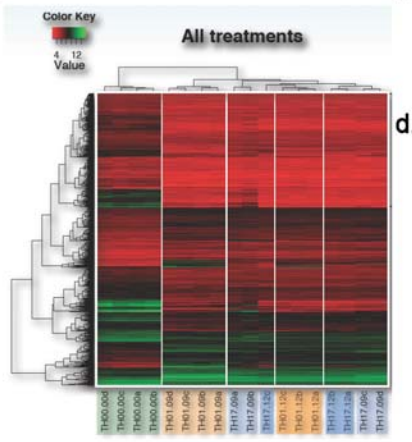
In conclusion, we identified for the first time that Rev-Erbα is expressed in *in vitro* polarized Th17 but not in Th1 cells. Though our data demonstrated that this nuclear receptor had no impact in Th17 differentiation, it might have important roles in the biology of this T cell lineage, which will need to be addressed in the future.

5.2 - RESULTS

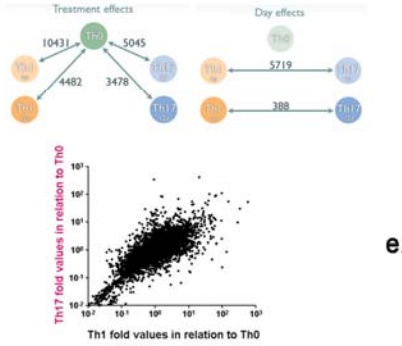
5.2.1 IDENTIFICATION OF REV-ERBα AND ITS EXPRESSION IN CD4$^+$ T CELL SUBSETS

A microarray analysis of *in vitro* polarized Th1 and Th17 cells, both in the activated (day 9, day 3 of second polarization) and resting state (day 12, day 6 of second polarization) was performed in order to identify potential differences between these two T cell subsets. Data obtained was compared to those from naive T cells ("Th0") (**Figure 5.1a**). Out of around 40000 probes used, approximately 5700 were considered to be differently regulated between activated Th1 and Th17 cells (**Figures 5.1b, c**). A first analysis confirmed the expression of signature genes of these two populations, such as IFNγ, IL-17A, IL-17F, T-bet and RORγt, only expressed in their known cell type (**Figure 5.1c**). We also observed that many genes only regulated in Th17 cells comprised chemokine ligands and receptors, emphasizing a migratory role for these cells. On the other hand, it was interesting to notice many cell death related molecules only regulated in Th1 cells, which reinforced our idea of a cytotoxicity capacity for Th1 cells (see chapter 4) (**Figure 5.1d**). Additionally, the pathway analysis identified new molecules that were still not described in T cells and were found to be related with Th17-related genes. This was the case for the nuclear receptors Rev-Erbα (Nr1d1) and its target gene Bmal1 (Arntl1) (**Figure 5.1e**).

a.



b.



c.

Symbol	TH01_9	TH01_12	TH17_9	TH17_12	Definition
Ifng	24.38	1.17	-4.30	-5.07	Mus musculus interferon gamma (Ifng)
Tbx21	15.01	5.65	-2.07	-1.99	Mus musculus T-box 21 (Tbx21)
Il17f	-1.07	-1.14	26.80	4.83	Mus musculus interleukin 17F (Il17f)
Rorc	-1.11	-1.67	6.01	4.63	Mus musculus RAR-related orphan receptor gamma

d.

Symbol	TH01_9	TH01_12	TH17_9	TH17_12	Definition
Tff1	1.40	1.16	30.82	1.85	Mus musculus trefoil factor 1 (Tff1)
Rora	1.00	-1.23	2.09	1.75	Mus musculus mRNA for nuclear orphan receptor ROR-alpha 4.
Ccl9	3.32	1.80	9.60	5.59	Mus musculus chemokine (C-C motif) ligand 9 (Ccl9)
Cxcl4	1.09	1.08	4.82	3.65	Mus musculus chemokine (C-X-C motif) ligand 4 (Cxcl4)
Ccr8	1.07	-1.88	3.71	1.74	Mus musculus chemokine (C-C motif) receptor 8 (Ccr8)
Mmp12	1.14	1.70	3.34	3.99	Mus musculus matrix metalloproteinase 12 (Mmp12)
Ccr6	-1.96	-2.83	4.01	1.63	Mus musculus chemokine (C-C motif) receptor 6 (Ccr6)
Ccl6	-1.18	-1.16	3.12	1.72	Mus musculus chemokine (C-C motif) ligand 6 (Ccl6)
Ccl17	-1.16	-1.09	1.96	1.47	Mus musculus chemokine (C-C motif) ligand 17 (Ccl17)
Ccr4	-1.30	-1.30	1.82	1.40	Mus musculus chemokine (C-C motif) receptor 4 (Ccr4)
Arntl	-1.18	-1.40	1.69	1.63	Mus musculus aryl hydrocarbon receptor nuclear translocator-
Nr1d1	-1.69	-1.80	2.22	1.68	Mus musculus nuclear receptor subfamily 1
Klf2	-2.12	-5.87	-8.19	-7.04	Mus musculus Kruppel-like factor 2 (lung) (Klf2)
Il8ra	50.38	7.59	1.39	1.44	Mus musculus interleukin 8 receptor
Ccr5	23.08	8.99	2.67	1.09	Mus musculus chemokine (C-C motif) receptor 5 (Ccr5)
Il12rb2	16.33	6.33	1.75	1.26	Mus musculus interleukin 12 receptor
Il2ra	9.28	2.35	2.60	1.74	Mus musculus interleukin 2 receptor
Il24	4.27	-1.13	1.34	-1.03	Mus musculus interleukin 24 (Il24)
Icam1	2.38	1.27	1.12	1.02	Mus musculus intercellular adhesion molecule (Icam1)
Ebi3	7.48	5.48	3.59	2.81	Mus musculus Epstein-Barr virus induced gene 3 (Ebi3)
Casp3	3.74	3.22	1.67	1.41	Mus musculus caspase 3
Casp4	4.14	2.34	1.66	1.27	Mus musculus caspase 4
Gzme	7.71	3.15	1.07	-1.03	Mus musculus granzyme E (Gzme)
Klrk1	5.62	2.75	1.12	1.01	Mus musculus killer cell lectin-like receptor subfamily K
Pdcd1lg2	5.01	1.75	1.93	1.01	Mus musculus programmed cell death 1 ligand 2 (Pdcd1lg2)
Tnfrsf13c	2.88	-1.38	1.03	-2.44	Mus musculus tumor necrosis factor receptor superfamily
Tp53i11	2.06	1.14	1.02	1.16	Mus musculus tumor protein p53 inducible protein 11
Traf1	3.92	3.12	1.67	1.02	Mus musculus Tnf receptor-associated factor 1 (Traf1)
Nkg7	5.25	1.64	-4.18	-5.28	Mus musculus natural killer cell group 7 sequence (Nkg7)
Gzmd	4.93	3.60	-3.65	-1.01	Mus musculus granzyme D (Gzmd)
Klrk1	3.92	1.35	-3.09	-1.41	Mus musculus killer cell lectin-like receptor subfamily K
Prf1	1.90	-1.76	-4.18	-5.28	Mus musculus perforin 1 (pore forming protein) (Prf1)
Bcl2l11	1.53	-1.26	-2.12	-2.16	Mus musculus BCL2-like 11 (apoptosis facilitator) (Bcl2l11)
Tnf	2.19	1.56	-1.03	1.80	Mus musculus tumor necrosis factor (Tnf)
Csf1	7.64	3.60	-1.23	1.04	Mus musculus colony stimulating factor 1 (macrophage) (Csf1)
Sell	-1.32	-1.29	-4.48	-5.21	Mus musculus selectin lymphocyte (Sell, CD62L)

e.

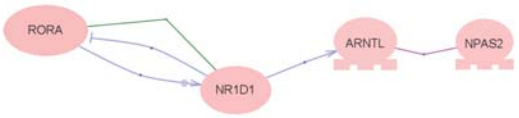


Figure 5.1 – Microarray analysis of Th1 *versus* Th17 cells. Naive CD4$^+$ T cells ("Th0") and activated (day 9) and resting (day 12) polarized Th1 and Th17 cells were prepared for gene expression analysis in an Ilumina microarray plataform. A. Heat map displaying the differentially expressed transcripts. B. Quantification of transcripts differently regulated in the different conditions (upper panels) and normalized microarray values obtained for Th1 and Th17 cells in relation to naive T cells of all the transcripts analyzed (lower panel). C. Microarray data of known signature genes of Th1 and Th17 cells. D. Microarray data of selected genes found to be differently regulated by Th1 and Th17 cells. E. Detail of the pathway analysis demonstrating the connection between RORα, Rev-Erbα and Bmal1.

Gene expression analysis by real-time PCR confirmed that Rev-Erbα and Bmal1 are constitutively expressed in naive T cells and, upon polarization, are only up-regulated in Th17 cells but not in Th1 nor in Th0 cells (**Figure 5.2**).

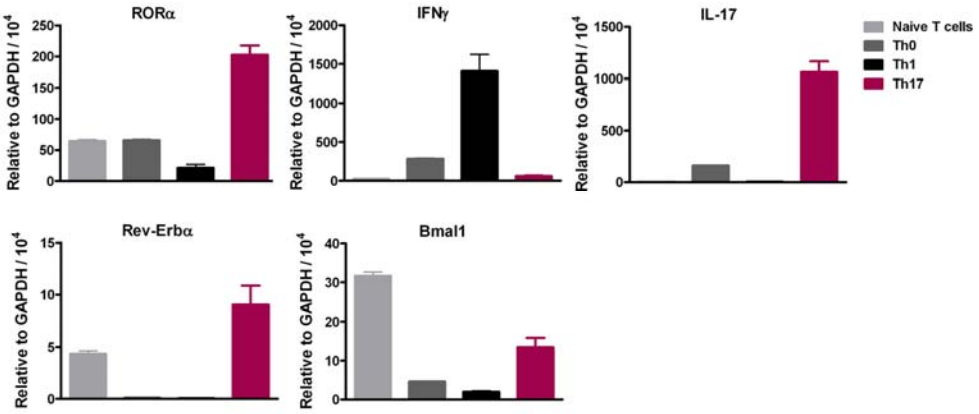


Figure 5.2 – Polarized Th17 but not Th1 cells express the orphan nuclear receptor Rev-Erbα. A pathway analysis of the microarray data was performed and Rev-Erbα and Bmal1 were selected for validation by real-time PCR. Naive $CD4^+$ T cells and activated polarized Th0, Th1 and Th17 cells were analyzed for gene expression of Rev-Erbα and its target gene Bmal1, together with their signature molecules, RORa and IL-17, and IFNγ.

To evaluate gene expression of Rev-Erbα during T cell polarization, in a first approach we preformed gene expression kinetics every 24 hours during 6 days of Th17 polarization and observed that Rev-Erbα is expressed at constitutive levels in naive T cells but, after T cell activation it is down-regulated in the first days to later up-regulate. This expression pattern seemed to be opposite to the one described by the Th17-specific transcription factors RORγt and RORα, which lead to IL-17 expression (**Figure 5.3a**). Later, we compared the gene expression kinetics of these genes every 3 days for two rounds of polarization (12 days) of Th17, Th1 and Th0 cells (**Figure 5.3b**). Indeed, we confirmed that RORγt, RORα and Rev-Erbα are only modulated in Th17 cells, suggesting also a role for the latter in Th17 cells. Interestingly, Rev-Erbα is down-regulated immediately after T cell activation and when T cells become committed to a specific phenotype, at around days 5-6, Rev-Erbα is up-regulated in Th17 cells but not in Th1 or Th0 cells. Of notice, the expression curve of Rev-Erbα during Th17 differentiation

is opposite to the ones of RORα and RORγt, suggesting an interaction between these three receptors.

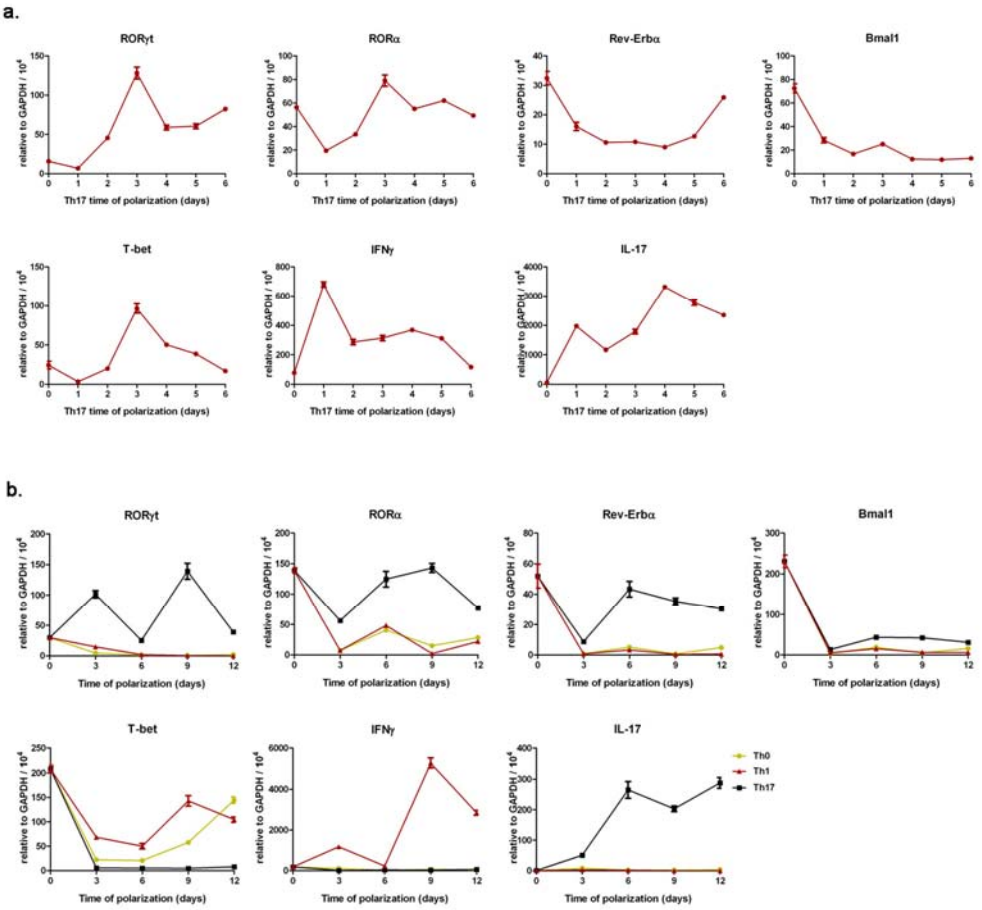


Figure 5.3 – Rev-Erbα expression kinetics during helper T cell differentiation. A. 2D2 CD4$^+$ T cells were activated in Th17 conditions and cells were collected every 24h during 6 days (one stimulation) for RNA isolation and gene expression analysis of RORγt, RORα, Rev-Erbα, Bmal-1, T-bet, IFNγ and IL-17. B. 2D2 CD4$^+$ T cells were activated in Th0, Th1 and Th17 conditions and cells were collected every 3 during 12 days (two stimulations) for RNA isolation and gene expression analysis of RORγt, RORα, Rev-Erbα, Bmal-1, T-bet, IFNγ and IL-17.

5.2.2 RELEVANCE OF REV-ERBα IN EAE AND TH17 DIFFERENTIATION

We addressed Rev-Erbα expression during EAE. The analysis of spleen and CNS infiltrating cells from MOG immunized EAE sick mice showed higher expression of Rev-Erbα and IL-17 in the CNS infiltrating cells (**Figure 5.4**). This suggests that Rev-Erbα is probably correlated with the re-activation of T cells in the CNS and higher percentage of IL-17 positive cells.

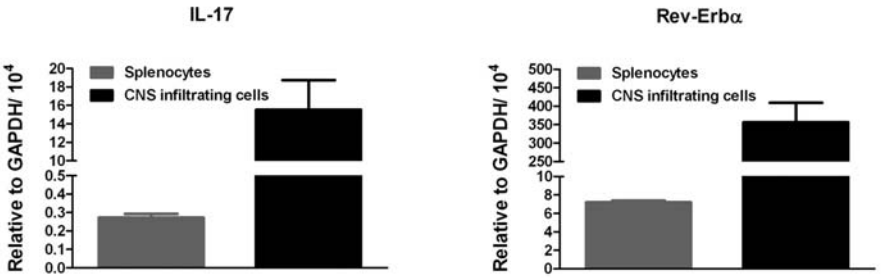


Figure 5.4 – Expression of Rev-Erbα in infiltrating cells that mediate EAE. Mice were immunized with recombinant rat MOG and at the peak of disease, splenocytes and CNS infiltrating cells were isolated for gene expression analysis of IL-17 and Rev-Erbα.

However, this data did not prove a correlation between these two molecules. Heme was recently described to be the novel ligand for Rev-Erbα. Therefore, we evaluated T cell polarization in the presence of heme and its synthesis inhibitor, succinyl acetone (SA). A titration of heme and succinyl acetone concentrations during Th17 differentiation revealed that heme enhaces IL-17 but not IFNg production. In opposition, succinyl decreased IL-17 but not IFNg production. FACS and ELISA data showed that 10 μM heme increased IL-17 production during Th17 differentiation and 1mM SA had an opposite effect, but both had no important effect in Th0 and Th1 cells (**Figures 5.5b,c**). However, gene expression analysis showed that heme influenced not only IL-17 but also IFNγ, either in T cells in polarization to Th1 and Th17 phenotype (**Figure 5.5d**) or in pre-polarized Th17 cells (**Figure 5.5e**).

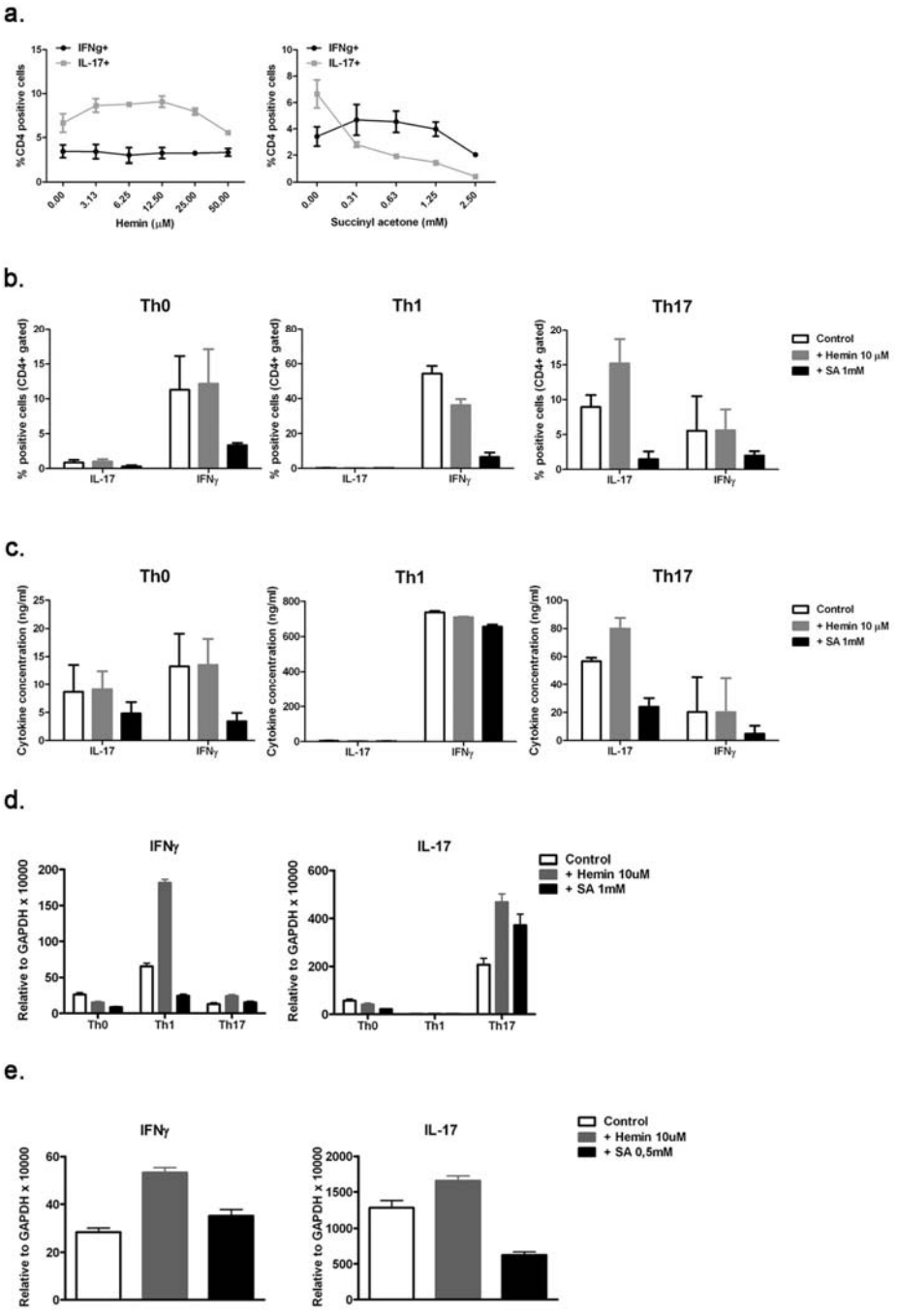

a.
IFNg+
IL-17+
%CD4 positive cells
Hemin (μM)
Succinyl acetone (mM)
b.
Th0
Th1
Th17
% positive cells (CD4+ gated)
IL-17
IFNγ
Control
+ Hemin 10 μM
+ SA 1mM
c.
Cytokine concentration (ng/ml)
d.
IFNγ
IL-17
Relative to GAPDH x 10000
Th0
Th1
Th17
Control
+ Hemin 10uM
+ SA 1mM
e.
+ SA 0,5mM

Figure 5.5 – Effect of heme, Rev-Erbα ligand, in T cell differentiation. Hemin (Heme) and an inhibitor of its synthesis, succinyl acetone (SA), were tested to influence Th0, Th1 and Th17 polarization. A. Hemin and SA concentrations were first titrated during Th17 differentiation and IL-17 and IFNγ producing cells were quantified by intracellular cytokine staining. B. 10 μM Hemin and 1mM SA were applied to naive $CD4^+$ T cells under Th0, Th1 and Th17 differentiation. IFNγ and IL-17 expression was analyzed by intracellular cytokine staining at day 6 of polarization. Data shown was obtained on the gated $CD4^+$ population. C. Analysis of IFNγ and IL-17 production by ELISA from the respective culture supernatants from day 6 of polarization. D. IFNγ and IL-17 gene expression analysis by real-time PCR from the above mentioned polarized cells at day 6. E. IFNγ and IL-17 gene expression analysis by real-time PCR of pre-differentiated Th17 cells re-stimulated for another 6 days in the presence of 10 μM Hemin 0.5mM and SA.

To answer the question whether IL-17 could be a target gene of Rev-Erbα, the latter was tested to bind to a 2Kb fragment of the IL-17 promotor in a contruct encoding also for *Renilla* luciferase. EL-4 cells were transfected with pcDNA plasmid containg Rev-Erbα or the known transcription factors for Th17 differentiation, the human RORC and RORα. No significant activity was found on Rev-Erbα transfected EL4 cells. In addition, Rev-Erbα was able to suppress luciferase activity when co-transfected with RORα (**Figure 5.6**). This is was interesting finding since it is known that Rev-Erbα suppresses RORα activity in other cell types. Additionally, both RORα and Rev-Erbα are known to compete to common ROREs in the promoter region of target genes.

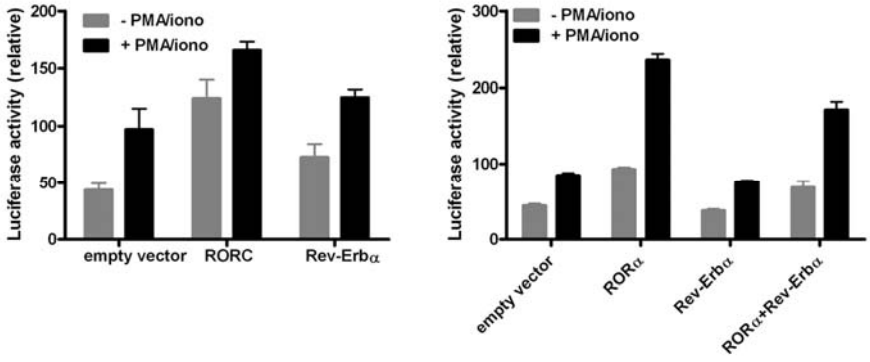


Figure 5.6 – Evaluation Rev-Erbα capacity to bind to the IL-17 promoter. EL4 cells were transfected by nucleofection with the reporter constructs enconding the 2kb fragment of the IL-17 promoter, the *Renilla* and *Firefly* luciferases and the pcDNA3 plasmids encoding human RORC, and mouse RORα and Rev-Erbα, either individually or in combination. After overnight incubation, cells were stimulated with PMA and ionomycin for 6h, lysed and tested for the dual luciferase assay.

Nevertheless, there was the possibility that Rev-Erbα could play an indirect role in IL-17 production. Therefore, we established two approaches. In the first one, we stably transfected the thymoma cell line EL-4 cells with pcDNA plasmids for Rev-Erbα and RORC as a control. PCR and ELISA data showed that only RORC was able to induce IL-17 expression and production but not Rev-Erbα nor its isoform Rev-Erbβ (**Figures 5.7a, b,d**). Interestingly, in co-transfection experiments Rev-Erbα abrogated IL-17 expression induced by RORC (**Figure 5.7c**).

Later, we evaluated the effect of Rev-Erbα by retrovirally transducing MOG-specific T cells with the pMSCV constructs enconding also GFP. When tranduced CD4$^+$ cells were polarized to Th17 conditions, only RORα and RORγt, but not Rev-Erbα nor Rev-Erbβ, were able to increase IL-17 production. In contrast, the dominant negative constructs for Rev-Erbα and Rev-ERbβ, which lack the LBD, induced slightly more IL-17 production than the wild-type constructs (**Figure 5.8**).

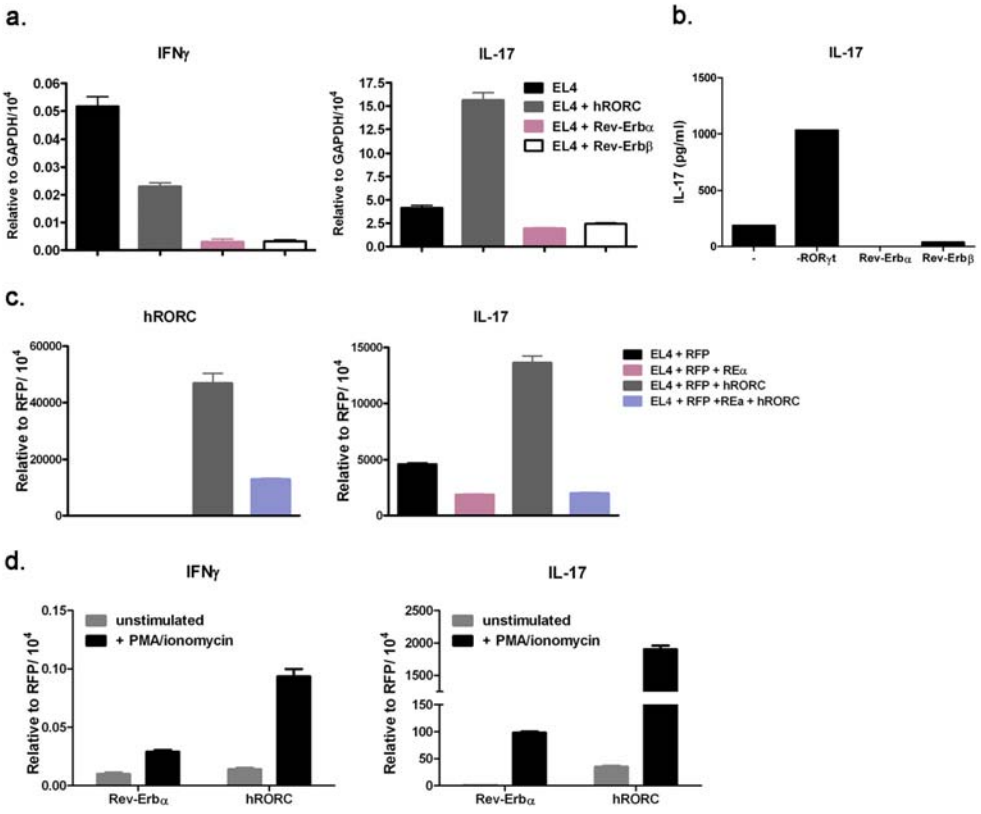


Figure 5.7 - Evaluation Rev-Erbα capacity to induce IL-17 production in transfected EL-4 cells. EL-4 cells were lipotransfected with the pcDNA3 plasmids encoding RFP, human RORC, and mouse Rev-Erbα and Rev-Erbβ, either individually or in combination, and later positively selected for neomycin resistance of for RFP positive cells. The positively transfected cells were maintained in culture for one week minimum to allow cytokine detection. A. IFNγ and IL-17 gene expression in single transfections. B. IL-17 production in single transfections. C. hRORC and IL-17 gene expression in co-transfections, normalized to RFP. D. IFNγ and IL-17 gene expression in single transfections with and without PMA and ionomycin stimulation.

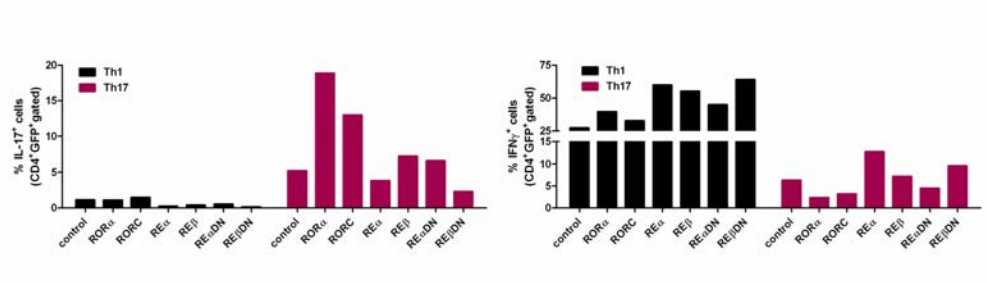


Figure 5.8 - Evaluation Rev-Erbα capacity to induce IL-17 production in transduced CD4$^+$ T cells. CD4$^+$ T cells were retrovirally transduced with the pMSCVneoIRES2eGFP plasmids encoding human RORC, and mouse RORα, Rev-Erbα, Rev-Erbα dominant negative (DN), Rev-Erbβ and Rev-ErbβDN. Cells were stimulated in Th1 and Th17 polarizing conditions and analysed 2-3 days post-transduction for IFNγ and IL-17 expression by FACS.

5.3 - DISCUSSION AND CONCLUSIONS

Gene microarrays are precious tools in the search for differently regulated molecules between two different conditions. Nevertheless, the task can be exhaustive, sometimes leading to non-end roads. In this Chapter, we demonstrated the identification of a nuclear receptor, Rev-Erbα, which is for the first time described to be expressed in T cells, in particular Th17 but not Th1 cells (Figures 5.1 and 5.2). Rev-Erbα is an orphan nuclear receptor of the nuclear hormone receptors family. Truly, it should no longer be called orphan receptor as its ligand was recently identified, the macromolecule heme. Rev-Erbα plays essential roles in lipid metabolism and circadian rhythm. Its close relationship with RORα, an essential transcription factor in Th17 differentiation (Yang et al., 2008), led us to pursue the validation of Rev-Erbα as an important molecule in Th17 cells. In fact, Rev-Erbα and RORα bind and compete to the same ROREs in the promoter region of target genes, being the first a suppressor and the second an enhancer (Forman et al., 1994;Raspe et al., 2002). This interplay between these two molecules

suggests that their target genes expression might be tightly regulated. In the case of the circadian rhythm related genes, this regulation by Rev-Erbα and RORα seems to be essential. It is possible that this tight crosstalk is also fundamental in Th17 cells biology. In fact, a close kinetics of gene expression during helper T cell differentiation, demonstrated that Rev-Erbα is expressed in naïve T cells but soon after activation it is down-regulated only up-regulating in Th17 cells (but not Th0 or Th1 cells) and when those become committed to the differentiated phenotype. Interestingly, the pattern of expression of Rev-Erbα is opposite to the one observed in RORα and RORγt. This suggests that Rev-Erbα is a repressor of RORα and possibly its initial down-regulation is necessary to promote RORγt and RORα up-regulation in order to promote Th17 differentiation. However, the numerous attempts to test Rev-Erbα importance in Th17 differentiation led to negative results. On one hand, we found Rev-Erbα does not bind to the IL-17 promoter (Figure 5.6). On the other hand, ectopic expression of Rev-Erbα, either by stably transfecting EL-4 cells or by retrovirally transducing $CD4^+$ T cells, did not show any role in promoting IL-17 production. In fact, though we cannot be completely sure, the values obtained with Rev-Erbα were always lower that the ones from control (Figures 5.7 and 5.8), suggesting that Rev-Erbα might have a negative impact in IL-17 production, probably in a regulatory mechanism to regulate IL-17 production, which is a very inflammatory cytokine. In addition, the data from the dominant negative construct, which lacks the LBD necessary to bind to heme and recruit co-repressor molecules, showed a very mild increase of IL-17 production in relation to control. We also performed indirect experiments by differentiating T cell in the presence of heme, Rev-Erbα ligand, and its biosynthesis inhibitor. However, we obtained inconclusive data, probably due to the fact that heme is a co-factor of many other proteins. Finally, we tried to silence Rev-Erbα expression in T cells by the use of siRNAs. However, the siRNAs available on the market were all theoretical sequences and upon testing did not induce differences in comparison with the scrambled control sequences. The same was true for the tested Morpholino oligomers against Rev-Erbα that do not block gene expression but protein translation (data not shown).

We found a higher Rev-Erbα and IL-17 expression in CNS infiltrating cells in comparison with splenocytes from EAE immunized sick mice. Though we cannot state a real relation

between these two molecules, it is our intention in the future to evaluate the EAE disease in Rev-Erbα deficient-mice, still not available in our animal facility.

In relation to the general microarray data, it was interesting to observe the expression of other genes, which were also differently regulated in Th1 and Th17 cells. In particular, many genes that were up-regulated in Th17 but not Th1 cells included chemokines and chemokine receptors, such as CCL9, CXCL4, CCR6, CCR4 and CCL17, reinforcing the capacity of these cells to influence and be influenced by migratory and inflammatory conditions. Also, numerous genes only highly up-regulated by Th1 cells included cytokines and cell death-related molecules, such as IL-8Ra, IL-12Rβ2, caspases 3 and 4, granzymes D and E, killer cell lectin-like receptor and TNF, which emphasizes the inflammatory and cytotoxic status of this T cell subset.

In summary, in this study we describe for the first time the expression of the nuclear receptor Rev-Erbα in Th17 but not in Th1 cells. We found that Rev-Erbα is regulated during Th17 differentiation but does not induce IL-17 production. However, we speculate that Rev-Erbα might behave as a negative regulator of RORα in Th17 cells, as described in other cell types when both molecules are co-expressed (Forman et al., 1994). Therefore, Rev-Erbα might indirectly control IL-17 production by Th17 cells. Additionally, RORα is also an enhancer of Rev-Erbα expression (Raspe et al., 2002), justifying therefore the up-regulated levels of Rev-Erbα in differentiated Th17 cells. Nevertheless, this hypothesis will need to be proved in the future with interaction studies. Also, the microarray data revealed many other interesting genes that should be addressed and validated in the future so as to help finding explanations for the different pathogenic functions of Th1 and Th17 cells in autoimmune responses in the CNS.

CHAPTER 6 GENERAL DISCUSSION AND MAIN CONCLUSIONS

The focus in CD4$^+$ T cell biology is at the moment of significant interest in the scientific community of the neuroimmunology field. During the development of this thesis, some reports have recently described similar data to ours, described in Chapter 3, though using different models (O'Connor et al., 2008;Kroenke et al., 2008;Cox et al., 2008;Luger et al., 2008;Stromnes et al., 2008). This fact reinforced the veracity of our data, while forcing us to explore other aspects not yet clarified. This thesis focused on three main projects which are highly interconnected.

In Chapter 3, entitled "*IN VIVO* ANALYSIS OF TH1 AND TH17 CELLS IN ADOPTIVE TRANSFER EAE", we were able to develop a system of adoptive transfer EAE in order to elucidate the effector roles of myelin-specific distinct CD4$^+$ T helper subsets, the Th1 and Th17 cells, in the pathogenesis of EAE. Our data indicate that both Th1 and Th17 cells alone are capable of inducing EAE, though differing in the clinical picture. While Th1 cell mediated disease induced a classical EAE phenotype, with progressing paralysis that affected mainly the spinal cord but also the cerebellum, approximately 50% of the cases in Th17 cells transfer exhibited a non-classical EAE phenotype characterized by an ataxic gait disturbance with affection also of the spinal cord but brain stem as well. Importantly, combinations of Th1 and Th17 cells displayed a higher encephalitogenic capacity than the two lineages transferred separately, indicating that these two distinct populations synergize to induce more severe EAE with earlier onset. Importantly, we observed that when Th17 cells were transferred to lymphopenic hosts, converted to a Th1 phenotype, emphasizing plasticity of Th17 but not Th1 cell subset.

In Chapter 4, entitled "EVALUATION OF CYTOTOXIC POTENTIAL OF HELPER CD4$^+$ T CELL SUBSETS ON BRAIN RESIDENT ASTROCYTES", we examined the capacity of MOG-specific Th1 and Th17 cells to kill primary astrocytes cultures based on early findings describing cytotoxicity to astrocytes by auto-reactive T cells that mediate EAE in rats (Sun and Wekerle, 1986). Our data showed that Th1 cells are capable of inducing astrocytes death whereas Th17 are not. Thought we were not able to identify specific molecules that mediate such cytotoxicity, we found that it is a process dependent on cell-to-cell

contact and that in a model where antigen presentation is enhanced, Th17 cells became cytotoxic too after converting to a Th1 phenotype.

In Chapter 5, entitled "MICROARRAY ANALYSIS IN T CELLS: Th1 *VS.* TH17", we decided to investigate the differential gene expression profiles in Th1 and Th17 cells as a consequence of our findings described in Chapter 3. To this goal, we performed microarray analysis of *in vitro* polarized Th1 and Th17 cells. Data analysis from such arrays led to the identification of potential differences of these two important T cell populations. A first analysis showed a confirmation of known genes with previously described data. Nevertheless, new genes either still not described or with unknown functions in T cells were also found. This was the case of the nuclear receptor Rev-Erbα, expressed in Th17 but not in Th1 cells, which was considered for validation and exploitation. We found that Rev-Erbα was regulated during Th17 differentiation but our data could not clearly state a defined role for this molecule in the modulation of IL-17 production. Based on the literature mentioned above, we can only speculate a possible role for Rev-Erbα in the modulation of RORα expression in Th17 cells since these molecules behave in a tight crosstalk to modulate their target gene expression.

Altogether, these observations lead us to conclude that, in contrary to what has been proposed recently, both $CD4^+$ T helper subsets, Th1 and Th17, can induce EAE. However, they seem to possess distinct effector mechanisms, as demonstrated by the different EAE clinical picture that these subsets induced, but also functional complementation. These data also emphasize a pathogenic and cytotoxic role for Th1 cells, which was underestimated in the last years. This is complemented with the microarray data showing a different chemokine and death-related molecules profiles in these two cell subsets. Despite the fact that we are not able to demonstrate with direct proofs, we could, based in our data, speculate about which different roles Th1 and Th17 cells could play in autoimmune responses. Though the immune context is not the same, auto-reactive Th1 and Th17 cells in principle do not differ from those reacting against foreign antigens. For that reason, if it is not abusive, one could establish some comparison with what happens in the infection models. For example, Khader and colleagues showed that in vaccination and subsequent *Mycobacterium tuberculosis* infection IL-17 producing cells are important triggering $CD4^+$ T cells in the recall

response. In the presence of IL-23, they can effectively mount an immune response to recruit several immune cells, such as neutrophils and IFNγ producing cells, to the local of infection through the release of several chemokines. On their side, IFNγ producing cells seem to be the effector CD4$^+$ T cells capable of restricting bacterial growth as IFNγ is essential in the activation of the microbicidal mechanisms of infected macrophages (Khader et al., 2007). In the active EAE model, heat-killed *Mycobacterium tuberculosis* is used as an adjuvant in CFA during immunization and there are evidences showing that mycobacteria during immunization trigger a strong Th17 response, therefore biasing this model to a specific T cell subset (Tigno-Aranjuez et al., 2009). This might explain why researchers reached the conclusion, based in the active EAE model and neutralization experiments, that IL-23 and Th17 cells, but not Th1 cells, were pathogenic in EAE. However, this might just mean that in active EAE Th17 cells are essential triggers of the autoimmune response and, without those, Th1 cells cannot properly be activated in order to infiltrate the CNS. In fact, our data and other recently described show in the adoptive transfer EAE model, where antigen-specific Th1 and Th17 cells are previously *in vitro* activated, that both T cell subsets can drive EAE or EAU (O'Connor et al., 2008;Kroenke et al., 2008;Cox et al., 2008;Luger et al., 2008). This clearly shows that depending on the model used, different conclusions can be achieved. However, altogether these different models can be important pieces of this complex puzzle to help understand which different roles each T cell subset can play in CNS autoimmunity.

Data shown in Chapter 4 nicely revived the almost lost “classic fame” of Th1 cells by demonstrating cytotoxicity of these cells on brain-derived astrocytes. These data and those regarding the pathogenic role of Th1 cells in passive EAE described in Chapter 3 suggest that IFNγ-producing CD4$^+$ T cells are, indeed, effector cells, capable of not only restrict bacterial growth but also detriment the host during auto-reactive immune responses. On the other hand, our data confirm Th17 cells pathogenicity in EAE and emphasize their capacity to proliferate and produce high amounts of chemokines, which might suggest a higher competence to recruit other immune cells, promoting therefore inflammation. In addition, in our EAE model and data from co-culture with

FT7.1 cells Th17 cells demonstrated a plastic capacity to convert to a Th1 phenotype by a MHC class II dependent presentation. However, T cell plasticity is a quite recent subject and still under warm discussion. Finally, our finding that Th1 and Th17 cells co-transfer in passive EAE display higher disease severity demonstrates synergism and cooperation of both subsets, highlighting the possibility that they have different roles in driving autoimmunity. The same can be concluded from the different clinical features obtained with the single EAE adoptive transfers.

In conclusion, this work goes in agreement with recently described data but also gives new interesting insights in the EAE field. This is still a very highly debatable issue but it will continue being so due to the fact that many disease and T cell biology mechanisms are still poorly understood. In particular, this work leaves still some open questions and unfinished tasks that will need to be pursued in the next future.

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NOTES