Immunomodulatory effects of hepatocyte growth factor in experimental autoimmune encephalomyelitis

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Immunomodulatory Effects of Hepatocyte Growth Factor in Experimental Autoimmune Encephalomyelitis

THÈSE

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ABBREVIATIONS

APC	Antigen-presenting cell
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
c-Met	Proto-oncogen protein c-Met
CNS	Central nervous system
DC	Dendritic cell
EAE	Experimental autoimmune encephalomyelitis
GA	Glatiramer acetate
HGF	Hepatocyte growth factor
HLA	Histocompatibility leukocyte antigen
MBP	Myelin basic protein
MHC	Major histocompatibility complex
MRI	Magnetic resonance imaging
MOG	Myelin oligodendrocyte glycoprotein
MS	Multiple sclerosis
NO	Nitric oxide
NK	Natural killer
OSP	Oligodendrocyte-specific glycoprotein
PBMC	Peripheral blood mononuclear cells
PLP	Proteolipid protein
PNS	Peripheral nervous system
PP-MS	Primary progressive multiple sclerosis
PTX	Pertusis toxin
RR-MS	Relapsing-remitting multiple sclerosis
SP-MS	Secondary progressive multiple sclerosis
TCR	T cell receptor
TGF	Transforming growth factor
Tg mice	Transgenic mice
Th	T-helper cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Immunoregulatory T cell

RESUME EN FRANÇAIS

La sclérose en plaques (SEP) est une maladie neurologique autoimmune chronique du système nerveux central (SNC). Elle est multifactorielle et ses manifestations cliniques sont liées à une démyélinisation des fibres nerveuses du SNC (cerveau et moelle). Sa prévalence montre d'importantes disparités géographiques, et elle touche majoritairement les femmes. L'étiologie de la SEP n'est pas encore établie, elle associe des facteurs génétiques et environnementaux (alimentaires, climatiques et viraux), l'opinion la plus largement acceptée concernant la pathogenèse de la maladie implique une réponse immunitaire cellulaire comme cause principale. Cette auto-immunité est associée à une perte de myéline et d'axone. La myéline peut être restaurée aux premiers stades de la maladie grâce à un processus de réparation endogène et le patient peut regagner tout ou une partie de son handicap neurologique. Ce processus de réparation devient de plus en plus inefficace avec l'avancement de la maladie. De nombreux traitements de la SEP visent à modifier le profil de la réponse inflammatoire, mais aucune de ces thérapies ont démontré des propriétés protectrices directement de l'axone.

L'encéphalomyélite autoimmune expérimentale (EAE) est un model animal de la SEP, carractérisée par une maladie autoimmune inflammatoire et démyélinisante du SNC. Un des modèles d'EAE le plus utilisé au laboratoire est la souris de souche C57BL/6 immunisée avec le peptide recombinant qui est une glycoprotéine myélinique d'oligodendrocytes (MOG $_{35\text{-}55}$ aa). Ce modèle présente un profil de maladie chronique proche de la SEP qui est caractérisé par la rupture de la barrière hémato-encéphalique, l'infiltration de cellules inflammatoires (des lymphocytes T spécifiques de la myéline et des cellules présentatrices d'antigène (APC)), une démyélinisation, une apoptose des oligodendrocytes ainsi que des lésions axonales. L'évolution clinique de ce modèle d'EAE comprend une phase aiguë suivie d'une phase chronique avec absence de rémission complète.

Le facteur de croissance hépatocytaire (HGF) est un facteur pléiotropique qui agit en se liant à son récepteur spécifique appelé c-Met. Ces fonctions incluent l'induction de la motilité, la prolifération, des modifications de la morphogenèse et la régénération de cellules épithéliales. Les souris dépourvues soit du HGF soit de son récepteur c-Met, meurent durant l'embryogenèse, avec des défauts de formation du placenta, du foie et du développement musculaire. Dans le SNC, c-Met est exprimé par les neurones, mais aussi par les cellules résidante du cerveau, telles que les oligodendrocytes, les astrocytes et les cellules microgliales. Il est connu que le HGF induit la croissance des axones et régule la

différenciation des différentes populations neuronales. De plus, HGF est capable d'induire la prolifération et la migration des cellules précurseurs des oligodendrocytes (OPC), les cellules à la base de la formation de la myéline, et est impliqué dans les processus de neuroprotection. L'effet immunomodulateur du HGF est plus anti-inflammatoire que pro-inflammatoire. Si le HGF est connu pour augmenter l'adhésion et la migration des cellules inflammatoires, il est egalement capable de modifier le profil de cytokines, d'inhiber la fonction des APC, de diminuer l'expression des chemokines inflammatoires, et de bloquer la fonction de NF-kappa B. Finallement, HGF joue un rôle immuno-protecteur dans plusieurs maladies inflammatoires comme la myocardite, la glomérulonéphrite, les maladies inflammatoires de l'intestin, l'arthrite et la fibrose pulmonaire.

Dans la première partie du projet de thèse, nous avons évalué l'effet de la surexpression du HGF dans le système nerveux centrale (SNC) de souris. Nous avons montré que HGF diminuait la sévérité de l'EAE. Cet effet est associé à une diminution de l'inflammation, de la démyélinisation et des lésions axonales dans le SNC. Nous avons montré aussi que HGF induit la tolérisation des cellules dendritiques (DCs) en inhibant leur fonction et en favorisant la sécrétion des cytokines de type Th2. De plus, HGF bloque la prolifération des lymphocytes T et induit la différenciation des cellules T regulatrices (Treg) sécrétrices d'IL-10. Ces résultats suggèrent que HGF représente un candidat d'intérêt pour le développement de nouveaux traitements des maladies autoimmunes démyélinisantes associées à une neuro-dégénérescence telle que la SEP.

Nous avons également montré durant ces années de thèse et dans le cadre de collaborations scientifiques que : i) les souris traitées continuellement avec la toxine pertusis développent une EAE moins sévère que les souris non traitées, cette protection étant due à une augmentation du nombre de cellules Treg qui secrètent l'IL-10, ii) les souris traitées avec l'antagoniste de l'IL-21 sont protégées contre l'EAE, iii) glatiramer acétate (GA) augmente le taux de l'IL-1Ra dans le sang et est capable de moduler la différentiation des lymphocytes T CD4 au niveau de leur stade de maturation chez les patients atteints de SEP, iv) le GA n'a pas d'effet sur la progression du lupus murin et finalement, v) l'interferon beta (IFN-β) a un effet potentiellement neuroprotecteur via l'induction de la sécretion du facteur neurotrophique provenant du cerveau (BDNF) chez les patients atteints de SEP.

SUMMARY

Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS). The pathogenesis of MS includes an auto-inflammatory process directed against the CNS, associated with demyelination and axonal loss. At early stages of the disease, the endogenous repair process may restore the myelin around the axons and a recovery of the neurological handicap. At later stages, this recovery process is often insufficient and patient progress to permanent disability. Actually, numerous MS-oriented therapies are aimed at modifying the profile of the inflammatory response toward a less inflammatory pattern. But, none of these therapies have demonstrated direct neuroprotective properties.

Experimental autoimmune encephalomyelitis (EAE) is the first identified and a well characterized animal model of immune-mediated CNS demyelination close to MS. One of the most common EAE model used in laboratory is the C57BL/6 mouse strain immunized with recombinant myelin oligodendrocyte glycoprotein (MOG) peptide (aa 35-55). It induces a chronic EAE with some similar pattern of MS including disruption of the blood brain barrier, infiltration of inflammatory cells (myelin specific T cells and antigen presenting cell (APC)), demyelination, oligodendrocyte apoptosis as well as neuro-axonal injury. The clinical evolution of this EAE model includes an acute stage followed by a chronic stage with absence of complete remission.

Hepatocyte growth factor (HGF), also called scatter factor, is a polypeptide growth factor which belongs to the plasminogen family. HGF is a pleiotropic factor that acts by binding to its specific receptor (c-Met) and can trigger motility, proliferation, morphogenesis and organ regeneration in a variety of epithelial cells. Mice lacking either HGF or its receptor die during embryogenesis, with defects in placenta, liver and muscle development. During EAE, both HGF and c-Met are expressed in the CNS and the latter is activated. c-Met is expressed in neurons but also in others brain-resident cells such as oligodendrocytes, astrocytes and microglia. In the CNS, HGF promotes axonal outgrowth and regulates the differentiation of various neuronal populations. In addition, HGF is able to induce proliferation and migration of oligodendrocyte precursor cells (OPC). Therefore, HGF is involved in the processes of neuroprotection, induces the proliferation and the generation of OPCs and thus could also participate to the process of remyelination.

The immunomodulatory effect of HGF in mostly anti-inflammatory although some a pro-inflammatory effects are described. On the one hand, HGF increases adhesion and migration

of inflammatory cells of both the adaptive and the innate immune system. On the other hand, HGF induces Th2/Th3 bystander deviation with increase of TGF-β and IL-10, inhibits APC function, down-regulates inflammatory chemokines, and blocks NF-kappa B function. HGF has immuno-protective effects in various animal models of inflammatory-mediated diseases including myocarditis, glomerulonephritis, inflammatory bowel disease, collagen-induced arthritis, and pulmonary fibrosis.

In the first part of the experiments presented in this thesis, we assessed the effect of an overexpression of HGF in the CNS of mice during EAE. We showed that HGF decreases the EAE clinical course which is associated with a decrease of inflammation, demyelination and less level of axonal loss in the CNS. We showed that HGF induces DC tolerization by inhibiting their function and induces Th2 bias cytokines. In addition, HGF blocks T lymphocyte proliferation and increases Treg differentiation with increase of IL-10 secretion. These results suggest that HGF is a candidate of high interest for the development of new treatments for immune-mediated demyelinating diseases associated with neurodegeneration such as MS.

In other collaboratorative projects, we showed that, i) continuous pertussis toxin treatment protect mice against EAE via expansion of Treg and elevated Th2 cytokines, ii) mice treated with IL-21 antagonist are protected against EAE, iii) glatiramer acetate (GA) enhances circulating sIL-1Ra levels in MS patients, and decreases Th1 differentiation of CD4 T cells in RR-MS patients at all stages of T-cell maturation, iv) GA has no effect on lupus mice model and finally, v) interferon beta (IFN-β) has the capacity of increasing brain derived neurotropic factor in peripheral blood mononuclear cells (PBMC) of RR-MS patient.

I. INTRODUCTION

A. Multiple sclerosis (MS)

A.1. Epidemiology

Multiple sclerosis (MS) is an inflammatory disease that affects the central nervous system (CNS), i.e., the brain and spinal cord. Onset usually starts between 20 and 40 years of age (1). Approximately 2.5 million individuals affected with MS worldwide, and 10000 in Switzerland. Women outnumber men by a ratio of approximately two to one (2), This bias towards females is also seen in other autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus and autoimmune thyroiditis. The distribution of MS cannot be explained on the basis of population genetics alone. Outside Europe, prevalence rates among Caucasians are half those documented for many parts of northern Europe. In Australia and New Zealand, there are gradients in frequency that do not follow genetic clines. Studies show increased MS incidence in families with a history of disease, relatively high risk in northern Europeans and relatively low risk in Africans, Asians, and native Americans (3).

A.2. Clinical presentation

Paralysis, sensory disturbulances, lack of coordination and visual impairment are common manifestations in MS. The disease often starts with an attack that can last up to several weeks; followed by remission where upon an individual can be disease free for several years. This cycle of relapse-remission involves 85% of MS patients (RR-MS) at disease onset, lasts several years in most individuals. Approximately 50% of them will enter a secondary progressive (SP-MS) phase after 8-15 years of evolution. In this secondary progressive phase, distinct attacks are rare or absent and the deterioration of health is gradually progressive. Evidence indicates that the earlier phase of disease, characterized by distinct attacks followed by remission, is mediated by an autoimmune reaction. The subsequent chronic phase of disease is due to the degeneration of both myelin sheath, which is synthesized by oligodendroglial cells, and the underlying axons, which emanate from the neuronal cell body some distance away. Indeed, it is axon loss in the spinal cord and spinal cord atrophy that correlate most strongly with the inability to walk and paralysis (4). The advent of magnetic resonance imaging (MRI), and its incorporation into current criteria for the diagnosis of MS, also permits an accurate diagnosis in a majority of patients with a first clinical demyelinating event (5). There is also heterogeneity in morphological alterations of the brain found by

histopathological evaluation (6). In 15% of MS patients however, clinical disability develops progressively without relapses, in which case the disease is called primary progressive MS (PP-MS).

A.3. Genetic influence

MS is considered as a complex polygenic disease characterised by a modest inherited risk for disease susceptibility (7). While the disease is not directly inherited, one can inherit a greater susceptibility to acquiring MS. A consistent MS-associated gene is the human leukocyte antigen (HLA)-DRB1 gene on chromosome 6p21 (8) which accounts for 16%-60% of the genetic susceptibility in MS (9). Although this association has reinforced the immune etiology of MS, possibly by affecting antigen presentation, the mechanism by which HLA-DRB1 contributes to disease susceptibility is unknown. Full genome screens of families with multiple cases of MS support a role for several additional unidentified genes, each with modest effect. Other genes within the HLA complex are involved in the pathogenesis of MS, including tumor necrosis factor (TNF), various components of the complement cascade, and myelin oligodendrocyte glycoprotein (MOG). Transcriptional profiling using gene microarrays and large-scale sequencing of transcripts from MS lesions have revealed genes involved in the pathogenesis of acute disease, like immunoglobulins and interleukin-6 (IL-6) (10), as well as genes like osteopontin (11) that play a role in the transition from relapsing remitting to chronic MS. The interleukin -7 (IL-7) receptor alpha chain and interleukin-2 receptor alpha chain (IL-7/2R alpha) have been identified as additional inheritable risk factors accounting for less than 0.4% of the variance in the risk for developing MS (12). It is clear from these studies that MS is a complex genetic disorder in which multiple interactions between polymorphic genes has low penetrances, and each exerts a small effect on the overall disease risk.

A.4. Role of the environment

Epidemiological, clusters or outbreaks, and migration studies have been widely used to illustrate potential environmental influences on MS. Although the interpretation of most of these studies has been difficult, in part due to the limited number of study participants in the individual reports, the results have been influential and do suggest a role for environmental factors in MS, and in some cases, they suggest the existence of critical time periods for exposure to putative environmental disease agents. A lot of environmental exposures have

been investigated. Including viral and bacterial infections, nutritional and dietary factors, trauma, pollution, chemical agents, and various occupational hazards. Common viruses are among the most frequently studied and biologically plausible putative infectious agents related to MS pathogenesis, and many have been proposed at one time or another to be the causative MS agent. Proposed candidates include the herpes viruses, including Epstein Barr virus (EBV), varicella zoster virus, and HHV6. Strong evidence for a role of EBV in particular, a ubiquitous herpesvirus with a worldwide distribution, has been indicated by epidemiologic (13) and laboratory studies (14). A higher risk of infectious mononucleosis (associated with relatively late EBV infection) with MS, and conversely, individuals never infected by EBV are at low MS risk. Study of EBV-specific cellular and humoral immune responses in the cerebrospinal fluid (CSF) of MS patients showed that the intrathecal responses to viral capsid antigen (VCA) and Epstein–Barr nuclear antigen-1 (EBNA-1) were increased in patients with early MS. This indicate that EBV may show more signs of reactivation in the CSF than in the blood at MS clinical onset (15). Environmental supplies of vitamin D have been proposed as a possible risk for developing MS (16). A recent study showed that the risk of MS is decreasing with increasing serum vitamin D levels (17). It has been shown also that newly diagnosed patients with MS have lower serum levels of vitamin D during MS relapses than in remission (18). Vitamin D was also able to inhibit experimental autoimmune encephalomyelitis (EAE) (19).

A.5. Physiopathology

A.5.1. Inflammation

a) T cells

Although the etiology of MS remains to be clearly established, the most widely accepted view of the pathogenesis of the disease implicates a cellular immune response as a central requirement (20). This view is supported by the histopathologic observations of the presence of activated T cells in the perivascular spaces (Figure 1) and the parenchyma in the early phases of the disease. These inflammatory lesions are the apparent substrates corresponding to the recurrent lesions seen with (MRI) (21). Studies of the EAE models have helped define the sequence of events involved in the development of autoimmune CNS-directed inflammatory disease. This initial sequence of immunopathological events can be

subdivided into two major phases: i) initial T-cell priming/activation and ii) subsequent recruitment and effector phase.

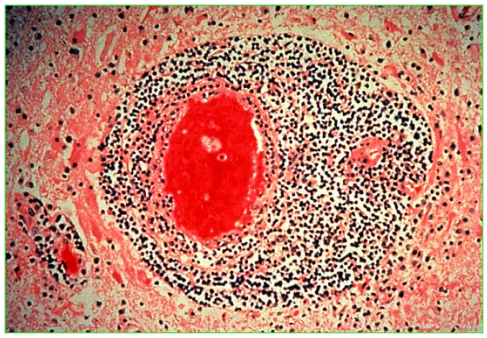

Figure 1: Acute perivascular inflammatory lesion in MS, (Hematoxilin- Eosine staining). Courtesy of Dr P.H. Lalive.

Antigen presentation is a crucial process during immunity for the generation of protective T cell responses against pathogens or other foreign structures. APCs are capable of engulfing foreign microorganisms and expressing the antigenic peptides in the major histocompatibility complex molecules on the cell surface. There are professional APCs equipped to initiate a primary immune response by the presentation of antigen to naïve T cells and non-professional APCs that can only stimulate a secondary response by the presentation of antigen to primed T cells. The dendritic cells, macrophages and microglia of the immune system possese the former ability, wereas B cells and certain stromal cells may engage in secondary T-cell responses (22). CD4 T cells recognize antigens that are associated with the MHC (major histocompatibility complex) class II molecules, whereas CD8 T cells bind antigen-MHC class I complexes. Both naïve CD4 and CD8 T cells become fully activated only when receiving additional co-stimulatory signals from the APC, whereas the activation of T cells that have already been “primed” or exposed to their antigens may be less dependent on co-stimulation (23). MS and EAE are thought to be mainly mediated by the actions of myelin-reactive CD4 T cells (24). This observation is based on the cellular composition of brain and CSF infiltrating cells, current evidence on the induction and perpetuation of MS still favors CD4 autoreactive T cells as a central factor for the autoimmune pathogenesis of MS by the following argument: i) CD4 T cells contribute to the CNS and CSF infiltrating inflammatory cells in MS, ii) genetic risk is to a substantial degree conferred by HLA-DR and -DQ molecules, iii) humanized transgenic mice expressing either HLA-DR or -DQ molecules are susceptible to EAE (25), and mice expressing both MS-associated HLA-DR molecules and MS patient–derived myelin basic protein (MBP) specific TCR develop spontaneous or

induced EAE (26), (iv) a therapeutic trial with an altered peptide ligand (APL) of MBP induced cross-reactive CD4 T cells with Th1 phenotype that led to disease exacerbations of MS patients (27). In MS, much less is known about CD8 T cells than CD4 T cells. CD8 T cells are much better suited than CD4 T cells to mediate CNS damage for the following reasons: i) except for microglia, none of the resident CNS cells express MHC class II; it can be induced on astrocytes by interferon gamma (IFN-γ) (28) but not on oligodendrocytes or neurons, and therefore the latter can only be recognized by CD8 T cells (29), ii) important oligoclonal expansions of CD8 memory T cells have been found in the CSF (30) and in MS brain tissue (31), and a persistence of CD8 T cell clone in CSF and blood (31), iii) CD8 T cells are more prevalent in MS brain tissue than are CD4 T cells (32), iv) a number of HLA class I–restricted myelin epitopes have been described for MBP, proteolipid protein (PLP) and MAG, and others (33), and the CD8 cytotoxic T cell response to MBP is increased in MS patients (33), v) CD8 myelin-specific T cells secrete chemoattractants (IL-16 and IP-10) for CD4 myelin-specific T cells (34). Both CD4 and CD8 T cell responses contribute to MS pathogenesis, albeit at different steps and with different roles.

b) B cells and antibodies

It has been shown that immunoglobulines (Igs) were elevated in the CSF of MS patients (35) which has been the most important and earliest evidence suggesting a role for B cells and antibodies (Abs) in the pathology of MS. B cells do not cross the intact blood brain barrier (BBB), however, once inflammation has started, B cells, Abs, and complement can enter the CNS. This production is local because the intrathecal-specific Abs are absent in the serum. B cells and Abs can contribute to MS disease pathogenesis in various ways. i) B cells can serve as APCs for autoreactive T cells, the encephalitogenic T cell epitopes, and the immunodominant T and B cell epitopes in humans often overlap (36), ii) B cells provide costimulation to autoreactive T cells, iii) B cells and tissue bound Ig can recruit autoreactive T cells to the CNS (37), iv) CSF Igs from MS patients may activate idiotope-specific T cells which sustain B cells that produce such idiotopes (38) and v) the production of myelin-specific Abs and the destruction of myelin within plaques appear to be an important way that B cells contribute to pathogenesis. B cells, plasma cells, and myelin-specific Abs are detected in MS plaques and in areas of active demyelination in MS patients (39). Abs can cause demyelination by opsonization of myelin for phagocytosis (40). Another Ab-mediated mechanism of demyelination acts via complement activation, leading to membrane attack

complex (MAC) deposition and complement-mediated cytolysis (41). MOG is a minor constituent specific to the CNS. The full-length protein contains 218 amino acids. The encephalitogenic properties of MOG is due to the extracellular location of its IgV-like domain which makes it an exposed target accessible to initial autoimmune attack on compact myelinated axons (42). Anti-MOG Abs are able to cause myelin destruction in EAE (43). Anti-MOG Abs have also been found in human MS lesions (39). It has been reported that serum anti-MOG Abs in patients with first CNS symptoms of MS and MRI lesions are predictive of subsequent exacerbations and the diagnosis of definitive MS (44), but later this result were rejected by a nother group which showed a lack of association between anti-MOG Abs and progression to MS (45). Both anti conformational MOG and anti galactocerebroside Abs have been described as potential biomarkers MS clinical stages (46-49). Both anti-PLP and anti-MBP Abs are not associated with an increase in myelin injury despite there increase numbers in the CSF of MS patients (50). Abs with specificity against minor myelin components, other autoantigens, lipids, and DNA are summarized in Table 1.

Table 1: Abs specificities against CNS components other than MOG, PLP and MBP, adapted from Lalive *et al*., 2008.

Target antigen	Remarks and references
Myelin-associated glycoprotein (MAG)	Low titers in MS; possible involvement in progression (51)
Oligodendrocyte-specific protein (OSP/Claudine-11).	Minor myelin component, anti-OSP detected in the CSF os MS patient (52)
2',3'-cyclic nucleotide 3' phosphodiesterase (CNPase)	Anti-CNP were present in sera and in CSF in high titre of MS patient (53)
Oligodendrocyte surface glycoprotein AN-2 (NG2)	Present in both serum and CSF of MS patient (54)
Galactocerebroside (Gal-C)	Major myelin lipid, anti-Gal-C has demyelinating activity in vitro and exacerbate EAE (55). AntiGal-C are found among the RR-MS subtype (49)
Gangliosides	Anti-GM1, GM3, GD1a, GD1b and GD3 Abs are higher in PP-MS and SP-MS (56)
Phosphatidylcholine	The presence of IgM anti-myelin/lipid has been found among oligoclonal bands in the CSF (57)
Neurofilament-L (NF-L)	Neurofilament light chain (NF-L) is a promising neuronal Ag biomarker, anti-NF-L Abs are increase in PP-MS and SP-MS (58)
Proteasome	auto-Abs directed against some specific subunits are increase in two thirds of MS patients (59)
Heat shock protein (HSP)	anti-HSP-60 protein have been associated with promotion of remyelination in animal and *in vitro* models (60), another report showed that anti-alpha-Bcrystallin Abs (a small HSP) was associated with an increase in relapse rate (61)
Nogo A (neurite outgrowth inhibitors)	IgM anti Nogo A was found to increase in both CSF and serum of RR-MS patients (62)
Lingo-1 (Nogo receptor-interacting)	Lingo-1 play a negative regulator of oligodendrocyte differentiation and myelination. Mice KO to Lingo-1 are protected against EAE (63)

c) Innate immune cells

The main note of the innate immune system is self-protection and maintenance of homeostasis. Nevertherless innate immune mechanisms can in some circumstances result in destructive autoimmunity. This part will summarize important findings and include observations that suggest a role for the following cells and molecules in the pathogenesis of MS: i) DCs are professional APC that play an important role in promoting activation and differentiation of naïve T cells, there are two distinguishes categories of DCs in human: myeloid (mDC) and lymphoid/plasmacytoid (pDC) (64). The interaction of DCs with T cells is crucial in determining T cell differentiation into either effector T cells (Th1, Th2, and Th17 cells) or Treg cells (natural Tregs and induced Tr1 cells). DCs can also stimulate natural killer (NK) cell mediated cytotoxicity or prime NK responses toward viral and bacterial pathogens (65). mDC-mediated inflammation is more pronounced in SP-MS patients than in RR-MS patients, these cells showed an enhanced of IL-12 in response to IFN-γ and LPS (66). This activated phenotype of DCs in both RR-MS and SP-MS patients is accompagnied by an enhanced secretion of TNF, IFN-γ, IL-6 and IL-23 (Th-17 bias cytokine) (67). pDCs are involved in both innate and adaptive immunity including protection from microbial infections and the generation of immunoregulatory immune responses. pDCs are assumed to play an important role in the immunoregulatory network in MS. pDCs are rarely detected in the CNS under nonpathologic conditions, but their number increase under neuroinflammatory conditions including MS in the CSF (68). ii) Microglia cells/macrophages, microglia are resident macrophages of the CNS, being involved in phagocytosis, antigen presentation and production of cytokines. There are rapidly activated in response to injury, neuro-degeneration, infection, tumors and inflammation. Until now, there are no unique markers distinguishing microglia cells from blood-derived macrophages in the CNS. Microglial cells and macrophages express all known toll like receptors (TLRs) and expression of these receptors is pivotal for generation of neuroimmune responses (69). Expression of TLRs is increased in brain lesions in EAE and MS (70). Macrophages and microglia cells are involved in demyelination and phagocytosis of the degraded myelin, which results in augmentation of the expression of myeloperoxidases, these enzymes and reactive oxygen species cause neuronal damage (71). Microglia cells can secrete TWEAK (TNF like weak inducer of apoptosis) molecule which is an inflammatory cytokine that can trigger proliferation, angiogenesis, inflammation and induction of cell death. The expression of TWEAK is upregulated in MS lesions by the cytokine milieu in CNS (72). Expression of TWEAK by microglia is involved

in extensive loss of myelin, neuronal damage and vascular abnormalities in cortical lesions. Microglia cells also express IL-17 and it receptor, IL-6, nitric oxide (NO), adhesion molecules, neurotropic factors and macrophage inflammatory proteins (73). Although the negative contribution of microglia/macrophage cells in MS or EAE pathology, there is evidence indicating a potential beneficial role of these cells by secreting anti-inflammatory cytokines (IL-10 and TGF-β) depending upon the inflammatory milieu in CNS (74). Switching their function from neuro-destructive to neuro-protective may be beneficial in preventing chronic demyelination and axonal loss and thus preventing progression or relapse of disease (75). iii) Natural killer (NK) Cells contribute to both effector and regulatory functions of the innate immune systeme via their cytotoxic activity mainly against viral infected cells or tumor cells and through their ability to secrete different cytokines (76). The actual role of NK cells in CNS autoimmunity is still unclear (77). *In vitro* NK cells show cytotoxic activity towards oligodendrocytes and other glial cells, such as astrocytes and microglial cells during inflammation by releasing of perforin (78). NK cells may also play a role in CNS protection and repair as these cells have the ability to produce neurotrophic factors such as brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3). iv) Mast cells are crucial component of allergic responses through the release of large quantities of histamine from their cytoplasm granules. Mast cells are present in the normal brain in the parenchyma and at the BBB and can interact with myelin (79). Studies have suggested several effects of mast cells in MS, elevated numbers in MS plaques were shown (80). Interestingly, mast cell–released mediators such as tryptase and histamine are increased in the CSF of MS patients. Mast cells and their mediators are involved in the BBB opening and increase CNS infiltration via inducing recruitment, adhesion, rolling, and extravasation of leukocytes through the chemokines/cytokines lymphotactin and IL-16, through TNF and IL-1-mediated induction of ICAM-1 and VCAM-1 expression. Mast cell proteases activate MMP precursors; Mast cell can also synthesize matrix metalloprotease (MMP-2 and MMP-9) directly. v) NK-T cells are a particular subset of T cells that share properties of NK cells and T cells and that recognize lipid antigen presented by CD1d by a T cells receptor of limited diversity. These cells can be either $CD4^+$ or $CD8^+$ or can be $CD4^-$ $CD8^-$. During MS, the number of total NK-T cells decreases with a prominent decrease of $CD4^-$ cell population and a modest decrease of $CD4^+$ NK-T cell subpopulation (81). The same study showed that long term cell lines derived from MS, enhanced of IL-4 and no differences in IFN-γ secretion, this Th2 bias of NK-T cells might be involved in mediating the remission phase of MS, highlighting the

immunoregulatory role of these cells in MS. vi) Gamma-delta T cells (γδT Cells) represent another distinct lymphocyte population that mediates host defense and immunoregulatory functions. Human γδT expresse NK cell inhibitory receptors which indicate there role in tumor immunity and autoimmunity. γδT cells exist under two fractions. One fraction expresses Vγ1 within epithelial tissues which provide a first line of defense against infections and cancer. The second fraction that expresses Vγ2 represents the majority of peripheral blood γδT cells. This fraction infiltrates chronic lesions and is detected in the CSF of MS patients (82). It is known that oligodendrocytes selectively stimulate the expansion of the Vγ2 subtype of γδT cells (83). Human γδT cells can lyse oligodendrocytes via perforin without the need for APCs, maybe through recognition of heat shock proteins (84), αB crystallin, or even non peptide antigens (85). These findings, together with EAE studies in which γδT cells appear to be important early mediators of damage (86), support a role for γδT cells in MS pathogenesis.

d) Cytokines and chemokines

Cytokines

To maintain homeostasis, a dynamic balance between pro- and anti-inflammatory cytokines is required. Pro-inflammatory cytokines are thought to play a role in the pathogenesis of MS via immune system activation in the periphery and/or by directly damaging the oligodendrocyte/myelin unit, in MS pro-inflammatory cytokines represented by (IFN-γ , TNF, IL-12, IL-17, and IL-23), and anti-inflammatory cytokines (IL-4, IL-10), and others exerting both effects as IL-6 (87). Pro-inflammatory cytokines can participate in the pathogenesis of MS at different points; elevated numbers of blood cells expressing TNF mRNA (88), serum TNF concentrations (89), and PBMC secreting TNF have been reported in MS patients (90). Nevertheless, therapy with a soluble TNF receptor Ig fusion protein or anti-TNF leads to increased and prolonged MS exacerbations (91). Results on blood IFN-γ in MS patients are conflicting. Although higher numbers of PBMC expressing IFN-γ mRNA and serum levels (89) have been found in MS, other studies found no differences (92). Higher numbers of mononuclear CSF cells expressing TNF and IFN-γ have been detected in MS patients. TNF has pro-inflammatory functions but is also involved in tissue repair in the brain. Pro-inflammatory cytokines have also been found in active MS lesions (93). The expression of TNF is elevated in active demyelinating lesions compared to inactive/remyelinating lesions (94). The addition of IFN-γ to cultured oligodendrocytes renders them susceptible to Fas

ligand–mediated apoptosis by inducing Fas expression on their surface (95). IL-12, a principal stimulator of IFN-γ has been implicated as a pro-inflammatory cytokine (96), but other data indicated that IL-23, a cytokine that shares the p40 chain with IL-12, is the main mediator of these effects (97). In MS, some studies have reported higher numbers of PBMC expressing IL-12 p40 mRNA (98), but other studies found no differences (99). IL-12 and IL-17 are also elevated in CSF and brain lesion of MS patients (100). Unexpectedly high numbers of cells expressing IL-4 mRNA have been observed in MS CSF lesions (101). Controvertial data on anti-inflammatory cytokines in MS described decreased numbers of PBMC secreting IL-10 and lower serum levels of IL-10 in MS (102). Moreover, investigators have described decreases in IL-10 expression but elevated numbers of PBMC expressing IL-10 mRNA before clinical relapses (103). Increased levels of IL-6, a cytokine with pro- and anti-inflammatory capacities, have been found in MS patient serum (90). Studies on IL-10 and IL-6 have been contradictory, at least two interpretations can be proposed: i) these cytokines, mainly IL-10, could be involved in MS pathogenesis by augmenting B cell proliferation, differentiation, and antibidy production. There is a correlation between IL-10 levels and IgG in the CSF of MS patients has been reported (104). ii) The presence of IL-4, IL-10, and TGF-β in the CSF or in MS brain parenchyma could reflect immunoregulatory effects that are initiated after disease aggravation and are important for disease resolution/prevention in EAE (105).

Chemokines

Chemokines and their receptors play an important role in the inflammatory recruitment of leukocytes and other cell types. Trafficking of inflammatory T cells into the CNS is a crucial step in MS and begins with weak adhesion and rolling on the endothelium of the BBB. Chemokines induce and activate leukocyte adhesion molecules that mediate firm adhesion to the endothelium and establish a chemotactic concentration gradient that results in recruitment across the endothelial monolayer. Chemokines mediate retention of leukocytes in the CNS. Numerous reports analyze the roles of chemokines and their receptors in intrathecal accumulation of T cells in MS (106). Among the various chemokine receptors, CCR5 and CXCR3 have received attention as key receptors on Th1 cells, as have CCR3 and CCR4 on Th2 cells. Furthermore, CCR7, an important marker for the capacity of mononuclear cells to migrate to secondary lymphoid organs, is also important. In MS chemokines were found in these compartements: <u>i) Blood:</u> CCR5 and CXCR3 expression is increased on circulating T

cells in MS patients (107). T cells expressing CCR5 and CXCR3 in MS produce high quantities of IFN-γ and TNF (108), and MBP-specific Th1 cells express high levels of CXCR3 and CXCR6 (109), ii) CSF: CCL5 (RANTES) and CXCL10 (IP-10) are elevated in MS CSF, whereas CCL2 (MCP-1) is significantly decreased (110). The increase of CXCL10 and decrease of CCL2 has been found during MS exacerbations and not during remissions (111). CCL3 (MIP-1α) has been found in the CSF of MS patients, as well as in other neuroinflammatory diseases. Higher proportion of CSF T cells that express CXCR3 and CCR5 has been found (110) compared to PBMC. Interestingly, CXCR3 expression probably facilitates the entry of T cells into the CSF, and CXCL10 mediates retention in the inflamed CNS. CCR5+ and CXCR3+ Th1 cells in the CSF also express CCR7 (112).

iii) Brain lesion: numerous chemokines and the corresponding receptors have been detected in MS brain lesions, CCL3, CCL4 (MIP-1β), and CCL5 are expressed within MS lesions, CCL4 in parenchymal inflammatory cells (macrophages and microglia), CCL3 also in parenchymal inflammatory cells and activated neuroglia (113), and CCL5 in perivascular inflammatory cells and less in astrocytes (114). Other chemokines in active MS lesions include CCL2, CCL7 (MCP-3), CCL8 (MCP-2), and CXCL10. CXCR3 is expressed on the majority of perivascular T cells in MS brain lesions. CCR1 has been found on newly infiltrating monocytes (115), CCR2 and CCR3 on macrophages, and CCR5 on infiltrating monocytes and activated microglia cells (114).

e) The blood brain barrier (BBB)

Penetration of the BBB by activated lymphocytes is a multistep process. There are specialized capillary endothelial cells in the CNS, which are nonfenestrated and connected by tight junctions. These capillary endothelial cells are induced to express vascular cellular adhesion molecule (V-CAM) and MHC class II by IFN-γ and TNF, which are released in the inflammatory response (116). Activated lymphocytes are able to extravasate through this barrier via adhesion molecules such as integrins, particularly alpha-4 integrin, which binds to V-CAM, and members of the Ig supergene family such as CD4, which bind to MHC class II. Any activated T cell expressing very late antigen (VLA-4) may bind to adhesion molecules on the surface of inflamed endothelium, which is the first component of the BBB and initiates transmigration. Blockade of VLA-4 reverses symptoms of clinical paralysis in acute EAE, and prevents further relapses in the chronic model. Once the activated lymphocytes have extravasated, they must pass through a barrier of extracellular matrix comprised of type IV

collagen before entering into the CNS. Alpha-1 integrin may play a role in binding to collagen type IV (117). After which, immune cells secrete enzymes such as MMP which allow the activated lymphocytes to gain access to the white matter surrounding axons of the CNS. MMP 2 and 9 also called gelatinase A and B, specifically degrade collagen type IV which surrounds inflamed brain endothelium. In MS, activated T cells, as well as NK cells secrete gelatinase A and B, allowing penetration through the extracellular matrix surrounding blood vessels in the CNS. IFN-β is a potent inhibitor of gelatinase B activity, and this may account for its success as a therapy for MS inhibition and may interfere with T cell migration into the CNS, as well as T cell secretion of TNF, a critical cytokine in the process of demyelination. It has also been demonstrated that TNF has neuroprotective functions (118, 119) and promotes proliferation of oligodendrocyte progenitors that aid in remyelination (120).

A.5.2. Demyelination and axonal degeneration

The traditional neuropathological view of MS highlights myelin loss (Figure 2) as the key event leading to impaired propagation of action potentials across the exposed region of the axon and ensuing neurological deficits. However, the early literature on MS already described substantial axonal damage in active lesions (121). Other histopathological studies reveal abundant transected and dystrophic axons in sites of active inflammation and demyelination, and confirm that partial or total axonal transection begins early in the disease process (4, 122). Axonal damage appears to take place in every newly formed lesion, and the cumulative axonal loss is considered now to be the reason for progressive and irreversible neurological disability in MS (123). Substantial numbers of axons (over 11000 per mm^3 of brain white matter) are transected in acute inflammatory demyelinating lesions (4) where the axon seems to be a target of the immunemediated demyelination (124). The axonal transection that produces early in MS is clinically silent because the human brain has a remarkable ability to compensate for neuronal and axonal loss (125). The transition of RR-MS to SP-MS is thought to occur when the brain exhausts its capacity to compensate for further axonal loss (126). In SP-MS, axonal degeneration of chronically demyelinated axons is supported by pathological and brain imaging studies. Pathological evidence for continuous neurodegeneration is based on macroscopic and microscopic observations. The presence of transected axons in chronic inactive lesions analysed post mortem provides histological evidence for degeneration of chronically demyelinated axons (127). The brains of many patients with SP-MS show progressive and substantial atrophy (Figure 3 and 4).

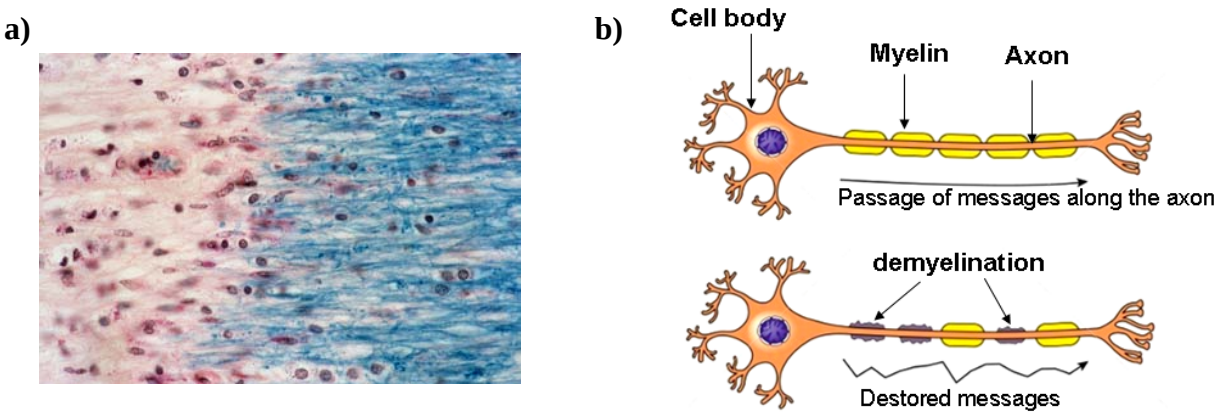

Figure 2: a) Demyelinated lesion (Luxol Fast Blue). Courtesy of Dr P.H. Lalive, and b) representation of myelin loss.

Different mechanisms of axonal injury in inflammatory lesions of the brain and spinal cord have been described including: adaptive immunity mediated through, i) cytotoxic CD8 T cells inducing axonal transection in active MS lesions (128), in EAE mice (129), and *in vitro* (130). Class II restricted T-cells may induce axonal injury, when they are directed against a protein component of the axon (131), CD4 T cells can induce tissue injury in an antigen independent way by inducing calcium oscillations and cell death in neurons (132), ii) auto-Abs against myelin components, and molecules expressed on the surface of axon, are thus potential candidates for mediating axonal or neuronal injury (133). Axonal injury can be mediated through innate immunity driven by iii) activated macrophages and microglia which produce a large array of toxic molecules, including, proteo-and lipolytic enzymes, cytotoxic cytokines, excitotoxins and reactive oxygen or nitric oxide intermediates (134). Pro-inflammatory cytokines released by activated T cells and activated microglia lead to further inflammatory cell recruitment, resulting in an aggressive immune attack against the myelin sheath. Following myelin destruction, axons have a higher demand for adenosine triphosphate (ATP) due to redistribution of sodium channels along the demyelinated segments (135). It has been shown that sodium channels play an important role in neurodegeneration during autoimmune CNS inflammation (136).

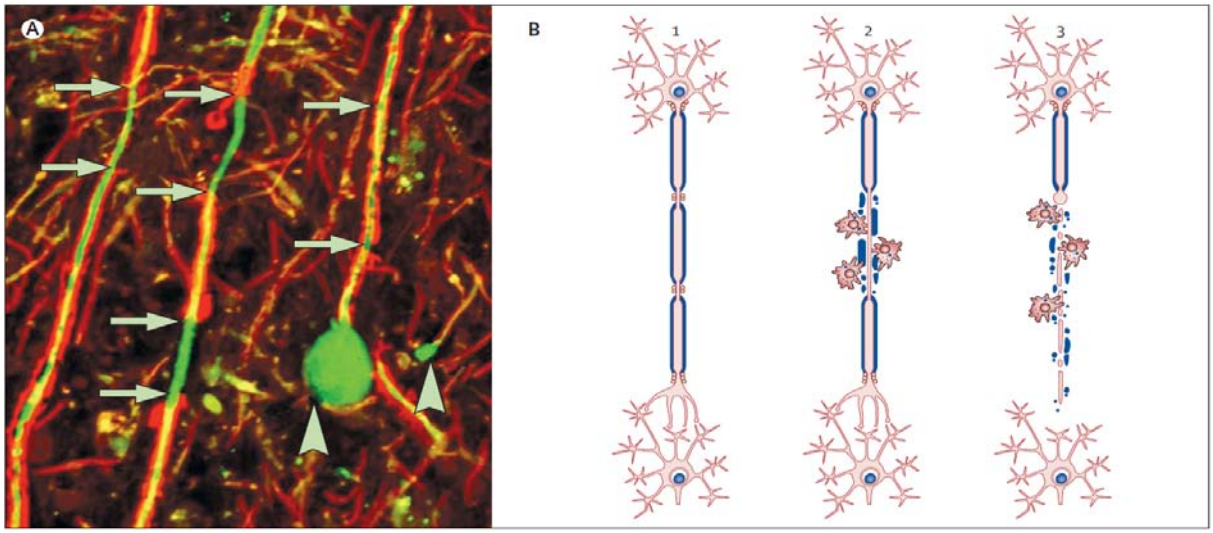

Figure 3*:* Transection of axons during inflammatory demyelination.
(A) Confocal image of an actively demyelinating MS lesion stained for myelin proteolipid protein (red) and non-phosphorylated neurofilaments (green). Three axons show areas of demyelination (arrows). Two axons end in swellings (arrowheads), called retraction bulbs, which are characteristic of the proximal ends of transected axons. Quantification of axonal retraction bulbs has established that significant axonal transection occurs in demyelinating lesions of MS. (B) Summary diagram of axonal responses during and after transection: (1) Normal myelinated axon. (2) Demyelination occurs due to an immune-mediated or immune cell-assisted process. (3) The distal end of the transected axon rapidly degenerates, whereas the proximal end connected to the neuronal cell body survives. After transection, the neuron continues to transport molecules and organelles down the axon, accumulating at the proximal site of the transection. Trapp *et al*., 2009.

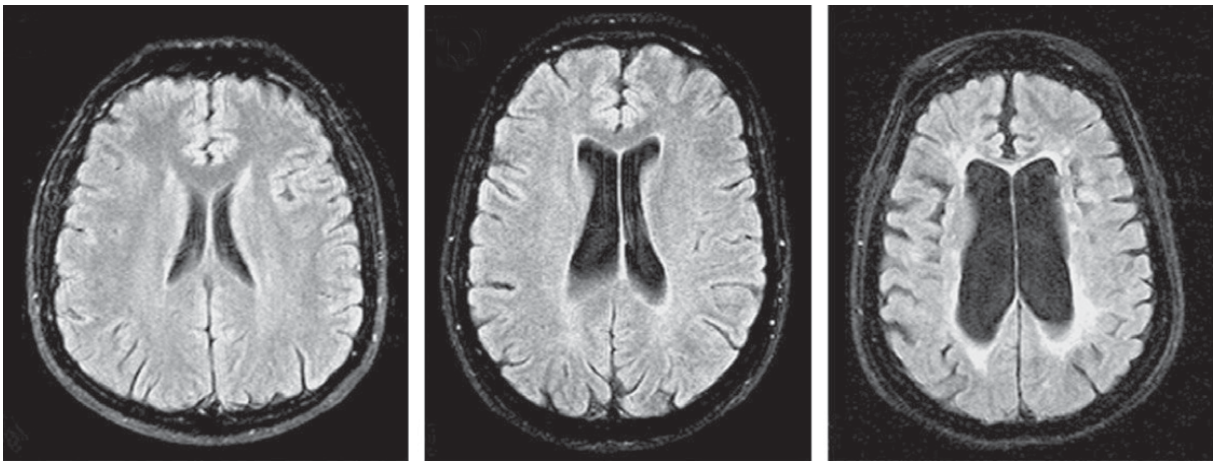

Figure 4: Cerebral atrophy in MS.
Chronically demyelinated axons degenerate due to loss of myelin trophic support and alterations in the chronic lesion microenvironment. Continuous and irreversible loss of brain tissue occurs during chronic MS, despite a dramatic reduction in new lesions. MRI scans are shown from (A) a normal brain, (B) the brain of a patient with RR-MS, and (C) the brain of a patient with SP-MS with end-stage disease. The progressive increase in ventricular volume indicates the brain atrophy that occur as most patients with MS age. Trapp *et al*., 2009.

In addition to the higher energy demand due to strong sodium concentrations in demyelinated axons, an impaired ATP production due to mitochondrial dysfunction has also been suggested (137). Defects of mitochondrial respiration chain complex IV have been described in MS patients (138), and have been associated with hypoxia-like tissue injury (6) and reduced in N-acetylaspartate (NAA) which is a sign for neuronal dysfunction and destruction (139). Increased energy demand and reduced ATP production due to demyelination leads to a vicious cycle in chronically demyelinated axons by the loss of Na^+/K^+-ATPase (140), which further contributes to an increased intracellular sodium concentration. Consequently, calcium is released from intracellular stores (141) and the direction of the Na^+/Ca^+-exchanger is reversed, resulting in additional extracellular calcium influx (142). The lethal increase in intracellular calcium then induces a variety of calcium-dependent enzymes like calpains, leading to cytoskeleton disruption (143). In addition, the proton-gated acid sensing ion channel-1 (ASIC1) has been associated with neurodegenerative processes in the context of neuroinflammation (144). The EAE model in ASIC1 deficient mice showed a reduced clinical deficit and reduced axonal degeneration compared to wild type mice. Axon pathology and the frequency of transected axons in MS lesions correlate with the degree of inflammation (4). The inflammatory microenvironment contains a variety of substances that induce axon damage. These factors include proteolytic enzymes, cytokines, oxidative products, and free radicals produced by activated immune and glial cells (145). iNOS, one of the principal enzymes involved in synthesis of NO, is upregulated in acute inflammatory MS lesions (146) by modifying the action of key ion channels, transporters, and glycolytic enzymes (147). NO and its derivative, peroxinitrite, also inhibit mitochondrial respiration (148) and limit the axon's ability to generate ATP.

Glutamate-mediated exitotoxicity is observed in many acute and chronic neurodegenerative conditions (149). Excitatory glutamatergic mechanisms, mediated by N-methyl-D aspartate (NMDA) and amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate receptors could be responsible for damage not only of oligodendrocytes but also of neurons. These excitotoxic mechanisms have been advocated to be further effectors of oligodendrocyte death and axonal damage and one of the major causes of neurological impairment in EAE (150). Several potential sources of glutamate were found in acute MS lesions and EAE including activated immune cells (151), axons (152), and astrocytes (153). Magnetic resonance spectroscopic studies of MS brains have detected elevated glutamate levels in acute MS lesions and in normal appearing white matter (154). A recent study by Werner *et al*. (155) confirmed the high level of glutaminase (glutamate-synthesizing enzyme)

expression in macrophages and microglia in close proximity to dystrophic neurons within active MS lesions, as seen in EAE. When released in excess, glutamate activates ionotropic and metabotropic receptors resulting in toxic cytoplasmic $Ca2^{+}$ accumulation and cell death.

A.6. Currently available MS therapies

Therapies developed so far work on several mechanisms such as immune modulations or inhibition of immune cell migration. Some of these therapies reduce the clinical disease activity and the progression of lesion load as determined by MRI. But so far no therapy is available to cure MS or even halt its progression. The search for more effective approches is therefore warranted.

Glucocorticoids (GCs)

GCs are known for their broad anti-inflammatory properties (156). Short courses of high dose intravenously administered synthetic GCs (i.e. methylprednisolone) are nowadays frequently used to reduce the duration and severity of acute MS relapses (157). However, the chronic use of GCs is unwanted as it usually causes severe side effects. The effects of GCs on MS relapses may be accounted for by direct effects on macrophage and microglia. GCs receptor is highly expressed in many macrophage populations, including microglia (158). It has been described that GCs down regulate TNF produced by activated microglia, prevent phosphorylation of p38 MAPK and decrease the expression of MHC class II molecules (159). GCs can change the cytokine profile and induce the shift from a Th1 into a Th2 response (160).

IFN-β (Rebif/Avonex/Betaferon®)

IFN-β is a type I interferon, which is used in the treatment of RR-MS and can reduce the frequency of clinical exacerbations (161). Still, IFN-β does not fully block lesion development, and can therefore only delay but not halt disease progression. Moreover, a large proportion of treated patients do not respond to the therapy which may be associated with the development of Abs against IFN-β (162). In addition, it may mediate a shift from a Th1 towards a Th2 response by reducing pro-inflammatory cytokines and induction of anti-inflammatory cytokines (163). Furthermore, the adhesion and migration of T cells, but also monocytes, into the CNS can be diminished because IFN- β inhibits the expression of

adhesion molecules on vascular endothelium as well (164). It was shown recently that IFN-β induces brain-derived neurotrophic factor (BDNF) in PBMC of MS patients and hypothesizes a potential indirect neuroprotective effect of IFN-β in RR-MS patients (165).

Glatiramer acetate (GA/Copaxone®)

GA (Copolymer 1 (Cop-1)/Copaxone®), is a synthetic random polymer of 4 amino acids (glutamate, lysine, alanine and tyrosine) in a ratio similar to that found in MBP (166). GA is one approved therapy for RR-MS and has shown to decrease the number of CNS lesions and the frequency of relapses. Initially, two main modes of action were suggested, i) T cells reacting to GA shift from a Th1 towards a Th2 phenotype (167), ii) GA may compete with MBP for binding to the CD11b (an integrin expressed on macrophages) as well as to the MHC class II of all APCs (168). GA would therefore inhibit the uptake and presentation of MBP and subsequently prevent the activation of MBP-reactive T cells. However GA appeared to directly induce type II anti-inflammatory monocytes in an MHC class II independent manner (169). These monocytes secrete high levels of IL-10 and TGF-β but low amounts of IL-12 and TNF in mice. Recent data further emphasize GA effects on monocytes by showing that treatment decreases the production of the pro-inflammatory cytokine IL-1β by chronically activated mouse and human macrophages (170). Simultaneously, the production of secreted IL-1 receptor antagonist (IL-1Ra), which prevents signalling via IL-1β, is increased. These recent studies indicate that the beneficial effects of glatiramer acetate exert some important modes of action via the myeloid compartment as well. Finally iii) GA is suggested to have neuroprotective actions, as demonstrated by the fact that GA-activated T cells can produce (BDNF) (171), and also prolonged induction of BDNF by CNS resident cells in different areas of the mouse brain during EAE (172).

Natalizumab/Tysabri®

Natalizumab is a humanized mAb directed against VLA-4, which is expressed on most leukocytes, and binds to the vascular cell adhesion molecule (VCAM)-1 on vascular endothelial cells. Natalizumab reduces relapse rates and disease progression in MS patients (173). It reduced not only the number of T cells, but also of macrophages and B cells in the cerebrospinal fluid in treated patients (174). Nevertherless, the utilisation of this treatment is limited to specific patient due to a risk of the opportunistic infection, the progressive multifocal leukoencephalopathy (PML).

Mitoxantrone (Novantrone®)

Mitoxantrone is an anti-neoplastic agent interfering with DNA synthesis and consequently cell division, mitoxantrone is used for many years to treat malignancies as breast and prostate cancer, and leukemia. Its potential usefulness in the treatment of MS was considered when mitoxantrone appeared to have immune suppressive effects as well. It blocks myelin degradation by macrophages (175). In MS, clinical trials have shown that treatment with mitoxantrone reduced gadolinium-enhanced lesions on MRI as well as relapse rate and disability progression (176), it has been shown that mitoxantrone caused a marked reduction in the proliferation of B cells, as well as a reduced ability of T cells to induce an immune response and it is expected that mitoxantrone inhibit microglia proliferation.

B. Experimental autoimmune encephalomyelitis (EAE)

EAE was described over 50 years ago as an important model of CNS autoimmune inflammation (177). The origins of EAE date back to the 1920s, when Koritschoner and Schweinburg induced spinal cord inflammation in rabbits by inoculation with human spinal cord extract. In the 1930s, researchers attempted to reproduce the encephalitic complications associated with rabies vaccination by repetitive immunization of rhesus monkeys with CNS tissue (178). Since then, several animal models of EAE have been generated in animals such as rodents and primates, and from these studies it became clear that EAE can produce many of the clinical, neuropathological and immunological aspects of MS (179).

B.1. Modes of immunization

EAE has been actively induced in susceptible experimental animals via several routes. Initially, active immunization with CNS homogenates or myelin components suspended in strong adjuvant was used. Later, especially with the advent of T-cell line technology, EAE was passively transferred by *in vitro*-activated autoimmune T-cell lines. More recently, spontaneous EAE models have been developed with transgenic mice in which a large proportion of T cells are myelin-specific (Table 2 and 3).

B.1.1. Actively-induced EAE (aEAE)

aEAE is an inducible model that yields fast and robust results in studies screening the effects of drugs on autoimmune inflammation or the function of particular genes in transgenic/knock out mice. These models are important for studying the encephalitogenic potential of myelin. MOG makes up less than 0.5% of all myelin proteins and is situated on the surface of myelin sheaths. MOG_{35-55} peptide is strongly encephalitogenic in C75BL/6 mice, the strain providing the genetic background of most transgenic mice (180). Disease induction with MOG_{35-55} in C57BL/6 mice requires the use of pertusis toxin (PTX) as part of the induction regimen. PTX has been hypothesized to facilitate immune cell entry into the CNS (181). However, PTX has other biological effects that could contribute to its activity in EAE, such as breaking T cell tolerance and promoting clonal expansion and cytokine production by T cells (182). In most murine EAE models, after a prodromal interval of 10-20 days, paralysis of tail and hind legs, progressing to the forelimbs and weight loss develop, reflecting preferential targeting of inflammation to the spinal cord and to some extent the cerebellum. There are rodent strains particularly susceptible to aEAE, and others which are more resistant, but in each animal strain, EAE responsiveness depends critically on the nature of the autoantigen applied. Interestingly, the encephalitogenic potential of CNS proteins appears to be strain dependent. Lewis rats, for example, are highly responsive to autoimmunization against myelin basic protein (MBP) (183), but respond poorly to MOG (184), while reactivity of C57BL/6 mice is the exact opposite (185). Myelin and non-myelin proteins were studied as potential autoantigens on MS and EAE including myelin antigens (MBP, PLP, MOG, MOBP, MAG, OSP, NogoA, GNPase), glial antigens (GFAP, S100b, αβ-crystallin), and neuronal antigens (Neurofilament-L, Neurofilament-M, b-synuclein, Contactin-2, Neurofascin) (131). More recently, C57BL/6 mice have become the animal of choice, especially for studies involving transgenic mice. Lewis rat and and C57BL/6 mice develop either self limited monophasic or chronic EAE. In addition to these models, EAE with interchanging relapses and remissions, reminiscent of early human MS, can be induced in SJL/J or Biozzi ABH mice. Finally, primate models of EAE have been used as they are genetically and physiologically closer to humans, and share clinical and pathological similarities to MS (186).

B.1.2. Passively transferred EAE (pEAE)

pEAE has also been useful in drug screening and functional gene characterization (Table 3). EAE can be adoptively transferred to naïve mice by injection of *in vitro* neuroantigen-activated T cells isolated from primed donors (187). The most common adoptive transfer models involve MHC class II-restricted CD4 T helper cells (188), while only a few reports describe EAE transfer by CD8 T cells (189). Many of the pathogenic CD4 T cells respond to activation by secreting IFN-γ and TNF, but not IL-4 or IL-5, qualifying them as Th1 cells. Recent studies, however, suggest that this population includes a subset of CD4 T cells that preferentially produce IL-17 (Th17 cells), by modifying T cell effector functions *in vitro* before transfer, Langrish *et al.* confirmed that IL-23-driven IL-17+ T cells are highly encephalitogenic (190). These data suggest that both Th1 and Th17 cells have pathogenic potential with respect to EAE induction (191). However Becher *et al.* have shown that neither the T cell driven overexpression of IL-17A nor its complete loss had a major impact on the development of EAE, by consequence Th17 T cells may only marginally contribute to the development of autoimmune CNS disease (192).

Table 2. Active and passive EAE models and their applications. Krishnamourthy *et al*., 2009.

	Known encephalitogenic autoantigen/epitopes		Studies and applications
Species/strains	aEAE	pEAE	
Mice			
C57BL/6	$MOG_{35–55}$ (185) $OSP_{179–207}$ (193) $rNogo_{-66}$ (194)	$NF\text{-}M_{18–30}$ (131) $MOG_{35–55}$ (185)	Commonly used strain for transgenic mice construction; Th1/Th17 CD4 T-cell mediated pathogenesis; pre-clinical validation of therapeutic compounds, CD8 T-cell mediated CNS damage
SJL/J	MOG, $MOG_{92–106}$ (195) $PLP_{139–151}$ (197) $PLP_{178–191}$ (199) $PLP_{104–117}$ (201) $OSP_{57–72}$ (203) $MOBP_{37–60}$ (205) $MOBP_{15–36}$ (204) MBP (207), $MBP_{89–101}$ (208), $Nogo_{-66}$ (194)	MOG (196) $PLP_{139–151}$ (198) $PLP_{178–191}$ (200) OSP (202) $MOBP_{15–36}$ (204) $MBP_{89–101}$ (206)	Study of relapse mechanisms; genetic control of autoimmune disease; epitope spreading; Abs mediated demyelination, gender influence on autoimmunity, pre-clinical validation of therapeutic compounds
Biozzi ABH	MOG, $MOG_{1–22}$, $MOG_{43–57}$ $MOG_{134–148}$ (195) $PLP_{56–70}$ (209) $MBP_{21–35}$ (208) $MAG_{97–112}$ (203) $\alpha\beta crystallin_{1–16}$ (210) NF-L (211), GFAP (212)		Study of relapse mechanisms; antibody mediated demyelination, pre-clinical validation of therapeutic compounds
B10.PL and PL/J	MBP Ac1–11 (213) $MOG_{35–55}$ (214)	MBP $Ac_{1–11}$ $MOG_{35–55}$	Study of Treg; molecular mimicry and environmental triggers, T-cell self-tolerance, pre-clinical validation of therapeutic compounds
C3H/HeJ	$PLP_{215–232}$, PLP (200)	$MBP_{79–87}$ (129)	CD8 T-cell-mediated CNS damage, T-cell self tolerance
Rat			
Lewis	MBP, $MBP_{29–84}$, MBP 61–82, $MBP_{80–105}$ $MBP_{170–186}$ (183) $\beta\text{-Synuclein}_{93–111}$ (216)	MBP (183, 215) $\beta\text{-Synuclein}_{93–111}$ (216)	Study of migratory behaviour of autoimmune T cells, genetic control of autoimmunity, pre-clinical validation of therapeutic compounds
DA, BN	MOG (217)	$MOG_{74–90}$, $MOG_{93–107}$ (218)	Antibody-mediated demyelination, molecular mimicry, genetic control of autoimmunity
Primates			
Rhesus monkey	MBP, $MOG_{34–56}$ (219)		Environmental triggers of CNS autoimmunity, axonal pathology, Abs-mediated demyelination, pre-clinical validation of therapeutic compounds
Common marmoset	MOG, $MOG_{14–36}$ (220)		Same as for rhesus monkey

Table 3. Spontaneous EAE mouse models. Krishnamourthy *et al.*, 2009.

Strain	Epitope	Model	Clinical charaterization	Applications
C57BL/6	MOG_{35-55}	CD4+TCR tg (221)	Paralytic EAE and optic neuritis (4 and 30%)	Study of autoimmune mechanisms developing without exogenous manipulation (spontaneous T-cell activation, B-cell responses, innate immune mechanisms, pre-clinical validation of therapeutic compounds)
C57BL/6	MOG_{35-55} and MOG	CD4+ TCR Tg x BCR tg (222)	Paralytic and optic neuritis (50%)	
B10.PL	MBP Ac_{1-9}	CD4+ TCR tg (223)	Paralytic EAE (100% in RAG-deficient background	
B10.PL	$MBPAc_{1-11}$	CD4+TCR tg (224)	Paralytic EAE (14-44%)	
SJL/J	$PLP_{139-151}$	CD4+ TCR tg (225)	Paralytic EAE (45-83%)	
SJL/J	$PLP_{139-151}$	CD4+ TCR tg (196)	Paralytic and ataxic EAE relapsing-remitting (60-90%)	
C57BL/6	Neo-self Antigen	ODC-OVA tg x OT-I (CD8+) TCR tg (226)	Paralytic EAE and locomotor defects (90% in normal Backround and 100% in RAG-deficient backround)	
Humanized Tg (HLA-DR2)	$hMBP_{84-102}$	Human CD4+ tg (with HLA DR2) (227)	Classical EAE (4% in normal background and 100% in RAG-deficient backround)	Study of human immune mechanisms in an in vivo context (genetic control, effector mechanisms).
Humanized Tg (HLA A3)	$hPLP_{45-53}$	Human CD8+ TCR tg (with HLA A3) (228)	Motor deficits (4%)	
Humanzed Tg (HLA-DR2)	$hMBP_{85-99}$	Human CD4+ TCR tg (with or without HLA-DR2) (229)	Progressive EAE (86%) without HLA-DR2 and relapsing EAE with HLA-DR2 (54%) in RAG-deficient bacground	

B.2. Physiopathology

In the brain, immune privilege has been attributed to two morphological peculiarities of this organ, the absence of classical lymph vessels and the BBB, a mechanical diffusion barrier for hydrophilic blood molecules built by specialized tight junctions in brain capillaries (230). However, antigens turned out to drain from brain to cervical lymph nodes (231) and leukocyte recruitment takes place in poscapillary venules (232). Immune tolerance is actively maintained involving various mechanisms such as peripheral depletion and tight control over the activation state of local APCs (233). The former is achieved by the constitutive expression of CD95 ligand (FasL) and Apo 1L allowing apoptotic elimination of activated T cells (234). Experimental model led us to understand the persistence of the immune-surveillance, first of

all autoreactive cells must escape selection in the thymus and occur naturally, despite the widespread expression of myelin autoantigens in the thymus gland (235). For example MBP-reactive T cells were first detected in the repertoire of naïve Lewis rats (236). Following focused on autoreactive MBP-specific T-cell responses from MS patients. Surprisingly, many similarities between MS patients and healthy controls were observed (237). A crucial control of autoreactive T cells is exerted by regulatory T cells, which shape and tune the recognition of the self-antigens (238). Lafaille *et al.* showed that even if all transgenic T cells bear myelin-specific TCRs, spontaneous EAE is still prevented by naturally occurring regulatory T cells, but will start as soon as the physiological mechanisms controlling T-cell homeostasis are disturbed (223). The paralleled evidence showed that T-cell regulation is disturbed in MS patients (239). Autoreactive T cells, present in the normal immune repertoire, must be activated and acquire the capacity to migrate across BBB (240). These activated T cells express a set of molecules and enzymes, ready to traverse the BBB. These autoreative T cells adhere to the endothelium via upregulated adhesion molecules as VLA-4/VCAM-1 which seem to play a dominant role (241). VLA-4 is the only molecule known to mediate both leukocyte rolling and arresting (242). Data from an EAE demonstrated both a reduced leukocyte migration into brain parenchyma and the development of EAE following repeated administration of monoclonal Abs (mAbs) known as Natalizumab (243) reversed towards the α4 chain of VLA-4 have proven to be very effective for MS treatment (244). For the transmigration, enzymes such as matrix metalloproteinases are crucial importance, based on their upregulation during EAE and therapeutic blockade (245). Once the T cells have passed the BBB they start local interaction with APC in the CNS (246). Clinical disease in EAE develops only when T cells that have entered the CNS are sufficiently re-activated in the CNS environment (247). The initial damage introduced by autoantigen specific T cells is a strong stimulus for further recruitment of macrophages and complement-mediated damage to oligodendrocytes and other structures in the CNS (248). Activated marcophages and microglia both produce a large number of deleterious soluble factors, which can induce functional blockade and/or structural damage in axons *in vitro*. Among these factors: NO, possibly in combination with reactive oxygen species (249), matrix metalloproteinases (250) and other molecules as exitotoxins (251) and proteases (252). The majority of T cells entering the brain are destroyed by apoptotic cell death, (253). Although the progress in the molecular techniques for apoptosis detection, the decisive death signals that induce T-cell apoptosis are only incompletely understood. There is a clear contribution of TNF-signalling (254), but also naturally occurring steroids may be involved (255). Phagocytosis of these apoptotic T-cell

fragments by microglia cells, so far characterized in cell culture of rodent and human glial cells (256). However physiological elimination of monocytic cells from the inflamed brain seems to occur rather by migration and not by cellular apoptosis. The rare apoptotic macrophages observed in EAE (257) do not suffice to explain the regression of macrophage infiltration.

B.2.1. Antigen presenting cells

In order for T lymphocytes to recognize and respond to antigen, an antigenic peptide has to be processed and presented on the surface of an APC in the context of MHC complexes. Competent professional APC for CD4 T cells, such as DC and macrophages, can capture and process antigen as a result of their ability to endocytose pathogens and cellular debris (258). Upon Ag-capture, DCs subsequently migrate to secondary lymphoid organs where they present the Ag, in the context of a MHC-II complex, to the CD4 T cells. If the interaction is strong enough, based on the specificity of the TCR, it can lead to T cell activation and proliferation (259). Although the selection process in the thymus should in theory eliminate all auto-aggressive cells, a certain proportion of mature, peripheral T cells carry a TCR that can recognize self-antigens. Although these self-reactive T cell are prime candidates for autoimmune disease initiators, the only presence of such auto-reactive T cells does not automatically lead to autoimmune disease as mechanisms of peripheral tolerance usually prevent such occurrence. Peripheral T cells require two types of signals from APC for activation and subsequent effectors function (Figure 5). One is the antigen-specific signal; the second is an antigen-independent signal provided by co-stimulatory molecules expressed by APCs. Several molecules have been implicated to have co-stimulatory potential. The most largely studied co-stimulatory receptor-ligand pair involves the B7-CD28 system. CD28 is expressed by T cells and its ligands are members of the B7 family (B7.1, CD80; B7.2, CD86) expressed on the APC (260). Peripheral T cells will be anergic when they encounter an antigen presented by an APC in the absence of a co-stimulatory signal, unable to proliferate and to produce cytokines upon challenge with antigen, or even induce apoptosis (261). The function of these molecules in autoimmunity has been extensively studied. In EAE neural Ag-directed immune responses and clinical disease are inhibited in an additive or synergistic manner by monoclonal Abs (mAbs) directed against B7.1 and B7.2 (262). The most effective APC of the systemic immune compartment are DCs which are currently being heavily investigated as they appear to conduct major control over the development of immunity.

Different subtypes of DCs has been identified and their distinct immune functions have been studied. There are three major sites at which encephalitogenic lymphocytes can encounter competent APCs: i) the systemic immune compartment with its secondary lymphoid tissues, ii) the CNS parenchyma harboring microglia and astrocytes, iii) the perivascular and meningeal space with its macrophages and DCs. It has been shown that myelin-specific lymphocytes transferred into rats first enter secondary lymphoid tissues, where the expression of activation markers is synchronized before CNS entry (246). An other group showed that the migration pattern of these encephalitogenic lymphocytes is not essential to ultimately invade the CNS, because in absence of lymphoid organs encephalitogenic lymphocytes still find their way into the CNS and initiate tissue destruction (263). Within the CNS, astrocytes and endothelial cells, in principle, are capable of expressing MHC class II molecules, but their capacity to act as competent APCs remains speculative (264), several groups showed that microglia and macrophages are generally considered the most prominent CNS-resident APCs and their capacity to present antigen *in vitro* has been repeatedly (265). In addition, it has also been shown that lymphocyte-induced activation of microglia *in vivo* is crucial for immune infiltration of the CNS and the maintenance of encephalitogenicity during the effector phase of EAE (266). Greter *et al*. postulated that a population of DCs associated with the meninges and CNS blood vessels allows encephalitogenic T cells to recognize their cognate antigen leading to CNS invasion, inflammation and ultimately neurologic deficit (263), they proposed that DCs at the interface of the CNS and the immune system are pivotal players in the development of CNS inflammation and could serve as a novel therapeutic target for the treatment of patients with MS and other inflammatory diseases of the CNS.

B.2.2. T cells

It is well established that myelin-reactive T cells are crucial for the induction of EAE (267). Earlier studies showed that myelin-Ag specific T cells with a Th1 phenotype transfer EAE, whereas Th2 cells were found to be incapable of transferring EAE (268). Recently, another subset of T cells, called Th17 cells, has been identified. Different from Th1 and Th2 cells, Th17 cells are generated from naive T cells by TGF-β and IL-6 (269) and are expanded and stabilized further by IL-23. Th17 cells are able to transfer EAE. It has been confirmed that IL-23-dependent IL-17-producing CD4 T cells are highly pathogenic and essential for the establishment of inflammation associated with CNS autoimmunity (190), another report found that only Th1 cells, but not Th17 cells, induce EAE (192, 270). These differences in the

observations might be due to differences in the capacity of Th1 vs Th17 cells to induce EAE. However, since each of these studies used a different T cell differentiation protocol and T cells of different TCR specificity, the conflicting data might be due to variations in the cytokine profiles of the T cell subsets or to different Ag specificities of the effector T cells. In addition to Th1, Th2, and Th17 cells, another effector T cell subset, Th9 cells, has recently been described (271). Driven by the combined effects of TGF-β and IL-4, Th9 cells produce large amounts of IL-9 and IL-10. It has been shown that IL-9 together with TGF-β can contribute to Th17 cell differentiation, and Th17 cells themselves can produce IL-9 (272). And that not only Th1 cells, but also Th17 and Th9 cells, are capable of inducing EAE upon adoptive transfer (273).

Regulatory T cells (Treg)

The study of EAE model led us to develop our understanding of how the immune system can regulate an autoimmune disease. The induction, expansion and maintenance of a putative suppressor/regulatory population $CD4^+CD25^+$ expressing the transcription factor $Foxp3^+$ (the master regulator of Treg function in EAE) has been the subject of intense study including both autoimmune disease and infection (274). It was shown that polyclonal $CD25^+$ T cells from naïve C57BL/6 mice were found to inhibit both IFN-γ production and proliferation of encephalitogenic cells from MOG_{35-55} immunized mice *in vitro* when co-cultured at high suppressor to responder ratios. Transfer of high numbers of these "naïve" $CD25^+$ Tregs could limit the severity of subsequent disease (275). *In vitro* suppression of MOG_{35-55}-reactive effector cell function by Tregs could be abrogated in part by addition of soluble IL-10 receptor. Furthermore, the failure of IL-10-deficient Tregs to reproduce this effect suggested a key role for this cytokine in Treg mediated suppression of EAE (276). Several studies have shown that Treg depletion prior to immunization for active disease could increase the severity of disease (277) and could enhance IFN-γ, IL-6 and IL-17 production (278) indicating that Tregs suppress the expansion of autoreactive effectors. This most likely occurs during priming in the draining lymph node. A recent report using two-photon imaging suggests that Tregs are able to maintain the thresholds for activation, even under inflammatory conditions may be through disrupting the capacity of DCs to prime naïve autoreactive T cells in the lymph node (279). Analysis of the movement of MBP-reactive naïve T cells and DCs showed that they formed more meaningful partnerships in the presence of MBP peptide within the node when Tregs were absent than when they were present.

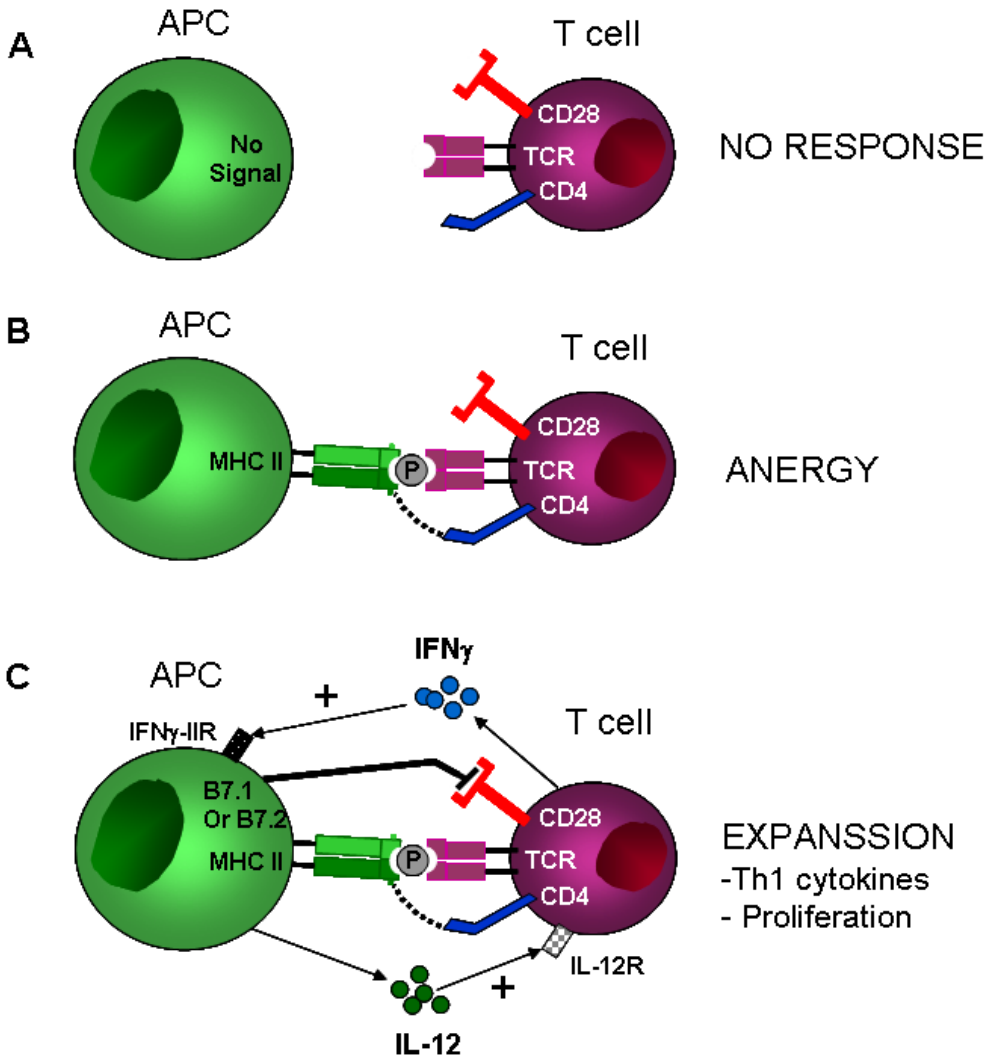

Figure 5: Two signals are required to successfully present Ags. CD4 T lymphocytes do not recognize native Ag and require Ag to be presented by APC in the context of MHC class II molecules. T cells that are presented with Ag in the presence of co-stimulatory signals (as B7.1-2) are usually rendered anergic (clonal tolerance). Delivery of both signals (TCR and CD28) leads to T cell expansion and activation. T cell-derived TNF and INF-γ leads to activation of more APCs that in turn can stimulate more T cells and induce Th1 differentiation via IL-12 secretion, (Becher *et al.*, 2000).

B.2.3. Cytokines as mediators of inflammation

It was shown that Th1 cells infiltrate the CNS in EAE in large numbers and produce their cytokines when CNS inflammation occurred. Purified Th1 cells could be transferred into naïve animals and mediate autoimmune disease. Conversely, Th2 cells were shown to exert beneficial effects in EAE (280). It was surprising at first that in mice none of the Th1 and Th1-inducing cytokines (IFN-γ, IL-12, IL-18, and TNF) could explain the importance of the Th1 effectors cells in the disease (281-284). It was reported that MOG-induced EAE in IFN-γ

cytokine knockout mice was not different than disease in mice with intact cytokine production (285). The conclusion was that IFN-γ is not crucial to the induction of EAE. An other study with IFN-γ R knockout mice supports this conclusion (281). In the same study they directly support a down-regulatory role for IFN-γ in both induction and effector phase of MOG-induced EAE. The role of IL-12 in EAE has been addressed based on the differential susceptibility of *p35-/-* mice and *p40-/-* mice to the development of EAE. They showed that the *p35-/-* animals develop severe EAE when immunized with MOG peptide, whereas *p40-/-* mice are resistant to disease development. Thus, IL-12 p70 cannot be required for the development of EAE. In contrast, IL-12 p70 was shown to be required for Th1 differentiation. IL-12 family member IL-23 instead of IL-12 itself is a prerequisite for the development of EAE (282). The central role of IL-23 in EAE was proven by Sedgewick and colleagues by the deletion of the p19 subunit of IL-23 (97). The particular impact connected to this finding was the description of a novel Th cell effector type, which showed IL-23-dependent expansion and expression of the pro-inflammatory cytokine IL-17A (190). IL-23- and IL-6-deficient mice showed impaired Th17 polarization. In consequence, it seems that such loss rendered the mice resistant to EAE (191). Some data was generated that correlated Th17 cells and IL-17A expression with the development of autoimmune inflammation in mice and humans (286). Neutralization of IL-17 in MOG induced EAE in C57BL/6 mice by Hofstetter *et al*. with IL-17-receptor-Fc-fusion soluble protein or with mAbs against IL-17A and F resulted in an attenuated clinical EAE course (287). It has been shown also that the development EAE was significantly suppressed in *IL-17-/-* mice (288).

C. Other animal models of demyelination

C.1. TMEV-induced hepatitis virus and Semliki

Semliki Forest virus (SFV) induces a CNS infection and is useful for understanding the potential viral etiology of MS (289). Intracerebral infection of susceptible strains of mice, such as SJL, with natural Theiler's murine encephalomyelitis virus (TMEV) leads to either rapidly fatal encephalomyelitis (high neurovirulence strains) or persistent CNS infection and immune-mediated demyelination (290) characterized by gait disturbances, spastic hind limb paralysis, and urinary incontinency start 30-40 days after infection. During the acute phase of the disease, virus replication is mainly in neurons, whereas during the chronic phase, TMEV persists predominantly in macrophages and glia (291). TMEV virus is known to cause demyelination in infected nude mice that cannot generate T lymphocytes (292), also pointing

to a direct viral effect on myelin damage. Upon TMEV infection, cytokines and chemokines are induced in primary astrocytes such as TNF, IL-1, IL-6, CCL2, and CCL5 (293). Inflammatory responses to TMEV infection seem to depend on TLR3 and TLR2 (294), as well as protein kinase signalling (295). CD8 T cells play an important role in the clearance of TMEV from CNS (296), however, there is also evidence that MHC-I restricted CD8 T may be directly cytotoxic to axons and mediate injury (297).

C.2. Cuprizone and lysolecithin, chemical-induced demyelination

Several toxin-based models of demyelination, including cuprizone and lysolecithin, while not attempting to accurately mimic the pathogenesis of autoimmune CNS inflammation, have proven very useful in studying the mechanisms of demyelination. In contrast to the systemically administered cuprizone, the membrane solubilising, glia-toxic lysolecithin has been used to create focal area of demyelination by direct injection into defined CNS white matter tracts, which allows for a better control of lesion size and location (298). Feeding of cuprizone (bis-cyclohexanone oxaldihydrazone) to young adult mice induces a consistent, synchronous, and anatomically reproducible demyelination. Furthermore, removal of cuprizone from the diet of mice leads to remyelination (299). Cuprizone is a chelator that binds copper, an essential component of metalloenzymes, like the mitochondrial cytochrome oxidase and monoamine oxidase. Cuprizone can induce demyelination in different strains of mice, among them in 8-10-weeks old C57BL/6 mice, the background most commonly used for knockout and transgenic mice. If cuprizone is overdosed, (mitochondrial) hepatopathy and weight loss are serious toxic effects (300). The regional pattern of demyelination includes white matter tracts, preferentially the corpus callosum, as well as the cerebellar peduncles (301). Recently, cortical demyelination has been detected (302), and demyelination in the hyppocampal formation has been associated with seizures in the cuprizone model (303). Substantial demyelination is present after 3 weeks of cuprizone, and by 4-5 weeks more than 90% of axons are demyelinated. Under continued exposure to cuprizone, about 50% of axons recover and are remyelinated by 6 weeks. During cuprizone toxicity, there is also demyelination-associated axonal loss which is more prominent in aged mice (304). During administration of cuprizone, the expression of the myelin gene in the brain decreases, and most of the mature oligodendrocytes in the corpus callosum, the site of the particular profound demyelination, undergo apoptosis (305). In contrast to EAE, the BBB seems to

remain intact (306) and T cells are almost completely absent, while microglia/macrophages accumulate and predominate in the demyelinated areas (307).

C.3. Model of lipopolysaccharide-induced demyelination

Some studies have examined the effects of lipopolysaccharide (LPS) injections into the CNS. It was describe that an inflammatory reaction, and after a delay of 5-7 days, a demyelinating lesions that occur following the injection of LPS into rat spinal white matter (308, 309) and persist between 9 and 14 days. Besides activation of resident microglia, CNS infiltrating phagocytes (neutrophils and monocytes) seem to contribute to the inflammatory response (310). It has been shown that microglial TLR4 is required for leukocyte recruitment into the brain in response to LPS (311). The exact mechanism of LPS induced demyelination is unknown. In primary mixed glial cultures, LPS induces a selective loss of oligodendrocyte precursors. Activation of microglia, but not astrocytes, was found to be required for LPS toxicity (312). TLR4 is necessary for LPS-induced oligodendrocyte injury in the CNS, although mRNA for LPS receptors TLR4/CD14 is either absent or only found at very low levels in oligodendrocyte precursors (312). Thus, LPS may not be directly toxic to oligodendrocytes but causes demyelination via factors derived through microglia or astrocytes. Recent evidence suggests that microglial peroxynitrite, or in the presence of astrocytes, activation of TNF/TNFR signalling, plays a key role in LPS-induced damage of developing oligodendrocytes *in vitro* (313).

D. Role of neuroprotective factors in EAE

Neurotrophic factors (NFs)

NFs comprise the large family of secreted peptides, originally described as target-derived growth factors crucial for neuronal differentiation and survival (314). The first known NF was nerve growth factor (NGF) described by Levi-Montalcini in the 50s. Originally, NGF was known by its anti-apoptotic activity and the role in neuronal development. Other structurally related proteins of similar function were later described and named “neurotrophins.” The group includes: NGF, BDNF, neurotrophin 3 (NT3), and neurotrophin 4/5 (NT4/5) (315). Recent data showed that BDNF can be secreted by PBMC from IFN-β treated RR-MS patients (165). Two additional trophic factors families has been described: neuropoietic cytokines (neurokines) and glial-cell line neurotrophic factor family ligands (GFLs). Glial derived neurotrophic factor (GDNF) was purified and characterized in 1993 as

a growth factor promoting the survival of the embryonic dopaminergic neurons of the midbrain-neurons degenerated in the Parkinson's disease (316). GFLs are distant member of TGF-β superfamily and currently consist of GDNF, neurturin, artemin and persephin (317). The second family of neuropoietic cytokines includes ciliary neurotrophic factors (CNTF), leukemia inhibitory factor (LIF), and other more pleiotropic cytokines (318). Neurotrophins have been found to stimulate growth and promote survival of neurons by their interaction with the tyrosine-related kinase (Trk) receptors. Trophic factors exhibit selective affinity for the Trk receptors: BDNF and NT4/5 bind to TrkB, NGF to TrkA, and NT3 to TrkC (319). All of the neurotrophins in their uncleaved state as proneurotrophins and in their mature form have also ability to bind to pan-neurotrophin receptor p75 (p75NTR). Interaction with this receptor may result in neuronal cell survival, cell death or myelination and depends on the state of the neurotrophin and whether p75NTR acts alone or with other receptors (320).

Evidence for the role of NFs in EAE

There is increasing evidence for the role of NFs in neurodegeneration and neuroprotection during EAE, supporting the hypothesis of a protective NGF role in rat EAE showed that NGF-deprived rats presented more severe clinical course of the disease which was associated with a significant reduction of NGF in the brain stem and spinal cord, whereas BDNF decreased only in the spinal cord (321). In the acute phase of EAE, CNTF inhibited inflammation in the spinal cord, resulting in ameliorating the clinical course of EAE during treatment (322). Recently, a novel delivery system for BDNF was developed using transformed bone marrow stem cells (BMSC) and undertook studies on mice EAE (323). They noted that mice receiving BMSC producing BDNF had reduced clinical impairment compared to control mice receiving BMSC without BDNF production. Both pathological examination and analysis of apoptosis of brain and spinal cord presented reduced inflammatory cells infiltrates in treated comparing to non-treated mice (323).

E. Hepatocyte growth factor (HGF)

E.1. Origin and function of HGF

Hepatocyte growth factor (HGF) is a pleiotropic factor able to evoke a wide array of cellular responses (Figure 6). HGF was discovered independently as fibroblast-derived effectors of epithelial movement and cell–cell interactions (scatter factor) (324, 325), as a growth factor for liver (326), and other epithelial cells (327) as an epithelial morphogen (328) as an inhibitor of tumour growth (329) and as a chemoattractant for motoneurons (330). The mature HGF molecule is a heterodimer consisting of a 69 kDa α-chain and a 34 kDa β-chain (331). The generation of mature HGF from the inactive pro-HGF is the result of the post-translational modification by a unique serine protease which produces enzymatic hydrolysis of the single chain pro-form to the mature heterodimeric form (326). HGF was the first member of the plasminogen-related growth factor family, a novel family of high-molecular-weight polypeptide growth factors whose domain structure and mechanism of activation resemble those of the blood protease plasminogen (332). Two plasminogen-related growth factors have been identified so far: HGF and HGF-like/macrophage stimulating protein (HGFl/MSP) (333, 334). Biologically active HGF is a α–β heterodimer produced by proteolytic cleavage of a single-chain inactive precursor of 728 amino acids. The larger chain (residues 1–494) contains a typical signal peptide, cleaved during secretion, followed by five distinct domains: an N-terminal (N) hairpin loop homologous to the activation peptide of plasminogen and four kringle (K) domains (335) (Figure 6). The smaller β chain (residues 495–728) resembles a typical serine protease domain. In addition HGF is able to bind to heparan sulfate proteoglycans (336).

E.2. HGF receptor (c-Met)

The receptor for HGF is the tyrosine kinase encoded by the c-*met* proto-oncogene (337). The c-Met receptor is expressed in the normal epithelium of the majority of tissues where it is primarily located at the intercellular junctions together with cell adhesions molecules such as E-cadherin (338). c-Met is a disulphide-linked heterodimer originated from the proteolytic cleavage of a single chain precursor. The heterodimer is formed by a single-pass transmembrane beta chain (145 kDa) and a completely extracellular alpha chain (50 kDa). c-Met activation leads to auto-phosphorylation of multiple tyrosine residues located in its cytoplasmic domain, specifically Y1151, Y1345 and Y1356. A binding site for multiple

substrates is located adjacent to these newly formed phosphotyrosines. Substrates that bind c-Met are multidomain proteins characterised by the presence of specific domains, including the Src homology 2 (SH2), the phosphotyrosine binding (PTB) and the Src homology 3 (SH3) domain. Some of the substrates that directly interact with c-Met include the growth factor receptor-bound protein (Grb) 2 (339), STAT3 (340), the p85 subunit of phosphatidylinositol 3-kinase (PI3K) (341), Shc (342), phospholipase C-γ, c-Src (339), (PLCγ) and Gab1 (343).

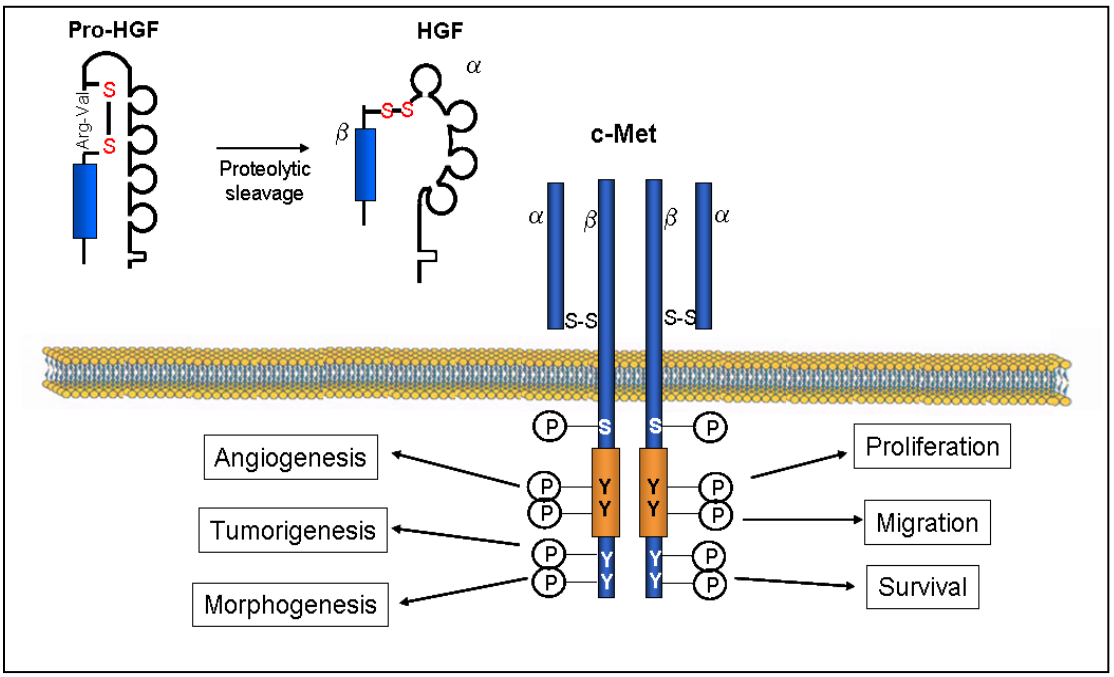

Figure 6: Representation of pro-HGF, HGF and the c-Met receptor. Proteolytic cleavage of pro-HGF generates the heavy (H) and light (L) chain of the active factor and is accompanied by a major conformational change. Active HGF binds to c-Met, inducing receptor dimerization and activation. The multidocking site at the C-terminus of the c-Met β subunit is generated by phosphorylation of tyrosine residues (Tyr1349 and Tyr1356) upon HGF binding. The multidocking site binds to various adaptor molecules that transmit the signal.

E.3. HGF/c-Met regulation

Mice lacking either HGF or its receptor die during embryogenesis with defect in placenta, liver and muscle. HGF plays an important role in liver regeneration since in animals, plasma levels of HGF and cellular mRNA are elevated after partial hepatectomy or liver damage induced by hepatotoxins (344). Thus, HGF and c-Met constitute a paracrine signalling systeme, as shown in embryogenesis studies (345). Several levels of regulation of HGF and c-Met have been identified that together form a complex and highly controlled pathway. The first level is transcriptional. In the majority of tissues, expression of the *HGF*

gene is restricted to certain cells (typically, fibroblasts or other mesenchymal cells), whereas expression of the c-*met* gene is confined to different cells that do not secrete the ligand including epithelial, skeletal muscle and motoneurons, c-Met is expressed also by various types of leukocytes including monocytes/macrophages (346), DCs (347). A second level of regulation is provided by the high-affinity binding of HGF to heparan sulphate proteoglycans (HSPGs). This ensures stockage of HGF in the proximity of secreted cells and thus a local mechanism of action. Studies indicated that HSPGs are not essential for HGF binding to the c-Met receptor and for signalling (348). It has been shown that HSPGs might be required for the agonistic activity of truncated forms of HGF, such as NK1 and NK2 (the products of alternatively spliced forms of the primary HGF transcript), and that they might potentiate the activity of full-length HGF at least on certain cell types (349). A final level of regulation is provided by proteolytic processing of the single-chain, precursor form of HGF (pro-HGF). Pro-HGF itself can bind to c-Met with high affinity but is unable to activate the receptor (350). Thus, proteolytic cleavage of pro-HGF is essential for activity. It is known that urokinase plasminogen activator (uPA) and tissue plasminogen activator (tPA) can convert pro-HGF into two-chain HGF *in vitro* (351). Certain epithelial cells also secrete two potent inhibitors of HGF activator (HAI-I and HAI-II) in tissue injury, both of which are members of the Kunitz-type family of serine protease inhibitors (352). These findings do not rule out the possibility that HGF processing plays an important role in specific moment, for example at certain stages of development or in the tissue response to injury.

E.4. Role of HGF in inflammation

The modulatory role of HGF in inflammation and in immune-mediated disorders is now well established. On the one hand HGF promotes adhesion and migration of B cells (353) and T cells (354) as well as DC migration (355). HGF frequently counteracts TGF-β, a potent immunosuppressive cytokine (356). Hence, these results indicate that HGF might be an immunopotentiator. On the other hand, recent studies clarified an immunosuppressive effect of HGF. HGF reduced acute and chronic rejection of the allograft with increased expression of TGF-β and IL-10, indicating that HGF might induce allograft tolerance (357). HGF attenuates allergic airway inflammation (358) in down-regulating Ag-induced Th1 and Th2 type immune responses and also with suppression of the Ag-presenting capacity of DCs in the lung. It has been reported that HGF is a potent anti-inflammatory cytokine that decreases the RNA expression of TNF and IFN-γ, two inflammatory cytokines, and directly suppresses pro-

inflammatory membrane co-factor protein 1 (MCP-1) and RANTES expression, probably via NF-κB in tubular epithelial cells (359). Interestingly, both chemokines are involved in EAE pathogenesis (360). HGF also offers protection in acute ischemic and inflammatory injuries by its anti-apoptotic and pro-angiogenic effects (361) by attenuating ischemia-induced cell death (362) and ischemia-or toxic-induced acute renal failure (363). HGF attenuates acute colitis by facilitating intestinal wound repair as well as inhibiting inflammation (364). It has been shown that activated polymorphonuclear leukocytes (PMNLs) in systemic inflammatory response syndrome patients increased HGF in their granules and demonstrate enhanced degranulation of HGF. The release of HGF from migrated PMNLs in the inflammatory tissue may play an important role in wound healing and organ regeneration under those conditions. DCs are highly specialized antigen presenting cells that integrate a variety of incoming signals and orchestrate the immune response (365), DCs play a major role in EAE by inducing CNS inflammation and clinical disease development (366). HGF has an anti-inflammatory effect via the suppression of DCs function (358).

E.5. Role of HGF in neuro-protection

Increasing evidence suggests that HGF plays an important role in the CNS development and neuroprotection. HGF and its receptor are expressed in the adult and developing nervous system (367). HGF promotes neuron survival and is a chemoattractant for motor neurons (330). In addition, HGF promotes neurite outgrowth from neocortical explants (368) and enhances sympathetic neuron survival and axonal growth (369). Exogenous HGF protects against neuronal death and reduces infarct volume after transient cerebral ischemia (370). Previous studies have revealed that HGF stimulates the proliferation of Schwann cells (371) and olfactory ensheathing cells (OECs) (372). OECs are thought to derive from precursor cells in the olfactory epithelium (373). Several reports demonstrated that transplanted OECs promote peripheral and CNS axonal regeneration (374), and remyelinate demyelinated CNS axons (375). HGF stimulates OPCs proliferation, migration and influences oligodendrocytes cytoskeleton organization (376). It has been shown recently that HGF was involved in the early remyelination of EAE by inducing migration of OPC cells into lesions (377). In the same study they showed that IFN-β, the main drug used to treat MS patients, also leads to HGF production by brain glial cells. In addition to its immunomodulatory role, IFN-β may have an indirect remyelinating and neuroprotective effect for MS patients and may contribute to CNS protection. It has been shown that overexpression of HGF in the nervous

system attenuates motoneuron death and axonal degeneration and prolongs the life span in a mice model of amyotrophic lateral sclerosis, a neurodegenerative disease of the nervous system. In this model HGF prevented induction of caspase-1 and inducible nitric oxide synthase (iNOS) in motoneurons and retained the levels of the glial-specific glutamate transporter (378). It has been shown that HGF directly protects cortical neurons against oxygen-glucose deprivation/reperfusion (OGD/R)-induced cell injury in a dose dependent manner, and HGF has a potent anti-apoptotic action on neurons exposed to OGD/R (379). HGF concentrations in CSF were significantly higher with diseases of the CNS than control diseases, especially in CNS demyelinating diseases like Acute Demyelinating Encephalomyelitis (ADEM) (380). Finally, HGF is implicated in the processor post-ischemic brain repair (381).

E.6. Role of HGF in inflammatory-mediated disease animal models

The interest of the relationship between HGF and autoimmune disorders is nowadays increasing. In fact, due to its immune-modulator effects, HGF seems to be involved in several pathogenic pathways of various autoimmune conditions. HGF has shown promising protective effects as a therapeutic agent for organ diseases such as renal injury, myocardial infraction, inflammatory bowel diseases and lung diseases.

a) Rheumatoid arthritis

Rheumatoid arthritis (RA) is an autoimmune disorder and a systemic chronic inflammatory disease characterized by persistent synovial cell proliferation with inflammatory cell infiltration and destruction of joints. RA has been assumed to be Th1 (382) and Th17 type disease (383). The role of HGF in RA has been reported in few cases. HGF and its receptor c-Met were found in the synovial tissue of patients with RA (384). HGF might suppress the development of Ag-induced arthritis. Its can diminished the severity and incidence of arthritis with up-regulation of IL-10 and suppression of IL-17 in experimental arthritis model.

b) Kidney diseases

HGF ameliorates chronic renal injury in a variety of models, including remnant kidney (385), unilateral ureteral obstruction (386), and diabetic nephropathy (387). Tubular epithelial cells (TEC) are a major target for HGF in the kidney, and several mechanisms have been proposed to explain this beneficial action, including antiapoptosis (388), promoting TEC

proliferation (389), prevention of epithelial-to-mesenchymal transition (390), increased activity of matrix degradation pathways (385), has an anti inflammatory effect via NF-kappaB inhibition (359). HGF accelerated glomerular repair through the growth of capillary endothelial cells and capillary regeneration in experimental progressive glomerulonephritis (391).

c) Cardiac diseases

Serum and myocardial concentrations of HGF and its receptor c-Met are substantially increased following acute myocardial infarction. HGF has been shown to be cardioprotective towards acute cardiac ischemia-reperfusion injury. Gene transfection of HGF into rat heart attenuats acute ischemia injury. Administration of HGF protein reduces infract size and increases cardiac performance in a rat model of acute ischemia/reperfusion. In contrast, acute blockade of endogenous HGF increases infract size and mortality. These beneficial effects of HGF appear to be related to angiogenic properties, anti-apoptotic mechanisms and to an induction of a bystander Th2 cytokine deviation (392). HGF is cardioprotective factor by increasing the tolerance of cardiomyocytes to ischemia, reducing cardiomyocyte apoptosis, increasing prosurvival Akt protein kinase, which improve function of dilated cardiomyopathy in mice (393).

d) Inflammatory bowel disease (IBD)

Inflammatory bowel disease (IBD) composed of ulcerative colitis and Crohn's disease. In IBD patients, elevated serum HGF and mucosal overexpression of HGF and its receptor c-Met have been reported (394) suggesting an association with intestinal tissue repair. In cell culture, HGF stimulates the growth of gastrointestinal epithelial cells (395). HGF improves colonic damage in rat IBD models. Its therapeutic effects are manifested via reduced inflammation, including that due to anti-apoptosis, rather than through epithelial cell proliferation. HGF may have the potential for clinical use in patients with IBD (396). Transfection with HGF cDNA markedly suppressed intestinal mRNA expression of Th1 cytokines such as IL-12, IL-1β, IFN-γ, and TNF. Numbers of total and CD4 T cells, neutrophils, and myloperoxidase activity in intestinal epithelium were diminished by HGF gene transfer, which also prevented weight loss, and improved survival. HGF might be useful for controlling IBD (397).

e) Lung disease

Pulmonary fibrosis is a common feature of interstitial pulmonary diseases and both apoptosis and fibrinogenesis play critical roles in its formation and development. HGF is a mitogenic and anti-apoptotic factor for alveolar and bronchial epithelial cells. It also stimulates the migration and morphogenesis of those cells (398). Yaekashiwa *et al.* (399) demonstrated that continuous systemic injection of recombinant HGF suppressed pulmonary fibrosis induced by bleomycin in the mice model. HGF had significant effect when it was administered simultaneously or subsequently to bleomycin treatment. It has been identified HGF as a key ligand to elicit myofibroblast apoptosis and extracellular matrix degradation in increasing activities of MMP-2/-9, predominant enzymes for breakdown of fibronectin, whereas activation of the HGF/c-Met system in fibrotic lungs may be considered a target to attenuate progression of chronic lung disorders. In addition, HGF attenuates an animal model of allergic airway inflammation and airway hyperresponsiveness (400).

f) Graft-versus-host disease (GVH)

It has been shown that HGF suppresses acute and chronic rejection in a murine model of cardiac transplantation by induction of tolerance and prevention of cardiac allograft vasculopathy (357). In addition, HGF ameliorate GVH disease in a murine model by reducing the number of splenic B cells, host B cell MHC class II expression, and serum levels of IgG and anti-DNA Abs. HGF decreases IL-4 mRNA expression in the spleen, liver, and kidneys (401).

E.7. HGF as therapeutic agent

HGF has shown promising protective effects as a therapeutic agent for diseases such as RA, myocardial infarction, chronic renal failure, IBD, and pulmonary fibrosis. HGF may present a novel strategy for the treatment of these diseases. Generally, exogenously administred HGF proteins vanish from organs within several hours (402). To study the different effects of HGF in these different animal model diseases, there are two trategies used to deliver efficiently HGF in the target organs or to maintain a high HGF serum level. The first strategy is to use a slow-releasing form recombinant HGF protein mounted in biodegradable gelatin hydrogels (403), as shown for example in allergic airway inflammation (358) and kidney model disease (404). The second one is to administrate plasmid DNA

containing HGF gene with a hydrodynamics-based transfection system (405), as shown in renal failure animal model. In contrast, whith clinical use of HGF, the possibility of promoting tumor progression should be considered.

All these data suggest that HGF is a candidate of high interest for the development of new treatments for immune-mediated demyelinating diseases with neurodegeneration such as MS.

F. Unresolved questions

EAE is an immune-mediated disease model of MS, characterized by the activation of APCs and the infiltration of autoreactive lymphocytes within the CNS, it is used to explore the pathogenesis of inflammation, demyelination and axonal loss. Numerous hypotheses have been raised to explain the lack of remyelination and the progression of MS disease including continual loss of oligodendrocyte precursor cells and irreversible axonal damage. But until now this equation is not yet resolved.

HGF is a pleiotropic factor known for both neuronal and oligodendrocytic protective properties (330), in addition, HGF shows immunomodulatory properties, affecting both the innate and the adaptive immunity. HGF plays a protective role via its immunosupressor effect in animal models of inflammatory-mediated diseases including myocarditis (392), glomerulonephritis (406), IBD (397), collagen-induced arthritis (407) and pulmonary fibrosis (408).

In this project, we proposed to study the role of HGF on the development of EAE. Thus, we hypothetize that HGF delivered in the CNS of mice may protect against EAE course. To reach this objective we studied the effect of an overexpression of HGF in the CNS on EAE. We used C57BL/6 mice carrying HGF transgene under the control of a neuron-specific enolase (NSE) promoter (HGF-Tg mice) leading to selective overexpression of HGF by neurons in the CNS (378). Thus, we assessed the immunomodulatory effect of HGF **a)** *in vivo*: i) in the periphery and ii) in the CNS compartment, and **b)** *in vitro* by using a specific antigen presentation model.

G. Overview of results

We showed that overexpression of HGF in the CNS of Tg mice decreases the EAE clinical course. EAE was induced by two differents methods: i) by immunization with MOG_{35-55} peptide and ii) by adoptive transfer of T cells from 2D2 transgenic mice (which express a T cell receptor (TCR) specific for MOG_{35-55}). This inhibition of EAE in HGF-Tg mice appears before peak disease was reached. Histology analysis was performed at two time points (peak disease and chronic phase); the observations showed a decrease of inflammatory lesions as well as less level of demyelination and axonal loss in HGF Tg mice if we compared them with littermate WT mice. At peak disease we showed by immunofluorescence and by flow cytometry a decrease of inflammatory cells (T lymphocyte and APCs) infiltrating the CNS (spinal cord) of HGF Tg mice, the only T cell population which increases is regulatory T

(Treg) $CD4^+CD25^+Foxp3^+$ cells. However, no differences were observed in the systemic compartment which was expected, the overexpression is in the CNS and not in the periphery. The reduction of the inflammation in the spinal cord of HGF Tg mice was associated with a decrease of pro-inflammatory Th1 cytokines (TNF, IFN-γ and IL-12p70) whereas we showed increase of Th2 cytokines especially sharp increase of IL-10 cytokine secretion and mRNA expression. To confirm these results *in vitro*, we used a functional assay based on the specific antigen presentation (ASR). In this assays, we showed that when we treated DCs with recombinant HGF protein we suppressed DCs function via down regulation of CD40 co-stimulatory molecule and also a decrease of IL-12p70 secretion. Interestingly, these DCs co-cultured whith CD4 T cells induced differentiation of IL-10-producing Treg cells, accompagnied whith a decrease of IL-17-producing T cells and a downregulation of surface markers of T cell activation. Collectively, these data indicated that HGF can inhibit the clinical course of EAE through DC tolerization and induction of Treg cell population. In addition, our results suggested that HGF is a candidate of high interest for the development of new treatments for immune-mediated demyelinating diseases associated with neurodegeneration such as MS by combining potentially neuroprotective and myelin repair properties as well as immunosuppressive effects.

II) MATERIALS & METHODS

RESULTS

II. A)
Immunomodulatory effects of hepatocyte growth factor in experimental autoimmune encephalomyelitis

Section II.A

Hepatocyte growth factor inhibits CNS autoimmunity by inducing tolerogenic dendritic cells and $CD25^{+}Foxp3^{+}$ regulatory T cells

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OBJECTIVES:

To examine the role of HGF as an immunomodulator and neuroprotective factor, in an EAE model. We have explored whether overexpression of HGF in the CNS of mice affected the clinical course of EAE either induced by MOG_{35-55} peptide or by adoptive transfer.

Hepatocyte growth factor inhibits CNS autoimmunity by inducing tolerogenic dendritic cells and $CD25^+Foxp3^+$ regulatory T cells

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Immune-mediated diseases of the CNS, such as multiple sclerosis and its animal model, experimental autoimmune encephalitis (EAE), are characterized by the activation of antigen-presenting cells and the infiltration of autoreactive lymphocytes within the CNS, leading to demyelination, axonal damage, and neurological deficits. Hepatocyte growth factor (HGF) is a pleiotropic factor known for both neuronal and oligodendrocytic protective properties. Here, we assess the effect of a selective overexpression of HGF by neurons in the CNS of C57BL/6 mice carrying an HGF transgene (HGF-Tg mice). EAE induced either by immunization with myelin oligodendrocyte glycoprotein peptide or by adoptive transfer of T cells was inhibited in HGF-Tg mice. Notably, the level of inflammatory cells infiltrating the CNS decreased, except for $CD25^+Foxp3^+$ regulatory T (T_{reg}) cells, which increased. A strong T-helper cell type 2 cytokine bias was observed: IFN-γ and IL-12p70 decreased in the spinal cord of HGF-Tg mice, whereas IL-4 and IL-10 increased. Antigen-specific response assays showed that HGF is a potent immunomodulatory factor that inhibits dendritic cell (DC) function along with differentiation of IL-10–producing T_{reg} cells, a decrease in IL-17–producing T cells, and down-regulation of surface markers of T-cell activation. These effects were reversed fully when DC were pretreated with anti-cMet (HGF receptor) antibodies. Our results suggest that, by combining both potentially neuroprotective and immunomodulatory effects, HGF is a promising candidate for the development of new treatments for immune-mediated demyelinating diseases associated with neurodegeneration such as multiple sclerosis.

cMet (HGF receptor) | experimental autoimmune encephalitis | immune tolerance | multiple sclerosis | neuroprotection

Hepatocyte growth factor (HGF), also called "scatter factor," is a polypeptide growth factor that belongs to the plasminogen family and consists of a 62-kDa alpha subunit and a 34-kDa beta subunit that form a disulfide-linked heterodimer (1). The molecule was discovered independently as a growth factor in the liver (2, 3) and as a fibroblast-derived effector of epithelial movement and cell–cell interaction (4–6). HGF is a pleiotropic factor that can trigger motility, proliferation, morphogenesis, and organ regeneration in a variety of epithelial cells (7). HGF is able to induce various responses in development (1) and in pathological situations, including tumor progression (8) and suppression of fibrosis (9). The receptor for HGF, cMet, is a tyrosine kinase encoded by the *cMet* proto-oncogene (10, 11). Mice lacking either HGF or its receptor die during embryogenesis, with defects in placenta, liver, and muscle development (12–14).

Both HGF and its receptor cMet are expressed during brain development and persist in the adult (15, 16). cMet is expressed in neurons but also in other brain-resident cells such as oligodendrocytes, astrocytes, and microglia (17–22). HGF promotes axonal outgrowth and regulates the differentiation of various neuronal populations, including sensory, sympathetic, and motor neurons (23, 24). The ability of HGF to promote survival of neurons is as potent as that of several neuroprotective factors, including brain-derived neurotrophic factor, ciliary neurotrophic factor, glial cell line-derived neurotrophic factor, and neurotrophin-3 (23). In addition, HGF is able to induce proliferation and migration of oligodendrocyte precursor cells (OPC) (17, 18, 22) as well as inhibition of the proapoptotic caspase-3 pathway in oligodendrocytes (21). Therefore, HGF could be involved in the processes of neuroprotection, attenuation of oligodendrocyte degeneration, and/or remyelination.

In animal studies, overexpression of HGF in the CNS delays disease progression and prolongs life span in a mouse model of amyotrophic lateral sclerosis (20), a neurodegenerative disease of the nervous system. In addition, HGF is involved in the process of postischemic brain repair (25). Increased concentrations of HGF are detected in the cerebrospinal fluid of patients with inflammatory and demyelinating diseases such as acute demyelinating encephalomyelitis and multiple sclerosis (26).

In addition to its action on the CNS, HGF shows immunomodulatory effects: on the one hand, HGF originally was reported to promote adhesion of B cells (27) and migration of T cells (28) as well as recruitment of dendritic cells (DC) (29). Moreover, HGF was reported to inhibit secretion of TGF-β (30), a potent antiinflammatory cytokine known to inhibit the progression of experimental autoimmune encephalomyelitis (EAE) (31). On the other hand, HGF was identified more recently as having protective effects in animal models of inflammatory-mediated diseases including myocarditis (32, 33), glomerulonephritis (30, 34), inflammatory bowel disease (35), collagen-induced arthritis (36), and pulmonary fibrosis (37).

In the present report, we assess the effect of an overexpression of HGF in the CNS of C57BL/6 mice carrying a HGF transgene under the control of a neuron-specific enolase (NSE) promoter (HGF-Tg mice) leading to selective overexpression of HGF by neurons in the CNS. In contrast, HGF serum levels were similar to those in WT littermate controls. Introduction of HGF under the control of the NSE promoter into mice leads to expression of HGF specifically in postnatal neurons of the CNS and subsequent extracellular secretion of HGF in the CNS, where it can act both on neurons and on other types of postnatal cells, such as glial and immune cells. In this experimental setup, the neural as

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well as the glial system have been found to be physiologically normal during development and in the adult (20, 38, 39).

EAE induced either by immunization with myelin oligodendrocyte glycoprotein (MOG) peptide consisting of amino acids 35–55 [MOG(35-55)] or by adoptive transfer of T cells from 2D2 transgenic mice that express a T-cell receptor (TCR) specific for MOG(35-55), (TCR^{MOG}) was inhibited before peak disease was reached in HGF-Tg mice. Notably, the level of inflammatory cells infiltrating the CNS decreased in these mice except for $CD25^+Foxp3^+$ regulatory T (T_{reg}) cells, which increased. In addition, a strong T-helper cell type 2 (Th2) cytokine bias was observed: TNF-α, IFN-γ, and IL-12p70 were decreased in the spinal cord of HGF-Tg mice, whereas IL-4 and IL-10 were increased. Antigen-specific response (ASR) assays showed that HGF is a potent immunomodulatory factor that inhibits DC function through down-regulation of their CD40 expression together with a decrease in IL-12p70 secretion. Furthermore, DC treated in vitro with recombinant mouse HGF (rHGF) induced differentiation of IL-10–producing T_{reg} cells, along with a decrease in IL-17–producing T cells and a down-regulation of surface markers of T-cell activation.

Collectively, our data strongly suggest that HGF can inhibit the clinical course of EAE through DC tolerization and induction of T_{reg}-cell population.

Results

MOG-Induced EAE Is Inhibited in HGF-Tg Mice. The HGF content in the spinal cord of HGF-Tg mice, as assessed by real-time PCR and ELISA, was significantly increased (*ca*. 2.5-fold) compared with WT littermates. In contrast, serum levels of HGF, determined by ELISA, did not differ significantly in HGF-Tg and WT littermate mice (Fig. 1*A*) (20). EAE was induced in HGF-Tg and WT littermate C57BL/6 mice using MOG(35-55) peptide. The clinical course of EAE was inhibited in HGF-Tg mice before peak disease was reached [day postimmunization (dpi) 18] until the chronic phase (dpi 45) (Fig. 1*B*). Histopathological analysis of spinal cord performed at peak disease (dpi 25) showed that there was less inflammatory infiltrate (determined by H&E staining) and demyelination (determined by luxol fast blue staining) in HGF-Tg mice, as demonstrated by a decrease in the mean number (± standard error of the mean, SEM) of lesions per slide (Fig. 1 *C* and *D*). Fewer inflammatory $CD4^+$ T-cell, $CD8^+$ T-cell, CD11b, and CD11c cell subtypes were observed in the spinal cord of HGF-Tg mice than in the spinal cord of WT mice (Fig. 1*E*). In addition, Bielschowsky's silver staining indicated a trend toward a decrease in axonal damage in HGF-Tg mice, but the difference did not reach statistical significance (Fig. 1 *C* and *D*).

CNS Overexpression of HGF Does Not Influence Splenocyte Function During EAE. Splenocytes of HGF-Tg mice and WT littermates were isolated at peak disease (dpi 25), and cell surface and intracellular markers were analyzed by flow cytometry. No differences between the two groups of mice were observed in the frequency of $CD4^+$, $CD8^+$, or $CD4^+CD25^+Foxp3^+$ T cells, $CD11c^-CD11b^+$ macrophages, or $CD11c^+$ DC (Fig. 2*A*). When proliferation assays were performed to evaluate the possible influence of CNS-specific overexpression of HGF on peripheral T cells, increased T-cell proliferation was observed with escalating concentrations of MOG peptide, but no difference was observed between HGF-Tg and WT mice (Fig. 2*B*). Analysis of splenocytes by FACS at EAE peak disease was performed with distinction of $CD4^+$ T cells for IFN-γ (T-helper type 1, Th1), IL-10 (Th2), and IL-17 (T-helper type 17, Th17) subsets. No distinction was observed in the spleen for these three $CD4^+$ T-cell subsets when WT and HGF-Tg mice were compared (Fig. 2*C*). To confirm the absence of specific splenic HGF increase in HGF-Tg mice, we analyzed the HGF content from whole spleen in WT and HGF-Tg mice. The results showed no difference in the splenic HGF content in normal and EAE peak-disease conditions (Fig. S1 *A* and *B*). To confirm further that HGF had no effect on splenocyte proliferation during EAE, a proliferation assay with increasing doses of HGF was performed. No proliferation of splenocytes was observed in either WT or HGF-Tg mice at various HGF concentrations (Fig. S1*C*).

Increase of $CD25^+Foxp3^+$ T_{reg} Cells and Induction of a Th2 Cytokine Bias in the Spinal Cord of HGF-Tg Mice During EAE. Inflammatory cell infiltrates from pooled spinal cords of HGF-Tg (n = six mice) and WT littermates (n = five mice) were isolated at peak disease (dpi 25) by Percoll gradient, stained for surface markers, and analyzed by flow cytometry. Compared with WT littermates, a

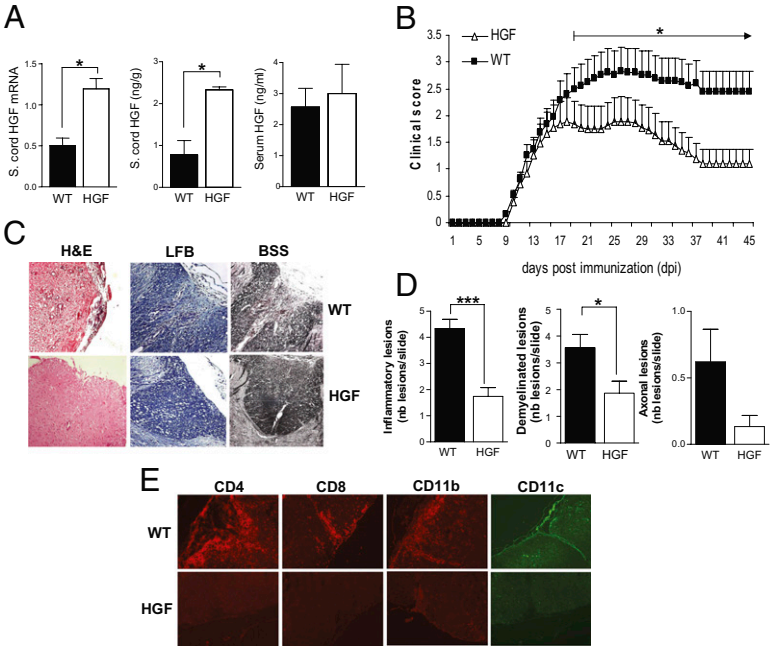

Fig. 1. MOG(35-55)-induced EAE is inhibited in HGF-Tg mice. (*A*) HGF in the spinal cord of HGF-Tg mice versus WT littermates (n = 3 per group) as measured (*Left*) by real-time PCR (relative HGF mRNA expression) or (*Center*) by ELISA (ng/g of spinal cord) (mean ± SEM). *, $P < 0.05$. (*Right*) HGF measured in the serum by ELISA (ng/ml; mean ± SEM). (*B*) EAE scores were determined daily after disease onset in HGF-Tg mice (triangles; n = 17) and WT littermates (squares; n = 18). Shown are mean EAE score ± SEM. The difference was significant before peak disease was reached (dpi 18) and persisted until the chronic phase (dpi 45). *, $P < 0.05$. (*C*) Histopathology of paraffin-embedded spinal cord sections from PBS-perfused WT and HGF-Tg mice at peak disease (dpi 25). Sections were stained with H&E, luxol fast blue (LFB), or Bielschowsky's silver staining (BSS) (magnification 200×). (*D*) Fifteen sections of spinal cord per mouse (n = 6 per group) were analyzed, and the mean number of lesions per slide (±SEM) are presented as histograms. *, $P < 0.05$; ***, $P < 0.001$. (*E*) Representative sections of spinal cord were analyzed. Shown are T-lymphocyte (CD4 and CD8) (red), macrophage (CD11b) (red), and DC (CD11c) (green) infiltration (magnification: 100×).

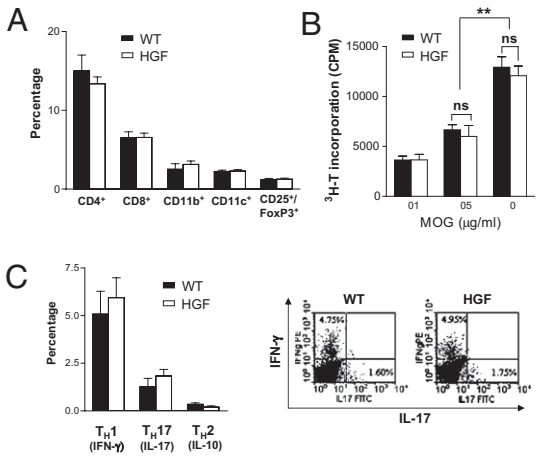

Fig. 2. Unchanged characteristics of splenocyte populations in HGF-Tg mice during EAE. (*A*) Splenocytes were isolated from HGF-Tg mice and WT littermates during EAE and stained for CD4, CD8, CD11b, CD11c, and CD25-FoxP3. Bars show percentages of total splenocytes ± SEM (n = 3 per group). (*B*) Proliferation assay with MOG(35-55) of splenocytes from HGF-Tg mice and WT littermates (mean cpm ± SEM of triplicate experiments; n = 3). **, $P < 0.01$; ns, not significant. (*C*) Analysis by intracellular cytokine staining of the production of IFN-γ (T_H1), IL-10 (T_H2), and IL-17 (T_H17) in splenocytes at peak disease. (*Left*) Bars show percentages of CD4 subtypes ± SEM (n = 6 per group). (*Right*) Representative dot blots of IFN-γ and IL-17 analyses.

decrease of $CD4^+$ and $CD8^+$ T cells, $CD11c^-CD11b^+$ macrophages, and $CD11c^+$ DC was observed in HGF-Tg spinal cords (Fig. 3*A*). In contrast, an increase of $CD25^+Foxp3^+$ T_{reg} cells was observed in the spinal cords of HGF mice (Fig. 3*B*). Intracellular staining for IL-17 production in $CD4^+$ T cells was lower in the spinal cord of HGF-Tg mice than in WT littermates (Fig. 3*C*). In a subsequent experiment, we analyzed by ELISA the cytokines detected in the supernatant of spinal cord homogenate of the two groups of mice. We observed a decrease in TNF-α, IFN-γ, and IL-12p70 in HGF-Tg mice, whereas IL-4 and IL-10 were increased (Fig. 3*D*). The sharp increase in IL-10 (*ca*. 8-fold) was confirmed further by real-time PCR (Fig. 3*E*).

HGF Inhibits Antigen-Presenting Function of DC in Vitro and Induces a Th2 Cytokine Bias. To assess the ability of HGF to influence DC function and T-cell proliferation, we performed a mixed lymphocyte reaction with DC obtained from BALB/c splenocytes and T cells from C57BL/6 spleen cells. In parallel, we confirmed that the cMet receptor was present on the surface of $CD11c^+$ DC of C57BL/6 or BALB/c mice but was not expressed by $CD4^+$ T cells (Fig. S2). After incubation with rHGF at various concentrations, DC were cocultured with T cells, and a proliferation assay was performed using ^{3}H-thymidine incorporation. T-cell proliferation was inhibited in a dose-dependant manner when allogeneic DC were pretreated with rHGF (Fig. S3). We further evaluated the ability of rHGF to inhibit DC function in an in vitro model closer to EAE, i.e., an ASR assay performed with MOG(35-55). In this experiment, DC were purified from C57BL/6 mouse splenocytes and then were treated with rHGF at various concentrations (1–100 ng/mL) or were left untreated and finally were pulsed with MOG(35-55) peptide (20 μg/mL). In parallel, TCR^{MOG} T cells were obtained from splenocytes of 2D2 mice. After 48 h of coculture, T-cell proliferation was measured. A significant inhibition of T-cell proliferation was observed with increasing concentrations of rHGF (10–100 ng/mL), with the maximum effect starting at 30 ng/mL (Fig. 4*A*). To confirm that the inhibition of DC function was indeed mediated through the HGF–cMet pathway, an anti-HGF receptor (α-cMet) neutralizing antibody (10 μg/mL) was added to the DC before incubation with rHGF. Inhibition of T-cell proliferation was abrogated completely by preincubation of DC with the α-cMet antibody across the entire range of tested rHGF concentration (Fig. 4*A*). Furthermore, we examined the expression of T-cell activation markers during ASR assays and found that rHGF-treated DC pulsed with MOG(35-55) were not capable of activating T cells, which remained in a low state of activation ($CD44^{low}CD62L^+$ and $CD44^{intermediate}CD62L^+$). In contrast, when the ASR assays were performed without rHGF pretreatment, T cells were highly activated ($CD44^{high}CD62L^+$ and $CD4^{high}CD62L^-$) (Fig. 4*B*). We then analyzed by ELISA the cytokine secretion profiles in the ASR assay supernatants after DC had been cocultured with TCR^{MOG} T cells. We observed a decrease in TNF-α, IFN-γ, and IL-12p70, whereas IL-4, IL-10 and, to a lesser extent, TGF-β were increased when DC were pretreated with rHGF at 30 ng/mL (Fig. 5*A*). In a subsequent ASR assay, we analyzed intracellular IFN-γ and IL-17 production in $CD4^+$ T cells by flow cytometry and found that IL-17 production was inhibited when $CD4^+$ T cells were cocultured with rHGF-treated DC. In contrast, there was no change in T-cell production of IFN-γ (Fig. 5*B*). Finally, the expression of costimulatory surface molecules on DC (CD40, CD80, CD86, and MHC class II) was measured by flow cytometry during ASR assays. Unlike CD80, CD86, and MHC class II, a decrease in CD40 expression was observed under rHGF influence and was reversed when DC were pretreated with the α-cMet receptor antibody (Fig. S4).

HGF-Treated DC Induce Expansion of $CD25^+Foxp3^+$ T_{reg} Cells in Vitro with Increased IL-10 Production. Because with rHGF pretreatment we observed a strong increase of IL-10 in both the spinal cord of HGF-Tg mice and the DC plus T-cell culture supernatant from ASR assays, we also examined by flow cytometry whether rHGF-treated DC could promote the induction of $CD25^+FoxP3^+$ T_{reg} cells, known to be the major IL-10–producing subtype of T cells. Indeed, a strong increase in $CD25^+FoxP3^+$ T_{reg} cells was observed when rHGF-treated DC, pulsed with MOG(35-55), were

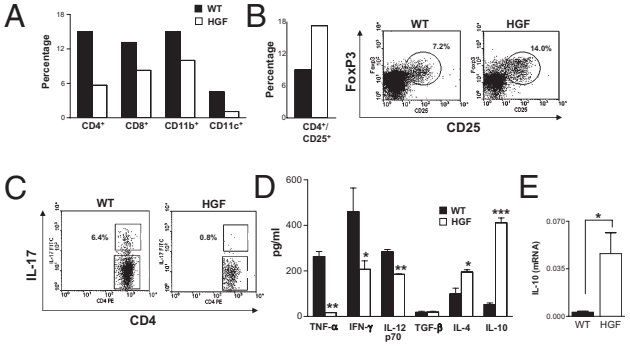

Fig. 3. Spinal cord inflammation of HGF-Tg mice is characterized by a Th2 bias and an increase in T_{reg} cells. (*A*) Inflammatory cells were isolated by Percoll gradient from pooled spinal cords of HGF-Tg (n = 6) versus WT littermates (n = 5) at EAE peak disease (dpi 25) and were stained for CD4, CD8, CD11b, and CD11c. Bars show percentages of total cells. Spinal cord inflammatory cells were stained further (*B*) for CD4-CD25 (histogram; percentage of total $CD4^+$ T cells) and CD4-CD25-FoxP3 (dot blots) or (*C*) for IL-17. (*D*) Spinal cord supernatants from HGF-Tg mice and WT littermates (n = 3 per group) from a subsequent EAE were analyzed by ELISA for cytokines. Shown are the mean ± SEM of experiments performed in triplicate. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (*E*) The increase of IL-10 detected by ELISA in the spinal cord of HGF-Tg mice at peak disease was confirmed by real-time PCR analysis. *, $P < 0.05$.

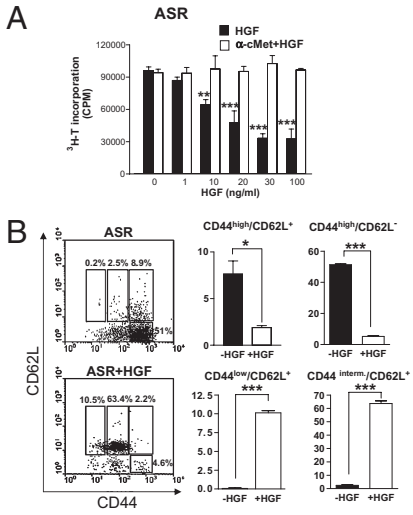

Fig. 4. HGF inhibits in vitro CD11c$^+$ DC function and T-cell activation markers. (*A*) ASR assay performed with C57BL/6 CD11c$^+$ cells stimulated with MOG(35-55) and coincubated with TCR^{MOG} T cells. T-cell proliferation was analyzed after CD11c$^+$ cells were pretreated with α-cMet-blocking antibody or were left untreated and then were preincubated with increasing concentrations of rHGF. **, $P < 0.01$; ***, $P < 0.001$. (*B*) FACS staining for CD44 and CD62L coreceptors of CD4$^+$ T cells after CD11c$^+$ cells were preincubated with rHGF (30 ng/mL) or were not preincubated. Results shown are the mean of three to five independent experiments ± SEM. *, $P < 0.05$; ***, $P < 0.001$.

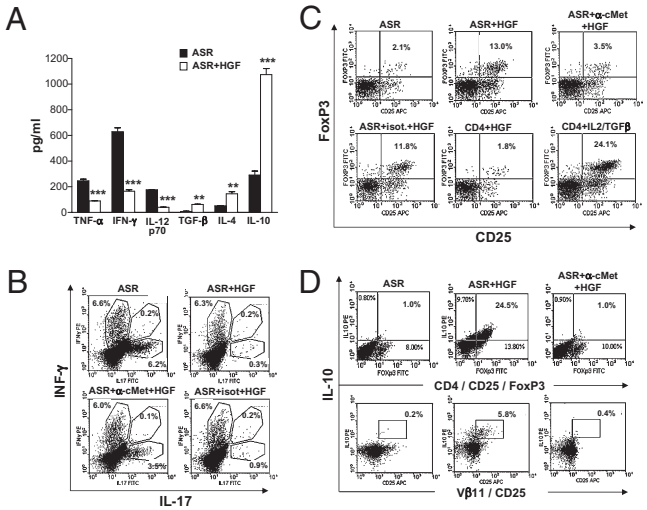

Fig. 5. HGF-treated DC induce a Th2 cytokine bias and CD25$^+$FoxP3$^+$ T$_{reg}$-dependant IL-10 production but decrease IL-17. (*A*) ELISA analysis of cytokines from ASR supernatant: TNF-α, IFN-γ, and IL12p70 are decreased, whereas TGF-β, IL-4, and IL-10 are increased. Results shown are mean ± SEM of triplicate experiments; rHGF added at 30 ng/mL. **, $P < 0.01$; ***, $P < 0.001$. (*B*) Intracellular staining of splenocytes from ASR experiments: CD4$^+$ T-cell–dependant production of IL-17 was inhibited by rHGF-treated CD11c$^+$ cells. Figures are representative of four independent experiments. (*C*) CD4$^+$ T cells were stained for CD25-FoxP3 after CD11c$^+$ cells were treated with rHGF (30 ng/mL) or left untreated. HGF-treated DC induce a strong increase in T$_{reg}$ cells. The effect of HGF on CD11c$^+$ cells was inhibited by preincubation of CD11c$^+$ cells with α-cMet–blocking antibody. HGF had no direct effect on CD4$^+$ T cells, but IL-2/TGFβ–treated CD4$^+$ T cells induced the production of CD25$^+$FoxP3$^+$ T$_{reg}$ cells (positive control). (*D*) IL-10 production (intracellular staining) was increased in CD25$^+$FoxP3$^+$ T$_{reg}$ cells and Vβ11(TCR^{MOG}) CD25 T cells when T cells were cocultured with rHGF-treated CD11c$^+$ cells. This effect was inhibited when CD11c$^+$ cells were preincubated with α-cMet–blocking antibody. Figures are representative of four to six independent ASR experiments.

cocultured with TCR^{MOG} T cells (Fig. 5*C*). This effect was abrogated when DC were pretreated with α-cMet antibody. Also, HGF had no direct influence on CD4$^+$ T cells (in contrast to IL-2 plus TGF-β, which was used as positive control). Intracellular staining performed during ASR assays showed an increase of IL-10 in CD4$^+$, CD4$^+$CD25$^+$ (Fig. S5), and CD4$^+$CD25$^+$Foxp3$^+$ T cells (Fig. 5*D*) when DC were pretreated with rHGF. Again, this effect was reversed by preincubation of DC with α-cMet antibody. In addition, most IL-10$^+$CD25$^+$ T cells were TCR^{MOG} T cells (TCR Vβ11$^+$), demonstrating that MOG autoreactive transgenic T cells could be skewed toward a less inflammatory profile by rHGF-treated DC (Fig. 5*D*).

EAE Induced by Adoptive Transfer of TCR^{MOG} T Cells Is Inhibited in HGF-Tg Mice. To confirm our results further, we performed EAE experiments with adoptive transfer of TCR^{MOG} T cells from 2D2 transgenic mice. EAE was inhibited significantly in HGF-Tg mice, compared with WT littermates, before peak disease was reached (dpi 15) (Fig. 6*A*). At peak disease (dpi 23), analysis of spinal cord-infiltrating inflammatory cells was performed by flow cytometry to evaluate CD25$^+$ IL10-producing T cells as well as surface markers of T-cell activation. The results showed a strong increase in the CD25$^+$IL10$^+$ T-cell population in HGF-Tg mice as compared with WT mice (Fig. 6*B*). Analysis of T-cell activation markers at EAE peak disease confirmed our previous in vitro data (ASR; Fig. 4*B*) by showing that spinal cord CD4$^+$ T cells were maintained in a state of low activation (CD44^{low}CD62L$^+$) in HGF-Tg mice, whereas CD4$^+$ T cells (CD44^{high}CD62L$^-$) were activated mostly in WT mice (Fig. 6*C*).

Discussion

HGF is a pleiotropic factor that acts by binding to the HGF tyrosine kinase receptor, cMet (11). HGF and cMet are expressed in brain-resident cells including neurons (24), mature oligodendrocytes (21, 22), OPC (17, 18, 22), and microglia (19). In addition to its neuroprotective effect, HGF is known to influence inflammation. However, whether its global immunomodulatory effect is pro- or antiinflammatory is still unclear. On the one hand, HGF is known to increase adhesion and migration of inflammatory cells of both the adaptive and the innate immune system (27–29). On the other hand, several antiinflammatory effects of HGF have been described, including (*i*) a Th2/T-helper cell type 3 (Th3) bystander deviation with increase of TGF-β and IL-10 (33, 40), (*ii*) inhibition of antigen-presenting cell (APC) function (40, 41), (*iii*) down-regulation of monocyte chemotactic protein-1 (MCP-1) and regulated upon activation, normal T cell expressed and secreted (RANTES) chemokines (42), and (*iv*) blocking of NF-κB function (42). All these effects could contribute to the protective action of HGF in EAE and/or multiple sclerosis (43, 44).

Here we show that overexpression of HGF in the CNS of transgenic mice inhibits the EAE clinical course by using two different methods of disease induction: MOG immunization and adoptive transfer of MOG-specific T cells. This finding is confirmed by histological observations that show a decrease of inflammatory lesions as well as a lower level of demyelination and axonal loss in HGF-Tg mice. Analyses by flow cytometry of inflammatory cell phenotypes in the spinal cord show that the total number of T cells and APCs was decreased in HGF-Tg mice during EAE, with the exception of the T$_{reg}$ cell population, which increased. Of note, HGF was up-regulated exclusively in the CNS and not in the systemic compartment, as demonstrated by the absence of a significant influence of the transgene on spleen cells. The reduction of CNS inflammation in HGF-Tg mice was associated with a decrease of proinflammatory (Th1) cytokines including TNF-α, INF-γ, and IL12p70, whereas the antiinflammatory (Th2) cytokine IL-10 was strongly increased. In vitro

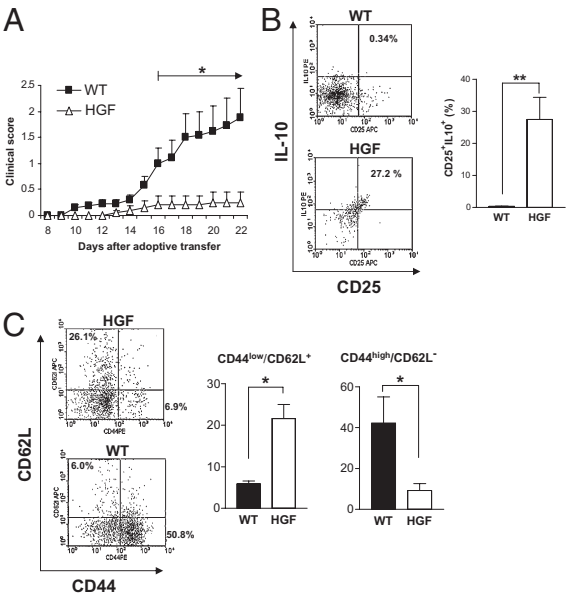

Fig. 6. EAE induced by adoptive transfer of TCR^{MOG}-specific (2D2) T cells is inhibited in HGF-Tg mice. Adoptive transfer of TCR^{MOG} T cells in HGF-Tg mice (n = 10) and WT littermates (n = 12). (*A*) EAE was inhibited in HGF-Tg mice before peak disease was reached. Shown is the mean clinical score ± SEM at day 15 after adoptive transfer. *, $P < 0.05$. (*B* and *C*) Inflammatory cells infiltrating the spinal cord of HGF-Tg mice and WT littermates (n = 5 per group) at EAE peak disease (day 23 after adoptive transfer) were isolated by Percoll gradient and stained by flow cytometry. (*B*) (*Left*) Representative dot blots of CD25 and IL-10 analyses. (*Right*) CD25-dependent IL-10 production was increased in the spinal cord of HGF-Tg mice (mean ± SEM). **, $P < 0.01$. (*C*) Staining for CD44 and CD62L coreceptors showed that $CD4^+$ T cells in the spinal cord of HGF-Tg mice were maintained in a low state of activation. (*Left*) Representative dot blots of CD62L CD44 analyses on T cells. (*Right*) Mean ± SEM, n = 3 per group. *, $P < 0.05$.

assays (i.e., ASR) mimicking immune response during EAE with DC stimulated by MOG with or without HGF and coincubated with TCR^{MOG} T cells demonstrated (*i*) inhibition of DC function (including a decrease in costimulatory CD40 on DC and IL12p70 secretion together with an inhibition of T-cell proliferation), (*ii*) a cytokine Th2 bias similar to that detected in the spinal cord of EAE mice, (*iii*) induction of $Foxp3^+$ T_{reg} cells with a high proportion of IL-10–secreting cells, and (*iv*) inhibition of surface markers of T-cell activation. All these effects were driven by HGF-tolerized DC, and HGF had no direct effect on $CD4^+$ T cells. In addition, the effects of HGF on DC were abrogated when DC were pretreated with blocking antibody directed against cMet, the HGF receptor, thereby indicating that the observed effects were related specifically to DC activation through the HGF–cMet pathway. Finally, EAE experiments were repeated using adoptive transfer of TCR^{MOG} $CD4^+$ T cells, and the results confirmed that inhibition of EAE in HGF-Tg mice is driven specifically through an increase of IL-10–producing T_{reg} cells and maintenance of T cells in a low state of activation.

HGF has been shown previously to be protective in various animal models of immune-mediated diseases such as myocarditis (32, 33), glomerulonephritis (30, 34), inflammatory bowel disease (35), collagen-induced arthritis (36), pulmonary fibrosis (37), allogeneic heart transplantation (45), graft-vs.-host disease (46), and asthma (47). According to these studies, HGF may ameliorate both Th1-type–dominated (33, 36) and Th2-type–dominated (40, 47) autoimmune responses. Interestingly, a recent report demonstrated that, in an experimental model of airway allergy, HGF might repress DC function without up-regulation of IL-10 (40). In contrast, our data along with recent reports on murine models of cardiac transplantation (45) and autoimmune myocarditis (33) clearly show an HGF-dependant induction of both T_{reg} cells and IL-10.

The HGF receptor cMet is not expressed on $CD4^+$ and $CD8^+$ T cells but can be detected on APC including $CD11b^+$ monocyte-macrophages and $CD11c^+$ DC. Of note, DC are the most efficient APC and are crucial in the EAE model, because the presence of DC alone is sufficient to present antigen in vivo to primed myelin-reactive T cells and to mediate CNS inflammation and development of clinical disease (43). Thus, a selective tolerization of DC as demonstrated in the present report could be sufficient to explain the inhibition of EAE. However, because cMet also is expressed on other APC, we cannot exclude completely the possibility that the protective effect of HGF observed in EAE also could be mediated by tolerized monocytes/macrophages.

Two plasminogen-related growth factors have been identified so far: HGF and HGF-like/macrophage-stimulating protein (HGFl/MSP) (48). The receptor for HGFl/MSP is a tyrosine receptor kinase, recepteur d'origine nantais (RON) (49), closely related to cMet, thereby suggesting a coevolution of these growth factors and their receptors. Of note, mice lacking RON show an exacerbation of symptoms during EAE, with overall worsened disease severity, increased demyelination, axonal loss, and neuroinflammation (50). Hence, these data suggest that both HGF and HGFl/MSP are protective in EAE.

In addition to its immunomodulatory effect, the role of HGF in regulating the survival, the differentiation, and the promotion of axonal outgrowth of various neuronal populations, including sensory, sympathetic, and motor neurons, has been firmly established (24). HGF is known to be a potent neuroprotective factor and also is able to induce differentiation of OPC into new myelin-forming cells (18). We recently demonstrated that HGF can be produced by microglia under TGF-β stimulation and can act as a chemotactic factor inducing migration of myelin-forming cells (i.e., OPC) into demyelinated lesions (17). Given that c-Met is expressed and phosphorylated in both OPCs and oligodendrocytes (22) and that activation of caspase-3 in oligodendrocytes can be attenuated by HGF application in the animal model of spinal cord injury (21), we cannot exclude the possibility that the beneficial effects of HGF overexpression in the CNS might be mediated partially via the prevention of oligodendrocytic cell degeneration. The importance of these mechanisms in the attenuation of EAE lesions in HGF-Tg mice remains to be determined.

In conclusion, our data demonstrate that HGF is a strong immunomodulator that inhibits EAE, a model of CNS autoimmunity closely resembling multiple sclerosis. HGF is able to induce tolerization of DC and to inhibit T-cell function at different levels. Our data are in accordance with recent publications showing that the immunomodulatory effects of HGF are mediated through APC tolerization and induction of an antiinflammatory (Th2) cytokine pattern (33, 40, 41). Along with the previous observations that HGF is a strong neuroprotector (24) and potentially could trigger remyelination (17, 18), our results suggest that HGF might be a promising candidate for the development of treatments for immune-mediated demyelinating diseases associated with neurodegeneration, such as multiple sclerosis.

Methods

Mice, Induction of EAE, and Isolation of CNS-Infiltrating Mononuclear Cells. NSE-HGF-Tg mice were generously provided by H. Nakamura and T. Funakoshi (University of Osaka), and genotyping was performed as described (20). The transgenic founder mice (C57BL/6J background) were bred with WT C57BL/6J mice and backcrossed at least 15 times before EAE induction. 2D2 (TCR^{MOG}) transgenic mice were generously provided by V. Kuchroo (Harvard Medical School). All mice were bred in house under pathogen-free conditions and were used at 8–10 weeks of age. Animal experiments were approved by the local veterinary office (Geneva, Switzerland) according to Swiss ethical regulations. For active immunization, female HGF-Tg mice and WT littermates were immunized with MOG(35-55) as described (51). For

adoptive transfer, spleen cells from TCR^{MOG} transgenic mice were stimulated with MOG(35-55) and IL-12p70 (R&D Systems) for 4 days. At day 5, $CD4^+$ T cells were isolated and injected into recipient mice (7×10^6 cells per mouse). Animals received pertussis toxin (300 ng per mouse) on days 0 and 2 after T-cell transfer. Mice were assigned clinical scores daily as described (51). CNS mononuclear cells were isolated as described (52).

ASR Assays and T-Cell Proliferation Assay. $CD4^+$ T cells and $CD11c^+$ cells were selected from spleen cells using MicroBeads (Miltenyi Biotec) according to the manufacturer's protocol. DC were incubated with rHGF (eBioscience) (1–100 ng/mL) for 24 h. As a negative control, DC were preincubated with anti-cMet antibody (10 μg/mL; eBioscience). To examine the effect of HGF on Ag presentation, DC were pulsed with MOG(35-55) (20 μg/mL). After washing with PBS, DC (1×10^5/mL) were cocultured with $CD4^+$ T cells (1×10^6/mL) obtained from 2D2 (TCR^{MOG}) transgenic mice. Proliferation assays were performed as described elsewhere (43).
Methodologies are described in *SI Methods*.

RNA Isolation and Real-Time PCR. RNA was extracted from spinal cords of mice. The spinal cords were flushed with ice-cold PBS, and RNA was isolated using RNAeasy Mini Kits (Qiagen) following the manufacturer's instructions. The PCR was performed as described previously (20). Results were quantified relative to a standard curve generated with serial dilutions of a reference cDNA from a DC line and normalized using TATA-biding protein mRNA. Each experiment was repeated at least three times, and values were expressed as mean ± SEM.

Statistical Analysis. Statistical analysis was performed using two-tailed Student's *t* test. $P < 0.05$ was considered statistically significant.

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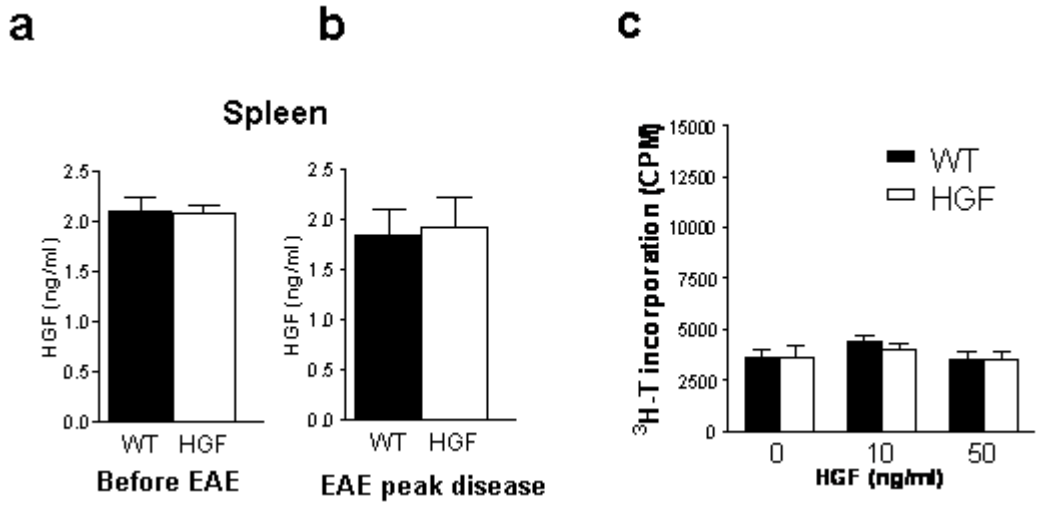
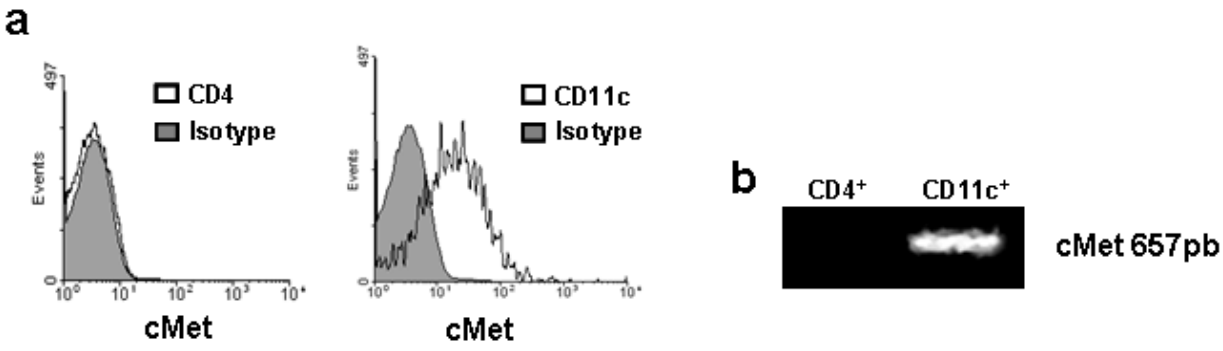
Benkhoucha *et al*. Supplementary Figure 1



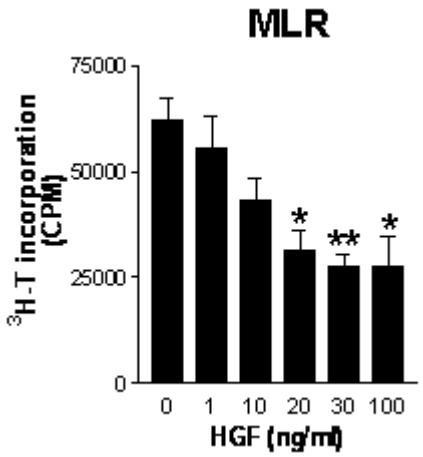
Supplementary Figure 1. HGF in not increase in the spleen and does not induce proliferation of splenic T cell during EAE. HGF measured by ELISA in spleen cells in WT versus HGF-Tg mice before **(a)** and during EAE peak disease **(b)** (n=3; ± SEM, each experiment in triplicate). Proliferation assay of splenocytes with rHGF (0 to 50 ng/ml) in HGF-Tg mice and WT littermates during EAE peak disease **(c)** (dpi 25; mean counts per minute ± SEM; n=3; each experiment in triplicate).

Benkhoucha *et al*. Supplementary Figure 2



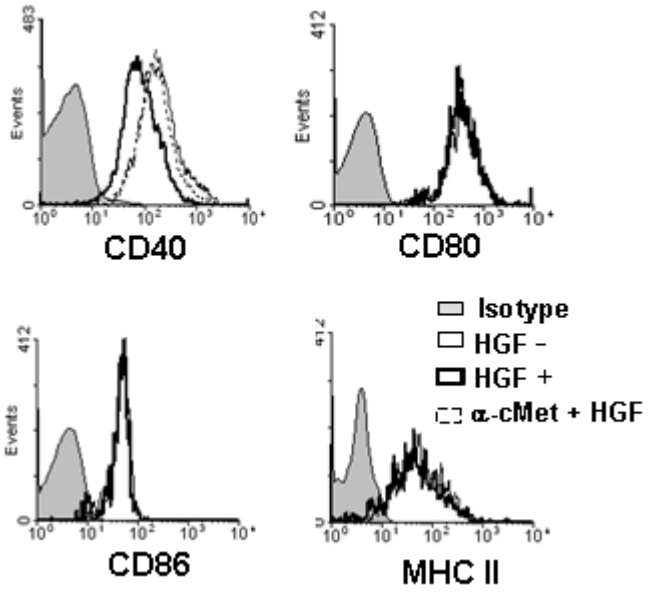
Supplementary Figure 2. The c-Met (HGF) receptor is present on the surface of CD11c$^+$ cells but not on CD4$^+$ T cells. (**a**) c-Met receptor staining on the surface of CD11c$^+$ and CD4$^+$ T cells from fresh C57BL/6 splenocytes (figures representative of four independent experiments). (**b**) Detection of the c-Met receptor by RT-PCR on CD11c$^+$ and CD4$^+$ T cells from fresh C57BL/6 splenocytes.

Benkhoucha *et al*. Supplementary Figure 3



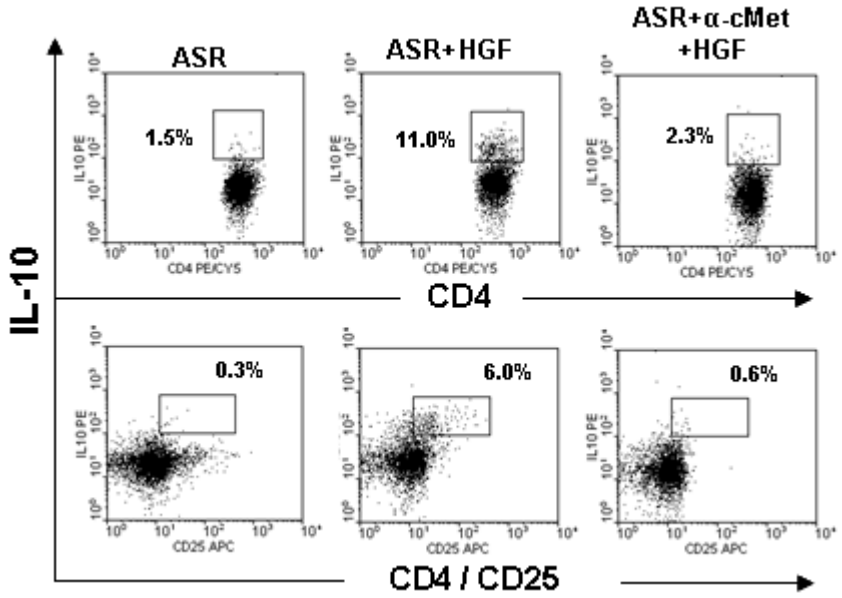
Supplementary Figure 3. Influence of HGF on DC function and T cell proliferation assessed by mixed lymphocyte reaction (MLR). MLR were performed through co-incubation of purified $CD11c^+$ cells (DCs) from BALB/c mice and purified $CD4^+$ T cells from C57BL/6 mice. T cell proliferation was analyzed after $CD11c^+$ cells were pre-incubated with increasing concentrations of rHGF (mean counts per minute ± SEM; triplicate wells).

Benkhoucha *et al*. Supplementary Figure 4



Supplementary Figure 4. HGF inhibits *in vitro* expression of CD40 in $CD11c^+$ DC. ASR staining for co-receptors of $CD11c^+$ cells including CD40, CD80, CD86, MHCII. CD40 expression was inhibited by rHGF (rHGF at 30 ng/ml; figures representatives of four independent experiments).

Benkhoucha *et al*. Supplementary Figure 5



Supplementary Figure 5. HGF-treated DCs induce IL-10 production in $CD4^+$ and $CD4^+CD25^+$ T cells. IL-10 intracellular staining for $CD4^+$ T cells and for $CD4^+CD25^+$ T cells from ASR experiments: IL-10 production was increased in both T cell subtypes when T cells were co-cultured with rHGF-treated $CD11c^+$ DC. HGF effect on $CD11c^+$ cells was inhibited by pre-incubation of $CD11c^+$ cells with α-c-Met blocking Ab (figures representatives of four independent experiments).

II. B)
Effect of repetitive pertussis toxin and IL-21 antagonist in experimental autoimmune encephalitis

Section II.B.1

Repetitive pertussis toxin promotes development of regulatory T cells and prevents CNS autoimmune disease

Martin S. Weber, Mahdia Benkhoucha, Klaus Lehmann-Horn, Johann Sellner, Michel Chofflon, Scott S. Zamvil, Patrice H. Lalive

Submitted for publication

INTRODUCTION

Pertussis toxin (PTX) is a protein produced by *Bordetella pertussis*. PTX is included in many a cellular Bordetella pertussis vaccines under it chemically or genetically detoxified form. It is used as an adjuvant for EAE induction. The mechanism by which i.v. PTX administration facilitates EAE is complex and not entirely understood. Activity of PTX is mainly attributed to an increased permeabilization of the otherwise cell-restrictive blood-brain barrier leading to an influx of immune cells into the CNS and induces a CD4 Th1 cell-mediated inflammatory response in the CNS, PTX increases expression of cerebrovascular adhesion molecules and promotes maturation and functional capacity of APC, increases production and release of pro-inflammatory cytokines such as IL-12 and decreases secretion of anti-inflammatory IL-10. It has been shown that PTX reduces number and function of Treg cells when it's used as an adjuvant for EAE induction, whereas its promote development of encephalitogenic Th-17 cells. Regarding all these effects, PTX may use different mechanisms to promote induction of EAE. Bacteria may also play a role in resistance to autoimmune diseases.

The work presented in this section describe that mice continuously exposed to PTX are indeed protected from active EAE induction which is associated with important decrease of proliferation and pro-inflammatory differentiation of myelin-reactive T cells. Interestingly, we showed that PTX treatment prior to disease induction elevated serum levels for TGF-β and IL-10 and promoted expansion of Treg. *In vitro* experiment confirmed that these anti-inflammatory cytokines appeared to be specifically produced by PTX responsive T cells.

Our data joint other findings which indicate that certain bacteria may protect against the development of autoimmune diseases. These results also suggest the potential use for still-unidentified bacterial agents in the manipulation of certain autoimmune diseases.

OBJECTIVES

To investigated whether continuous PTX pre-treatment may exert an immunomodulatory effect and inhibit EAE course, by blocking T cell responses to Ag.

Repetitive pertussis toxin promotes development of regulatory T cells and prevents CNS autoimmune disease

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Abstract

Background: Pertussis toxin (PTx), the major virulence factor of *Bordetella pertussis* is widely used to enhance incidence and severity of experimental autoimmune encephalomyelitis (EAE), the animal model of multiple sclerosis (MS). Its adjuvant effect is mainly attributed to a facilitated migration of activated immune cells across the blood brain barrier (BBB) into the central nervous system (CNS).

Methods and Findings: We investigated the immunological effects of repetitive PTx administration using C57Bl/6 wild-type and MOG p35-55 T cell receptor transgenic mice. While weekly intravenous (i.v.) injections for up to six months did not alter non-specific immune responses or antigen (Ag)-specific T cell responses to PTx or myelin Ag, mice exposed repetitively to PTx were largely protected from subsequent EAE induction. EAE resistance was reflected by a decreased proliferation and pro-inflammatory differentiation of myelin-reactive T cells. Splenocytes isolated from these mice produced regulatory IL-10 upon re-stimulation with PTx, but not in response to myelin Ag or a non-specific T cell stimulus. Longitudinal analyses revealed that pre-exposure of mice to PTx had elevated serum levels for TGF-β and IL-10 prior to disease induction. Most strikingly, repetitive PTx treatment had promoted development of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Treg) as early as 6 weeks after treatment initiation.

Conclusions: These data suggest that repetitive PTx treatment protects mice from CNS autoimmune disease through expansion of $CD4^+CD25^+FoxP3^+$ Treg. Besides their therapeutic implications, our findings indicate that encounter of the immune system with microbial products may not only be part of CNS autoimmune disease pathogenesis but also of its regulation.

Introduction

Pertussis toxin (PTx) is used for over three decades as an adjuvant to induce experimental autoimmune encephalomyelitis (EAE) in combination with myelin Ag. The 105-kDa protein is produced by *Bordetella pertussis* and is the cause for whooping cough in humans. In a chemically or genetically detoxified form, PTx is included in many acellular Bordetella pertussis vaccines[1]. Structurally, PTx is composed of five proteins (S1, S2, S3, S4, and S5) and belongs to the A-B class of exotoxins[2]. The B subunit contains S2-S5 and binds to the surface of many eukaryotic cells. The A subunit S1, is subsequently released into the cytoplasm where it interferes with the inhibitory activity of Gi proteins unleashing intracellular signaling[3].

The mechanism by which i.v. PTx administration facilitates EAE is complex and not entirely understood. Activity of PTx is mainly attributed to an increased permeabilization of the otherwise cell-restrictive blood-brain barrier leading to an influx of immune cells into the CNS[4,5]. This assumption may not be conclusive as recent data suggest that PTx increases expression of cerebrovascular adhesion molecules[6,7], proposing an alternative mechanism by which PTx may facilitate leukocyte migration into the brain. PTx further promotes maturation and functional capacity of antigen presenting cells (APC)[8], increases production and release of pro-inflammatory cytokines such as IL-12[9] and decreases secretion of anti-inflammatory IL-10[10]. When used as an adjuvant for EAE induction, PTx reduces number and function of Treg[11,12], while promoting development of encephalitogenic Th-17 cells[13]. Taken together, PTx may utilize multiple mechanisms to promote development of EAE.

Several primarily pro-inflammatory bacterial agents including PTx apparently also have protective properties when the immune system encounters them under certain circumstances. In this regard, pre-exposure of mice to Bordetella pertussis surprisingly protected from EAE induction[14] which could be attributed to the toxin produced, as genetically altered PTx failed to suppress CNS autoimmune disease[15]. Notwithstanding these initial observations, they left unclear how PTx facilitates EAE in one setting but may hinder its induction in another setting. In our study, we demonstrate that mice continuously exposed to PTx are indeed protected from active EAE induction which is associated with a markedly decreased proliferation and pro-inflammatory differentiation of myelin-reactive T cells. PTx treatment prior to disease induction elevated serum levels for TGF-β and IL-10 and promoted expansion of Treg. These anti-inflammatory cytokines appeared to be specifically produced by PTx responsive T cells, as in vitro re-stimulation with PTx, but not with any other stimulus could trigger their release.

Materials and methods

Mice and EAE induction. C57BL/6 female mice, 5-8 weeks of age, were purchased from the Jackson Laboratory (Bar Harbor, MN). C57BL/6 MOG 35-55-specific T cell receptor transgenic mice [16] were kindly provided by Vijay K. Kuchroo (Harvard University). Mouse myelin oligodendrocyte glycoprotein (MOG) peptide 35-55 (MEVGWYRSPFSRVVHLYRNGK) was synthesized and purified (>99%) by Auspep (Parkville, Australia). Age-matched mice were immunized subcutaneously (s.c.) with 25μg MOG p35-55 in 0.1 ml of PBS emulsified in an equal volume of complete Freund's adjuvant (CFA) supplemented with 2 mg/ml of mycobacterium tuberculosis H37RA on day 0 (DIFCO Laboratories, Detroit, Michigan, USA). After immunization and 48 hrs later mice received an intravenous (i.v.) injection of 300 ng of bordetella pertussis toxin (PTx) in 0.2 ml of PBS[17,18]. Individual animals were observed daily and clinical scores were assessed in a blinded fashion as follows: 0 = no clinical disease, 1 = loss of tail tone only, 2 = mild monoparesis or paraparesis, 3 = severe paraparesis, 4 = paraplegia and/or quadraparesis, and 5 = moribund or death. At least three independent experiments were conducted with a minimum of 8 mice per group.

Pertussis toxin treatment. PTx was purchased from List, Biological Laboratories Inc. (Campbell, USA). Age-matched mice received weekly i.v. injections with 300 ng PTx or OVA 323-339 (Abgent, Inc., San Diego, USA) in 0.2 ml of PBS or PBS alone starting 10 weeks or 6 months prior to EAE induction, respectively. Mice were examined clinically every other day.

Proliferation Assays. Proliferative responses were measured using splenocytes. 5×10^5 spleen cells were cultured with antigen, in 0.2 ml RPMI medium supplemented with 5×10^{-5} M 2-mercaptoethanol, 2 mM glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin. Anti-mouse CD3/CD28 or phytohemagglutinin (PHA; BD Biosciences, Franklin Lakes, USA) were used as positive control. After 72 hr, cultures were pulsed with 1 μCi [^{3}H]-thymidine and harvested 16 hr later. Mean counts per minute (cpm) of [^{3}H] thymidine incorporation was calculated for triplicate cultures.

Cytokine analysis. Serum samples were obtained at the time-point indicated for cytokine analysis. Culture supernatants were collected at various time-points, 72-hr (TNF, IFN-γ, TGF-β), and 120-hr (IL-10). ELISA was performed using paired monoclonal antibodies specific for corresponding cytokines per manufacturer's recommendations (Pharmingen, San Diego, CA). The results for ELISA assays are expressed as an average of triplicate wells +/- SD. SOFTmax ELISA plate reader and software was used for data analysis (Molecular Devices Corporation, Sunnyvale, CA).

FACS analysis of $CD4^+CD25^+$ $FoxP3^+$ Treg. Development of $CD4^+CD25^+FoxP3^+$ Treg was evaluated using a FACS staining kit by eBiosience (San Diego, USA).

Histopathology. Brains and spinal cords of mice were fixed in 10% formalin. Sections were stained with Luxol fast blue-hematoxylin and eosin. Parenchymal inflammatory lesions were counted as described [19,20].

Statistical analysis. Data are presented as mean ± SEM. For clinical scores significance between groups was examined using the Mann-Whitney *U* test. A value of $p < 0.05$ was considered significant. All other statistical analysis was performed using a one-way multiple-range analysis of variance test (ANOVA) for multiple comparisons. A value of $p < 0.01$ was considered significant.

Results

Continuous PTx treatment is not immunosuppresive and does not induce tolerization

First, we investigated whether continuous PTx pre-treatment may exert a general specific immunosuppressive effect or may have tolerized mice for PTx, possibly hindering subsequent EAE induction using this adjuvant. Representative mice in both the PTx pre-treated group as well as in the control-treated group were sacrificed before EAE immunization and T cell responses to PTx, myelin Ag's and a mitogen were analysed **(Fig. 1a-d)**. Proliferation of splenocytes in response to stimulation with PTx was not significantly different between PTx pre-treated and control-treated mice. In both groups, maximal proliferation was obtained at a PTx concentration of 200 and 400 ng (**Fig. 1a**). Similarly, no difference in proliferation was observed upon non-specific stimulation with phytohaemagglutinin (PHA) between PTx pre-treated and non pre-treated mice **(Fig. 1b)**. Thus, continous PTx pre-treatment had no apparent tolerizing or immunosuppressive effect.

We next examined whether continuous PTX treatment may have generated myelin-specific T cell responses due to its presumed ability to increase permeabilization of the blood-brain-barrier. As shown in **figure 1c and d**, no peripheral proliferative response to myelin Ag was observed though, either in the group treated with PTx or in the control group. To further address the question whether repetitively PTx-facilitated transmigration of myelin-specific T cells may be sufficient to induce CNS autoimmune disease, MOG p35-55 T cell receptor transgenic mice[16] were injected with PTx weekly over six months **(Fig. 1e-f)**. None of these mice, which are highly susceptible to EAE, developed clinical signs of EAE (data not shown). Further, similar to wild-type mice, PTx treatment did not alter T cell responses to PTx **(Fig. 1e)** or MOG p35-55 **(Fig. 1f)** in MOG p35-55 transgenic mice.

Repetitive PTx pre-treatment protects mice against clinical and histological EAE

Mice treated weekly with PTx or control-treated mice were immunized with MOG p35-55 and evaluated for clinical signs of EAE. In comparison with the control-treated group, EAE was ameliorated in mice pre-treated with PTx (mean clinical score +/- SEM: 1.1 +/- 0.48 vs. 4.3 +/- 0.44; p=0.0001). Further, the onset of clinical symptoms was significantly delayed in the PTx pre-treated group (15 +/- 1 days) when compared to the control group (10 +/- 7 days) (p=0.011) **(Fig. 2a).** Representative sections of the spinal cord and brain were compared between PTx and control-treated mice. The number of inflammatory lesions as well as the extent of inflammatory infiltration was significantly lower in the PTx pre-treated group than

in the control group (mean number of inflammatory lesions per slide +/- SEM: 8,7 +/- 1,17 vs. 17 +/- 2,7). **(Fig. 2b)**. Luxol fast blue staining indicated less or no demyelination in PTx pre-treated group whereas extensive areas of myelin loss were observed in the control group **(Fig. 2c)**.

Repetitive PTx pre-treatment inhibits pro-inflammatory differentiation of myelin-specific T cells and promotes anti-inflammatory differentiation of PTx responsive cells.

We next investigated T cell responses to myelin Ag after EAE induction comparing PTx- and control-pretreated mice. T cell proliferation and cytokine release in response to non-specific stimulation was not different between the PTx-treated and the control-treated group (**Fig. 3a-d**). In contrast, in the PTx-treated group, T cell proliferation was markedly reduced in response to MOG 35-55, the Ag used for EAE induction (**Fig. 3e**). In addition, IFN-γ production, a Th1 cytokine was also only significantly decreased in the PTx pre-treated group upon re-stimulation with MOG p35-55 (**Fig. 3f**), but not with antiCD3/CD28 (**Fig. 3b**) or PTx (**Fig. 3j**). Release of TNF, a pro-inflammatory cytokine mainly secreted by activated antigen presenting cells and to a lesser degree by T cells, was also analyzed. In contrast to IFN-γ, TNF production was not different between both groups neither upon stimulation with antiCD3/CD28 (**Fig. 3c**), MOG 35-55 (**Fig. 3g**), or PTx (**Fig. 3k**).

As we had observed that PTx pre-treatment protected mice from subsequent EAE induction, we investigated whether this pre-treatment had promoted development of anti-inflammatory cytokines with regulative capacity. Splenocytes from PTx-pre-treated mice released significant amounts of IL-10 specifically after PTx re-stimulation (**Fig. 3l**), but not following re-stimulation with antiCD3/CD28 (**Fig. 3d**) or MOG 35-55 (**Fig. 3h**).

Repetitive PTx pre-treatment promotes development of $CD4^+CD25^+FoxP3^+$ Treg

Based upon this observation, we investigated next whether PTx treatment may enhance serum levels of anti-inflammatory cytokines without any further inflammatory stimulus. We thus injected mice weekly with PTx or the non-self Ag ovalbumin (OVA; 323-339). Serum cytokine levels were determined every 3 weeks after initiation of treatment. As shown in **figure 4a**, weekly injections with PTx elevated serum levels of IL-10 starting from week 9 after treatment initiation. Even more prominently, PTx injections raised serum levels of TGF-β **(Fig. 4b)** starting as early as 6 weeks after the first injection with PTx. In contrast, pro-inflammatory cytokines levels such as TNF **(Fig. 4c)** or IFN-γ **(Fig. 4d)** were not elevated.

As TGF−β, but also IL-10 are involved in development and maintenance of Treg, we analysed whether PTx pre-treatment was also associated with a longitudinally increased development of $CD4^+CD25^+FoxP3^+$ Treg. As shown in **figure 4e and f,** continuous PTx treatment strongly increased the frequency of splenic $CD4^+CD25^+FoxP3^+$ Treg, starting as early as 6 weeks after initiation of PTx treatment. Upon immunization (at week 10), the PTx-treated mice -as expected- developed significantly less severe EAE compared to the OVA-treated control group **(Fig. 5a).** At the peak of disease severity, clinical benefit was associated with a sustained increase of Treg **(Fig. 5b and c)** and elevated serum levels of IL-10 **(Fig. 5d)**.

Discussion

PTx is widely used as an adjuvant to induce EAE. Its co-administration with myelin Ag enhances incidence and severity of EAE symptoms and even renders otherwise resistant strains susceptible to EAE[21,22]. This EAE-facilitating effect is not restricted to PTx, but has also been demonstrated for other bacterial Ag's such as the superantigen staphylococcal enterotoxin B (SEB)[23-25]. In our study, we first administered PTx weekly for six months in a dose used for EAE induction into mice susceptible for MOG-induced EAE. We hypothesized that through repetitive permeabilization of the BBB peripherally activated immune cells could infiltrate into the CNS and lead to T cell recognition of myelin Ag. However, none of the mice continuously exposed to PTx developed myelin-specific T cell responses or showed any clinical EAE symptoms throughout the course of PTx treatment. Furthermore, in MOG p35-55 TCR transgenic mice, of which about 30% develop spontaneous optic neuritis[16], repetitive PTx alone could not generate myelin-specific T cell responses either and none of the mice treated for up to 6 months developed clinical symptoms of paralysis.

As reported in two independent earlier studies[14,15], mice repetitively pre-treated with PTx were in contrast largely protected from subsequent EAE induction. Accordingly, mice continuously exposed to PTx in our study exhibited markedly decreased proliferation and pro-inflammatory differentiation of myelin-reactive T cells upon immunization. So how could an agent with strongly pro-inflammatory, EAE-facilitating properties protect from the same disease when given repetitively prior to myelin Ag immunization? Several possibilities could account for this intuitively contradictive phenomenon. Most pro-inflammatory properties of PTx are frequently explained by its impairment of inhibitory G proteins. PTx was shown to enhance IL-12 and TNF production facilitating development of Th1 responses[9]. Similarly, through upregulation of IL-6, PTx used as an adjuvant promotes generation of IL-17-producing CD4 cells[13] and suppresses frequency of $CD4^+CD25^+FoxP3^+$ Treg[11,12]. One possible explanation for the opposing effect of repetitive application is that the continuous activation might lead to increased T cell apoptosis[26]. Data indicate that activated T cells are particularly susceptible to apoptosis[27,28], which could have decreased the frequency of self-reactive effector T cells before EAE induction. However, the unimpaired proliferation and pro-inflammatory differentiation of myelin-reactive T cells in repetitively PTx pre-treated MOG p35-55 TCR transgenic mice indicates that enhanced apoptosis of effector T cells may not account for EAE resistance. Alternatively, continuous exposure to PTx could have induced T cell anergy[29], thereby specifically abolishing PTx's T cell activating property at the

time of immunization. This assumption is fuelled by the fact that pre-treatment with Mycobacterium tuberculosis, which is also part of active EAE induction protocols, similarly confered protection against CNS autoimmune disease, whereas pretreatment with other bacterial components, such from Escheriachia coli, Shigella or Staphylococcus aureus failed to do so[14]. However, at least in the case of Mycobacterium tuberculosis, it had been demonstrated that the protective effect could be attributed to a 12-kDa protein which failed to activate encephalitogenic T lymphocytes[30], indicating that the domain conferring protection and the one facilitating EAE are not identical. The possibility of T cell anergy as the explanation for EAE resistance conferred by chronic PTX treatment appears further unlikely in the light of the unaltered T cell response to PTx in both C57Bl/6 wild-type and MOG p35-55 TCR transgenic mice treated with PTx over several weeks.

Largely excluding these possibilities, PTx-mediated EAE protection thus might be indeed mediated through the observed expansion of $CD4^+CD25^+FoxP3^+$ Treg. Development of Treg was associated with elevated serum levels for TGF-β and IL-10, two cytokines centrally involved in development and maintenance of Treg[31,32]. These regulatory cytokines are produced by a variety of immune cells, including T cells as well as various APC. Although it remains to further studies to investigate which cell(s) released IL-10 and TGF-β in response to repetitive PTx exposure, our current study provides a hint. Splenocytes isolated from PTx-treated mice secreted enhanced amounts of IL-10 specifically upon re-stimulation with PTx, but not upon Ag non-specific T cell activation. These findings suggest that IL-10 may not have been produced by T cells. In this regard, it has been demonstrated that various APC populations produce increased amounts of IL-10 when stimulated with PTx and that APC producing IL-10 and TGF-b are very potently promoting expansion of $CD4^+CD25^+FoxP3^+$ Treg[17].

In our study, we identified repetitive PTx treatment as a method to increase the frequency of Treg which was associated with protection from CNS autoimmune disease. Besides its therapeutic implication, this finding indicates that some microbial products may not only be involved in the pathogenesis of CNS autoimmune disease but also in its regulation.

Acknowledgements

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Figure legends

Figure 1. C57Bl/6 wild-type (**a-d**) or MOG p35-55 TCR transgenic mice **(e, f)** received weekly i.v. injections with 300 ng PTx in 200 ul of PBS or PBS alone for six months. Splenocytes were cultured in the presence of various concentrations of **(a, e)** pertussis toxin (PTx), **(b)** phytohemagglutinin (PHA), **(c, f)** MOG p35-55 or **(d)** MBP Ac1-11.

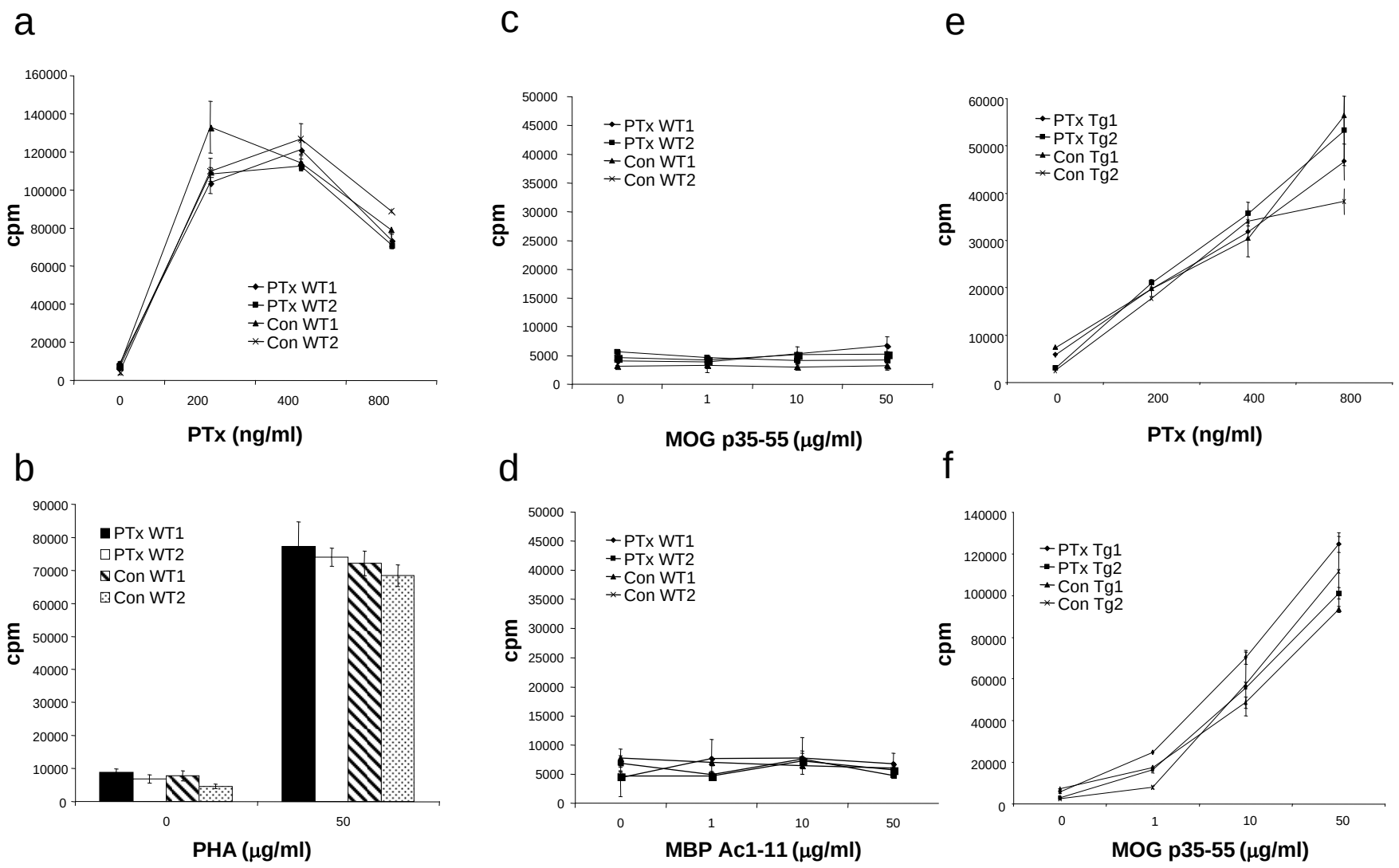
Figure 2. C57Bl/6 mice received weekly i.v. injections with 300 ng PTx in 200 ul of PBS or PBS alone. After six months of PTx injection, mice were immunized with MOG p35-55 in CFA and injected i.v. with 300 ng PTx in PBS immediately following the immunization and 48h later. **(a)** Mice were followed for clinical signs of EAE (mean severity as group average +/- SEM, * indicates $p<0.05$). (**b+c**) 50 days after EAE induction, CNS tissue from all mice was analysed for histological signs of EAE. Spinal cords were isolated and representative cervical, lumbal and thoracical sections were evaluated for inflammatory lesions. **(b)** Indicated is the average number of lesions per slide +/- SEM in each group (* indicates $p<0.05$). **(c)** Shown are representative slides stained with H&E (upper panels) or LFB (lower panels, magnification X100).

Figure 3. C57Bl/6 mice received weekly i.v. injections with 300 ng PTx in 200 ul of PBS or PBS alone. After six months, mice were immunized with MOG p35-55 in CFA and injected i.v. with 300 ng PTx in PBS immediately following the immunization and 48h later. 50 days after EAE induction, isolated splenocytes were cultured with anti-CD3 (0,5 μg/ml) and anti-CD28 (1 μg/ml) **(a-d)**, MOG p35-55 **(e-f)** or PTx **(i-l)** and evaluated for proliferation **(a, e, i)**, secretion of IFN-γ **(b, f, j)**, TNF (**c, g, k)** and IL-10 (**d, h, l)**.

Figure 4. C57Bl/6 mice received weekly i.v. injections with 300 ng PTx or OVA 323-339 (control) in PBS. Serum cytokine IL-10 **(a)**, TGF-β **(b)**, TNF **(c)** and INF-γ **(d)** were determined at week 0-3-6-9 post PTx treatment. $CD4^+CD25^+FoxP3^+$ T reg were analysed by flow cytometry at week 3 to 9 (**e** and **f**) (*indicates $p<0.05$).

Figure 5. C57Bl/6 mice received weekly i.v. injections with 300 ng PTx or OVA 323-339 (control) in PBS. After 10 weeks, EAE was induced with MOG p35-55. PTx pre-treatment significantly ameliorated disease severity **(a)**. Clinical benefit was associated with expansion of $FoxP3^+$ Treg cells (**b** and **c**) and elevated serum levels for IL-10 (**d**) (*indicates $p<0.05$, ** indicates $p<0.001$).

Fig. 1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PTx (ng/ml)
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40000
35000
30000
25000
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PTx WT2
Con WT1
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PTx (ng/ml)
b
90000
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10000
0
cpm
PTx WT1
PTx WT2
Con WT1
Con WT2
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PHA (μg/ml)
d
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25000
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10000
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Con WT2
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50
MBP Ac1-11 (μg/ml)
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140000
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100000
80000
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40000
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10
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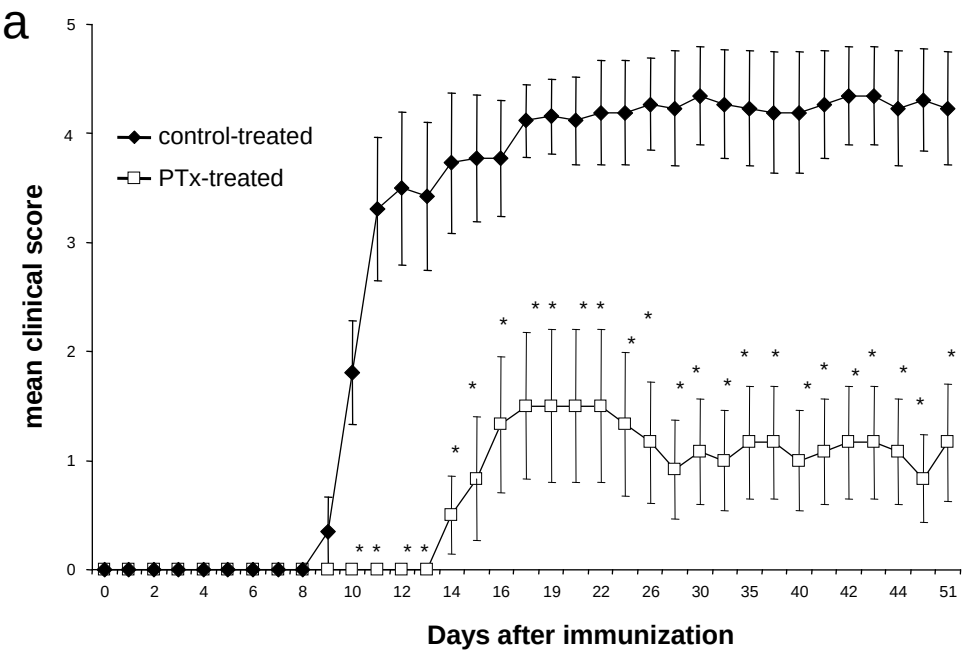
MOG p35-55 (μg/ml)

Fig. 2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mean clinical score
control-treated
PTx-treated
Days after immunization
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4
6
8
10
12
14
16
19
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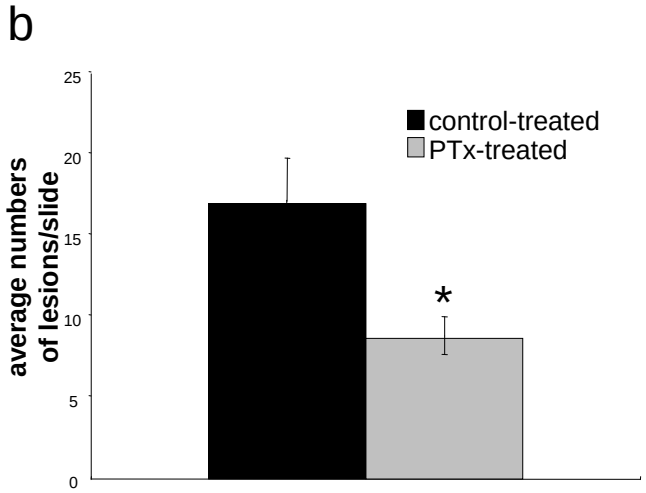
26
30
35
40
42
44
51


b
average numbers of lesions/slide
control-treated
PTx-treated
*

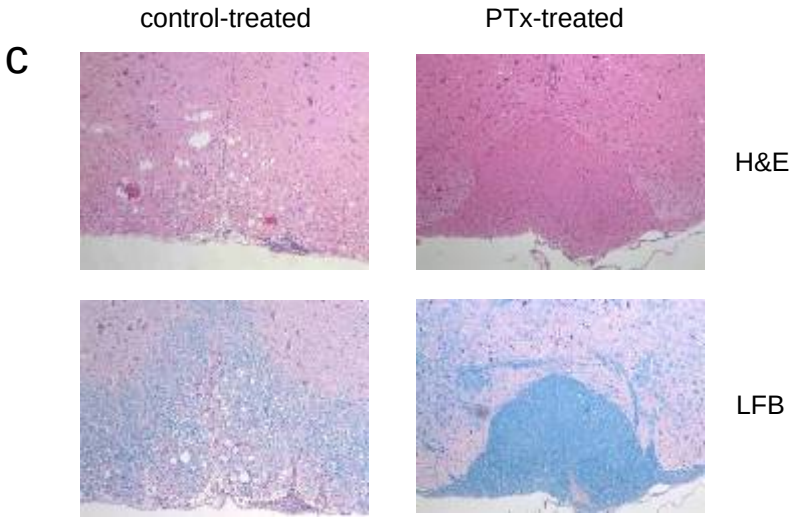
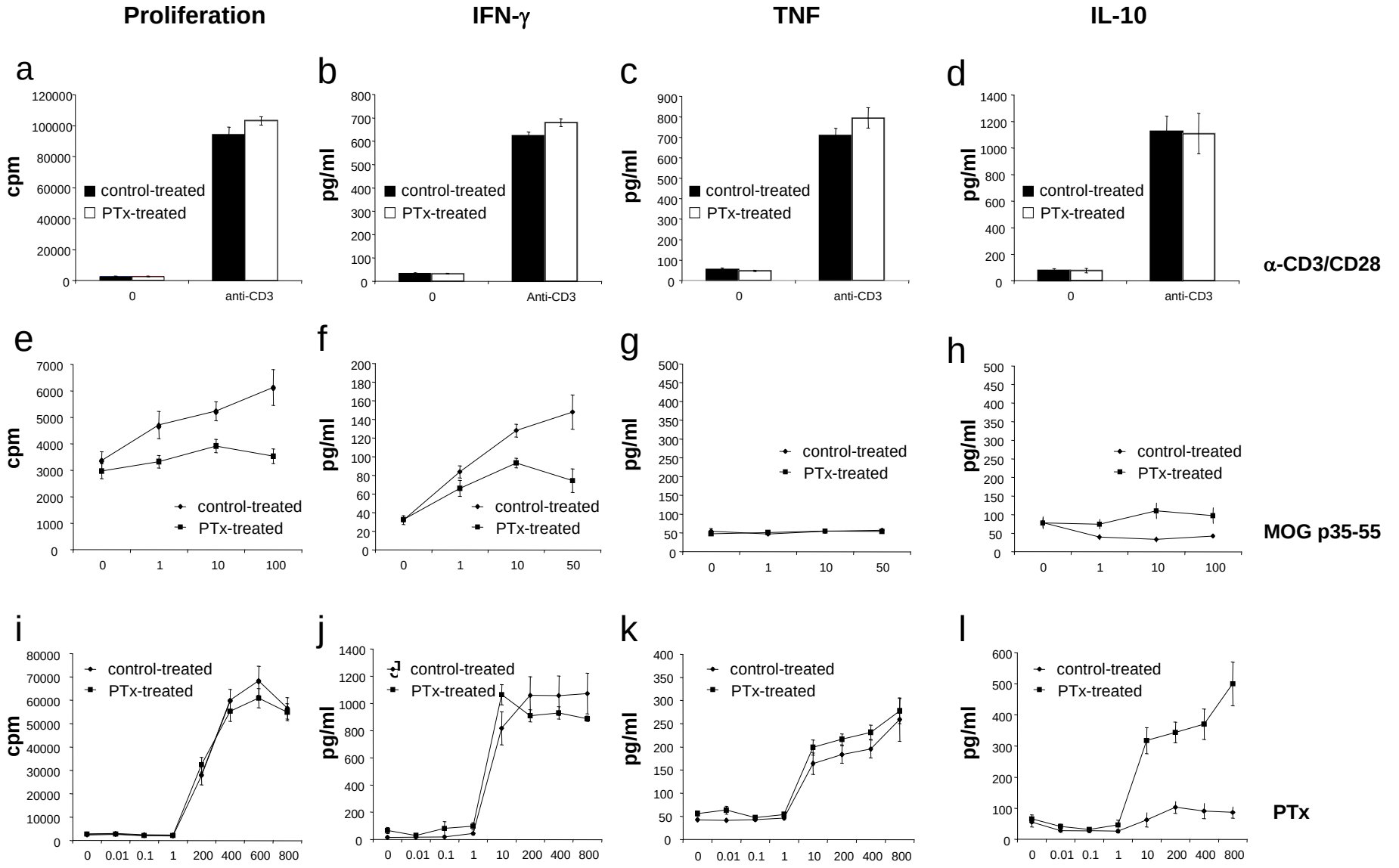

c
control-treated
PTx-treated
H&E
LFB

Fig. 3


Proliferation
IFN-γ
TNF
IL-10
a
b
c
d
cpm
pg/ml
control-treated
PTx-treated
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anti-CD3
Anti-CD3
α-CD3/CD28
e
f
g
h
MOG p35-55
i
j
k
l
PTx

Fig. 4

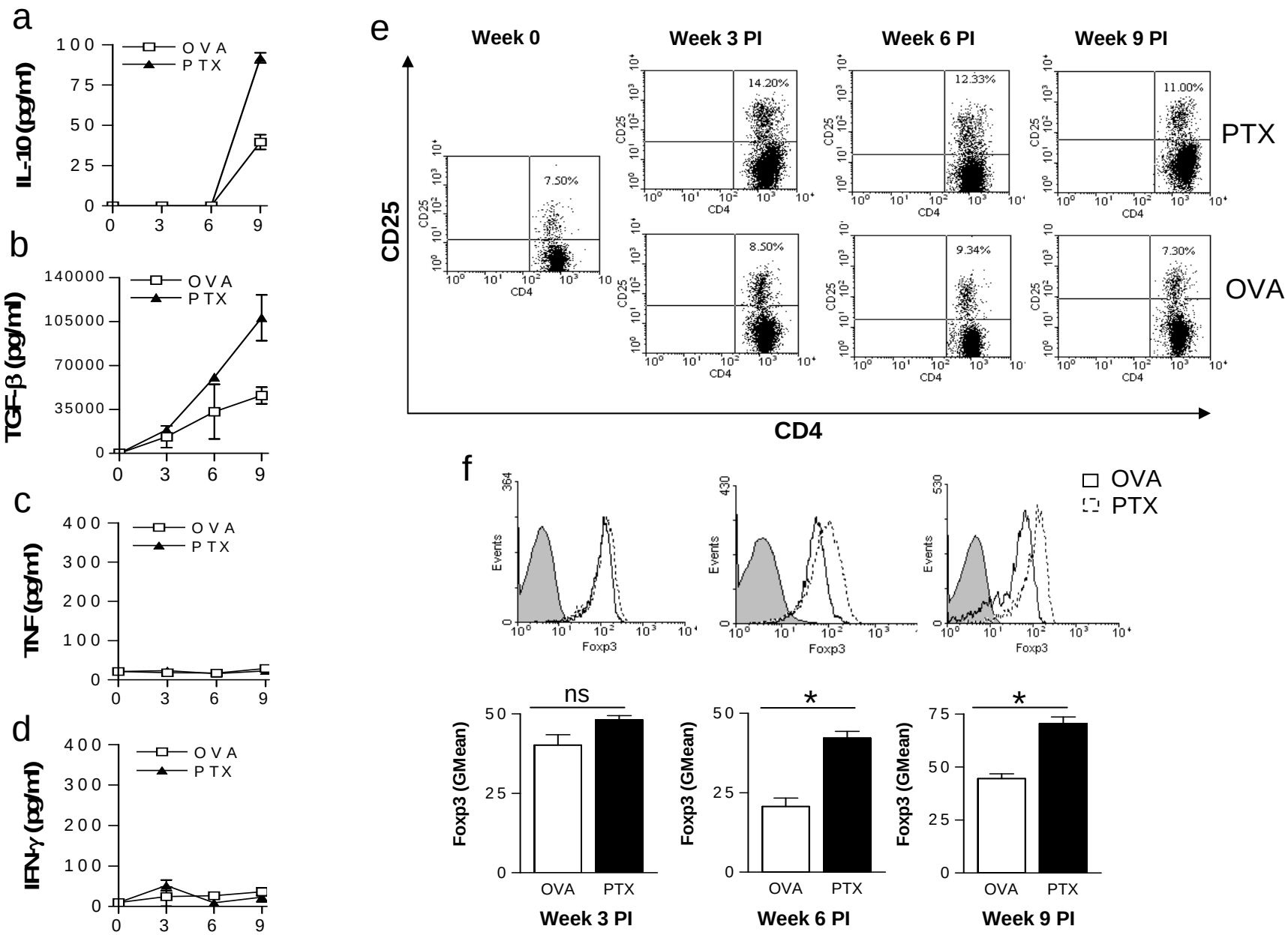
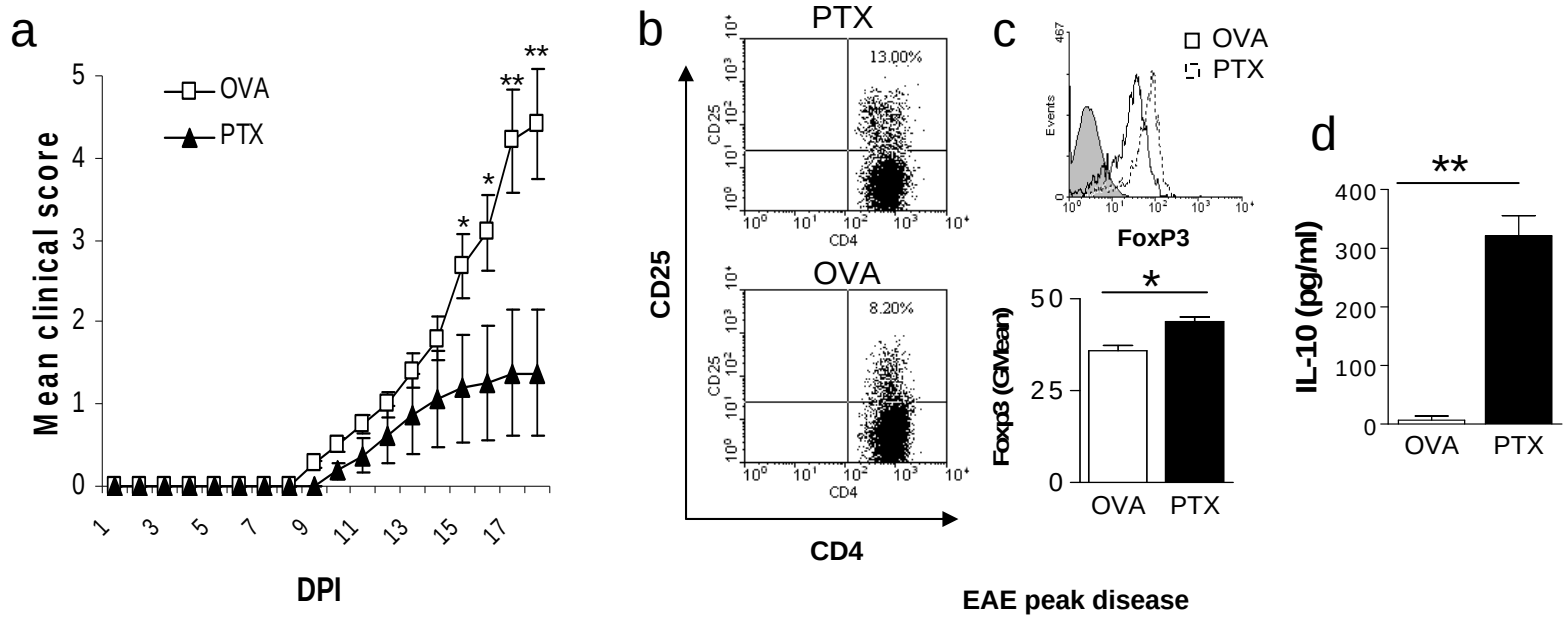

a
IL-10 (pg/ml)
OVA
PTX
b
TGF-β (pg/ml)
c
TNF (pg/ml)
d
IFN-γ (pg/ml)
e
Week 0
Week 3 PI
Week 6 PI
Week 9 PI
CD25
CD4
7.50%
14.20%
12.33%
11.00%
8.50%
9.34%
7.30%
PTX
OVA
f
Events
Foxp3
ns
*
Foxp3 (GMean)
Week 3 PI
Week 6 PI
Week 9 PI

Fig. 5


a
Mean clinical score
OVA
PTX
DPI
b
PTX
13.00%
OVA
8.20%
CD25
CD4
c
OVA
PTX
Events
FoxP3
Foxp3 (GMean)
d
IL-10 (pg/ml)
OVA
PTX
EAE peak disease

Section II.B.2

Opposite effects of IL-21 inhibition by a novel receptor antagonist in two murine models of autoimmune disease

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Submitted for publication

INTRODUCTION

IL-21 is a key T-cell growth factor (TCGF) involved in both innate and adaptive immune responses. IL-21 contributes to the proliferation of naive, but not memory T lymphocytes and play major and distinct parts in T-cell activation, it is may be involved in hematopoietic cell differentiation, proliferation of B cells or natural killer cells. IL-21 is produced exclusively by activated CD4 T cells and NK-T cells. However, the full spectrum of its activity on T cells is not well understood. IL-21 activity is mediated by the IL-21 receptor, member of type I cytokine receptors IL-21R expression in the lymphoid tissues, i.e. T cells, B cells and NK. The expression of IL-21R on the surface of myeloid DCs. Several studies of animal models suggest that IL-21 might play a role in autoimmunity. The progression of the EAE disease in IL-21 and IL-21R KO mice is greatly reduced owing to a dramatic reduction in Th17 cells. Resulting in a mutant with the same binding affinity to the IL-21R chain but that does not activate the transduction system, i.e. an IL-21 antagonist, and the mutated protein was fused with the constant region of murine IgG2a. IL-21 antagonist was generated with mutation of glutamine residues at the C-terminus of the 4-helix structures of IL-21, this mutant has the same binding affinity to the IL-21R chain but that does not activate the transduction system, and the mutated protein was fused with the constant region of murine IgG2a. *In vivo*, the Fc portion of this fusion protein confers longevity and is designed to target IL-21R-bearing cells such as activated T cells, DCs and synoviocytes. Big evidence suggesting that activated T cells play an important part in the progression of MS, we hypothesized that inactivation of IL-21R$^+$ cells might have a beneficial effect in the treatment of this disease.

OBJECTIVES

Given the implication of IL-21 the acute inflammation, we examined the effect of IL-21 antagonist on the development of EAE.

Opposite effects of IL-21 inhibition by a novel receptor antagonist in two murine models of autoimmune disease

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Abstract

IL-21 is a key T-cell growth factor (TCGF) involved in both innate and adaptive immune responses and it contributes to the proliferation of naive, but not memory, T lymphocytes. However, the full spectrum of its activity on T cells remains unclear, the development of biological agents targeting the receptor and signaling elements of IL-21 may open a new perspective in the treatment of pathologies such as autoimmune diseases which could be associated with an increased expression of IL-21.

We have developed a new human IL-21 mutant Fcγ2a fusion protein, referred to as IL-21 antagonist. This IL-21 antagonist blocks specifically the proliferation of human T lymphocytes induced by IL-21. In further tests targeting IL-21R *in vivo*, the effects of human IL-21 antagonist were assessed in terms of prevention and treatment of experimental autoimmune encephalitis (EAE). Upon immunization with MOG, mice developed severe inflammation and limb paralysis. Treatment with this IL-21 antagonist at the time of MOG challenge markedly inhibited the incidence, severity and progression of clinical symptoms as compared to control animals. We observed however that IL-21 antagonist had a dual effect in that it enhanced both inflammation and T-cell response in a murine model of Th1 contact hypersensitivity.

These findings confirm conclusively that IL-21 plays a part in innate and adaptive immune response. Targeting of IL-21 could become a new means of inhibiting and preventing autoimmunity, but its mode of action should be carefully evaluated as it may enhance inflammation like it did in the model of contact hypersensitivity.

Key words: Cytokines; Cytokine antagonist; IL-21; Autoimmunity

Abbreviations: TCGF, T-cell growth factor; rIL-21, recombinant interleukin-21; EAE, experimental autoimmune encephalitis; DTH, delayed-type hypersensitivity.

1. Introduction

The primordial goal in designing new therapies for autoimmune diseases or transplantation is the induction and maintenance of a state of specific immunologic tolerance. In both situations, $CD4^+$ T cells play a crucial part in initiating the immune response that ultimately leads to effector mechanisms mediating autoimmune disease or allograft rejection. Of the large number of cytokines released during the onset of the immune response, TCGFs such as interleukin-2 (IL-2), IL-15 and IL-21 play major and distinct parts in T-cell activation. Like IL-2, IL-21 is produced exclusively by activated $CD4^+$ T cells [1, 2] and NK-T cells [3]. IL-21 may be involved in hematopoietic cell differentiation, proliferation of B cells or natural killer cells [4] and co-stimulation of naive, but not memory, T cells, suggesting that IL-21 may have an autocrine/paracrine role in regulating the immune response [2, 5]. IL-21 activity is mediated by the IL-21 receptor which consists of a single IL-21R chain which is related to the IL-2Rβ chain, the IL-4Rα and the common γ chain [1]. IL-21R expression was first thought to be restricted to lymphoid tissues, i.e. T cells, B cells and NK cells, but recent reports characterized the expression of IL-21R on the surface of myeloid DCs [6], extending its occurrence to non-lymphoid tissues and other tissues such as epithelium, synovium, or transformed cells that may become capable of expressing IL-21R [7-9]. In *IL-21r*$^{-/-}$ mice the number of $CD4^+$ T cells proved normal in both thymus and peripheral lymphoid organ [1]. Although differentiation experiments *in vitro* using WT *vs*. IL-21R KO $CD4^+$ T cells did not reveal any differences in terms of Th1 *vs*. Th2 polarization [5], the capacity of IL-21R KO $CD4^+$ T cells for differentiation into Th17 cells proved to be reduced [10, 11]. Several studies of animal models suggest that IL-21 might play a role in autoimmunity, increased IL-21 levels being found in two mouse models of systemic lupus erythematosus [12, 13]. In addition, progression of EAE disease in IL-21 and IL-21R KO mice slowed down considerably owing to a dramatic reduction in Th17 cells [10, 14]. Finally, King et al. demonstrated that IL-21 may play a major part in the development of diabetes in NOD mice due to an increase in T-cell proliferation and turnover coupled with the absence of survival signals [15]. It thus appears that in mice IL-21 may contribute to the development of unstable, self-destructive T cells implicated in the pathogenesis of autoimmune disease [15].

A better understanding of the mechanisms underlying the biological activity of T cells is a fundamental step towards the development of specific molecules with improved

pharmacological and biological efficacy in treating T-cell-dependent autoimmune disorders. Based on the sequence homology between IL-21, IL-2 and IL-15, we achieved mutation of glutamine residues at the C-terminus of the 4-helix structures of IL-21, resulting in a mutant with the same binding affinity to the IL-21R chain but without activating the transduction system, i.e. an IL-21 antagonist [16-18], and the mutated protein was fused with the constant region of murine IgG2a. *In vivo*, the Fc portion of this fusion protein confers longevity and is designed to target IL-21R-bearing cells such as activated T cells, DCs and synoviocytes. This IL-21 antagonist should be able to bind the IL-21 receptor with high affinity without triggering the downstream signaling pathways.

In view of the large body of evidence suggesting that activated T cells play an important part in the progression of multiple sclerosis, we hypothesized that inactivation of IL-21R$^+$ cells might have a beneficial effect in the treatment of this disease. In the present study, using the murine model of experimental autoimmune encephalitis (EAE), we examined the effect of this novel IL-21 antagonist on the development of encephalitis. Because IL-21 has also been implicated in acute inflammation [19] we also determined the effect the IL-21 antagonist in a model of delayed-type hypersensitivity (DTH).

2. Materials and methods

2.1 Reagents and cytokines

RPMI-1640 medium, fetal calf serum (FCS), phorbol myristate acetate (PMA) and β-mercaptoethanol (β-ME) were purchased from Sigma Chemicals (St. Louis, MO). Phosphate-buffered saline (PBS), penicillin/streptomycin, L-glutamine, MEM non-essential amino-acids, sodium pyruvate and Zeocin were obtained from Gibco, Invitrogen (San Diego, CA). Human AB serum was provided by the Blood Bank of the University Hospital (Geneva, Switzerland). Ficoll-Paque^{TM} Plus was from Amersham Biosciences (Uppsala, Sweden). Serum-free Ultraculture media were purchased from BioWhittaker, Inc. (Walkerville, MD). Human recombinant IL-2 (rhIL-2) was obtained from Biogen, Inc. (Cambridge, MA), human recombinant IL-21 (rhIL-21) was a gift from Dr. D.C. Foster, Zymogenetics (Seattle, WA), murine recombinant IL-21 (rmIL-21) and murine IL-21R/Fc were obtained from R&D Systems, Inc. (Minneapolis, MN).

2.2 Genetic construction of an IL-21 antagonist (IL-21dm)

Human IL-21 cDNA was generated from mRNA extracted directly from PMA-stimulated human T cells. Murine IgG2a cDNA was generated, as previously described [16], using reverse-transcriptase Superscript II (Invitrogen) and synthetic oligo(dT) oligonucleotides (Promega). The IL-21 mutant cDNAs were designed to target selected glutamine residues (Q113 and Q118) within the 4^{th} α helix of human IL-21 to undergo mutation to aspartic acid *via* site-directed and PCR-assisted mutagenesis. For the construction of mutant plasmids, a 393-bp cDNA fragment encoding mature human IL-21 with relevant mutations at positions 114 and 119 was amplified by PCR utilizing synthetic sense: 5'-GTACGAAGCTTGCAAGATCGCCACATGATTAGAATGCGT-3' (*Hind*III site plus bases) and antisense oligonucleotides corresponding to the C-terminal fragment of human IL-21, followed by a *Bam*HI site (the mutated codons are underlined and in bold script): 5'*GGGATCC*CGAATCTTCACTTCCGTGTGTTCTAGAGGACAGATG**GTC**ATGAA TCATCTT**GTC**GAG-3'. This mutant IL-21 was then genetically linked to the constant region of murine IgG2a and cloned into the expressing vector pSecTagA (Invitrogen) [16]. Cos cells (American Type Culture Collection, Manassas, VA) were stably transfected with the expressing vector that carries the construct encoding the IL-21 mutant fusion protein (or IL-21 antagonist**)**. The transfected cells were cloned and cultured in serum-free Ultraculture media containing 100 μg/ml Zeocin. IL-21 protein from the culture supernatant was purified by protein A affinity chromatography (Pharmacia) and again by dialysis against PBS and 0.22 μm-filter sterilization and stored at -20^0C until use.

2.3 Isolation of human $CD4^+$ T lymphocytes and cultures

Peripheral blood mononuclear cells (PBMC) were isolated from normal young donors by density-gradient centrifugation. T cells were separated from PBMC by negative selection with Dynabeads Panmouse IgG (Dynal Biotech, Oslo, Norway) and a cocktail of antibodies to CD16, CD19, CD14 and CD8 for selection of $CD4^+$ T cells (Dako, Copenhagen, Denmark). After negative selection, cells were washed with PBS and T cells stained with PE-conjugated mAb to CD45RO and FITC-conjugated mAb CD4 (Dako). $CD45RA^+$ (naive) and $CD45RO^+$ (memory) $CD4^+$ T cells were subsequently isolated on a FACSvantage® sorter (BD PharMingen™ San Diego, CA). The selected

$CD4^+$ cells were cultured for up to 3 weeks in RPMI medium supplemented with 10% heat-inactivated (HI) FCS, 5% HI serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 1% MEM non-essential amino-acids and 0.1 mM sodium pyruvate, 5 mM βME (at $5x10^{-5}$M) (referred to as medium), autologous feeder cells irradiated at 3500 rad, and rIL-2 (10-50 ng/ml) or rIL-21 (10-50 ng/ml) in 5% CO_2-air humidified atmosphere at 37°C.

2.4 Western blot analysis

Size and specificity of purified IL-21 antagonist were validated by SDS-PAGE gel under reducing (+βME) and non-reducing (-βME) conditions, followed by Western blot analysis using polyclonal goat anti-human IL-21 (N-20) (Biotechnology, Inc., Santa Cruz, CA) and then by donkey anti-goat IgG-PER (Binding Site, Birmingham, UK) and detected by autoradiography using ECL^+ (Amersham Biosciences).

$CD45RA^+$ T lymphocytes were purified as described above and cultured for 7 days with IL-2 (25 ng/ml). The cells were then starved overnight at 37°C in RPMI medium supplemented with 1% heat-inactivated FCS. Cells were harvested and resuspended at 4 x 10^6 cells/ml in RPMI medium supplemented with 1% heat-inactivated FCS, and 500 µl was placed in 2-ml polypropylene tubes (Eppendorf) at 37°C. After 1 h, cells were stimulated with IL-2 (25 ng/ml), IL-21 (25 ng/ml) and/or IL-21 antagonist. After the indicated time of incubation, the reaction was stopped by the addition of 800 µl of ice-cold PBS and centrifugation. Total cell lysate was prepared and subjected to Western blot analysis [20]. The blots were probed with anti-phospho Tyr^{701} STAT1, anti-STAT3, anti-phospho Tyr^{703} STAT3, and anti-phospho Ser^{473} Akt (Upstate Biotechnology, Lake Placid, NY). Secondary HRP-conjugated goat anti-rabbit Abs were from Dako. Ab-bound proteins were detected by Uptilight hrp blot chemiluminescence substrate (Uptima).

2.5 Cell staining for flow cytometry

The following mouse anti-human mAbs and isotype-matched control mAbs were purchased from BD PharMingen™: Cy-chrome-conjugated anti-CD4, PE-conjugated anti-CD45RO, FITC-conjugated anti-CD45RO, FITC-conjugated anti-Annexin V, PE-conjugated PI and isotype controls. The following mouse anti-human mAbs and isotype-matched control mAbs were obtained from Dako: FITC-conjugated anti-CD4,

PE-conjugated anti-CD4, Cy-chrome-conjugated anti-CD45RA and irrelevant, isotype-matched controls. IL-21R was detected using the IL-21 antagonist (based on the detection method used for IL-15 mutant/Fcγ2a fusion protein or IL-15 antagonist) [16], followed by staining with FITC-conjugated goat anti-mouse IgG2a. Three-color immunofluorescence was performed to assess surface marker expression on T cells activated by IL-2 or IL-21. $CD4^+$ T lymphocytes cultured for up to 14 days were washed twice with PBS (completed with 2% serum AB and 1‰ NaN_3) and treated successively with FITC-, Cy-Chrome- and/or PE-conjugated mAbs on ice for 30 min, and washed with completed PBS. Cell staining was analyzed using FACSCalibur and Cell Quest software (BD PharMingen).

2.6 CFSE labeling and analysis of T-cell proliferation in vitro

$CD3^+$ T cells or $CD4^+$ T cells were isolated as above. The selected $CD4^+$ cells were labeled with fluorochrome 5-carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probe, Inc., Portland, OR), as described previously [21]. After staining, cells were plated for an in vitro assay as described below.

2.7 T-cell proliferation assay in vitro

$CD4^+$ T lymphocytes isolated as above were used for this purpose. Cells were plated at $25x10^5$ cells/well in U-bottom 96-well plates in enriched RPMI 1640 medium (as above), with autologous feeder cells and various concentrations of rhIL-2, rhIL-21 and the IL-21 antagonist. Cells were incubated in plates at 37°C for 72 h, followed by the addition of 1 μCi of (^{3}H) TdR (Perkin Elmer Life Sciences, Inc., Schwerzenbach, Switzerland) for an additional 12 h. Cells were then harvested, (^{3}H) TdR incorporation per well was measured, and the means assessed for each triplicate.

2.8 Specific T-cell proliferation assay in vitro

$CD4^+$ T lymphocytes from untreated mice and those treated with the IL-21 antagonist were isolated 15 - 20 days post immunization. T cells were plated at 25 x 10^5 cells/well in U-bottom 96-well plates in enriched DMEM medium (as above), with various concentrations of MOG (ranging between 1 and 50 ng/ml).

For specific T-cell proliferation, first feeder cells were isolated from the spleen of C57Bl/6 mice and incubated in 96-well plates for 24 h with MOG at 10 ng/ml. After 24

h, feeder cells were irradiated in the plates and $CD4^+$ T lymphocytes from 2d2 (C57BL/6 background) mice isolated as above were added at a concentration of 25 x 10^5 cells/well to the irradiated feeder cells for 72 h with rhIL-2, rhIL-21 and the IL-21 antagonist. In both experiments, cells were incubated at 37°C for 72 h before addition of 1 μCi of (^{3}H) TdR for 12 more hours. Cells were then harvested, (^{3}H) TdR incorporation per well was measured and the means assessed for each triplicate.

2.9 Contact hypersensitivity (DTH) in mice

C57BL/6 mice were sensitized by injecting hapten (TNBC) into one ear. Five days after sensitization, mice were treated with PBS (100μl/d), murine IgG2a (5 μg/d), recombinant murine IL-21 (5 μg/d) or IL-21 antagonist (5 μg/d) for 3 days. The same day, mice were challenged with TNBC (Sigma Chemicals) and local inflammation was measured using a thickness gauge (the controlateral ear was used as control). To challenge T cells of the Th_2 subset, another hapten was chosen: C57BL/6 mice were sensitized by injecting fluorescein isothiocyanate isomer I (FITC) (Sigma Chemicals) into one ear [22]. Three days after sensitization, mice were treated with PBS (100 μl/d), murine IgG2a (5 μg/d), recombinant murine IL-21 (5 μg/d) or IL-21 antagonist (5 μg/d) for 5 days. Five days after immunization, mice were challenged with FITC, and local inflammation was measured using a thickness gauge (the controlateral ear was used as control). Infiltration by mononuclear cells was determined by histopathology and immunohistochemistry.

2.10 Histopathology and immunohistology

Both ears were removed from mice 12 - 24 h post challenge and embedded in OCT compound (Tissue TCK, Miles Scientific, Elkhart, IN). Cryostat sections of the ears (n = 3/group) were fixed in paraformaldehyde-lysine-periodate for analysis of leukocyte antigens and stained by 4-layer peroxidase-antiperoxidase (PAP) method involving overnight incubation with mAb, followed by mouse Ig-absorbed goat anti-rat Ig, rabbit anti-goat Ig, goat PAP complexes and diaminobenzidine substrate. Rat anti-mouse monoclonal antibodies (mAbs) and isotype-matched control mAbs, purchased from Pharmingen (San Francisco, CA), consisted of mAbs to $CD4^+$ (H129.19) and $CD8^+$ (53-6.7) T cells. Sections were counterstained in hematoxylin and mounted. Isotype-matched mAbs and control Ab were analyzed for endogenous peroxidase activity in

each experiment. Samples were assigned a random number and processed and evaluated under blind conditions; each sample was evaluated at 2 - 3 different levels of sectioning.

2.11 Experimental autoimmune encephalitis

C57BL/6 mice were administered MOG to induce EAE. The mice were supplied by the animal core facility of our institution (Centre Médical Universitaire, Faculty of Medicine, Geneva). Wild-type C57BL/6 mice matched by age and gender were used as controls. Immunization was performed with $MOG_{aa35-55}$ peptide. MOG at 100μg/animal was emulsified in complete Freund's adjuvant (CFA) and immunization was carried out subcutaneously on the left flank (50 μl/animal) once at day 0. Pertussis toxin (PT) (300 ng) was injected i.v. (tail vein) at days 0 and 2. PT is required for this model to ensure that the blood-brain barrier is open and T-cell response is activated, prerequisite to EAE induction. The first examination consisted of a clinical assessment of the symptoms (occurrence of first symptoms, time after which disease reaches its peak). Mice were examined every other day to monitor the disease onset and the progression of clinical symptoms. Clinical symptoms were graded as follows: 0: absence of paralysis; 1: loss of tail tone; 2: weakness of hind limb; 3: paralysis of hind limb; 4: paralysis of both hind limbs and fore limbs; 5: moribund or dead. In untreated mice, clinical onset appears 8 - 15 days after MOG immunization and reach the peak disease (clinical score 3-5) between 20 - 30 days post immunization, after which the chronic phase set in. To determine the role of the IL-21/IL-21R pathway in EAE, the IL-21 antagonist was tested in a series of experiments. The preventive effect of the antagonist was assessed by injecting daily 1.5 - 5 μg i.p. of the IL-21 antagonist at (treatment protocol derived from previous experiments performed with the IL-15 antagonist, CRB-15, in two models of transplantation and in a model of collagen-induced arthritis) for 7 days before the induction of EAE and pursued until day 30 - 40 post induction. All the animal procedures were approved by the ethical committee of University of Geneva School of Medicine and the State of Geneva Veterinarian Office.

2.12 Cytokine determination

Samples of conditioned media were subjected to enzyme-linked immunosorbent assay (ELISA) for the determination of IFN-γ and TGF-β (R&D). The sensitivity of the assay was 10 to 30 pg/ml. To determine other cytokines a mouse cytokine 20-plex antibody

bead kit (Invitrogen™) was used according to manufacturer's instructions. Data were analyzed on a Bio-plex 200 System, Biorad, Luminex xMAP™ Technology.

2.13 Statistical analysis

Data were analyzed using the ANOVA test, *p <0.05 and **p <0.001 being considered significant (Statview 5.1, SAS Institute Inc., Cary, NC and GraphPad Prism 3.02).

3. Results

3.1 The IL-21 antagonist (IL-21dm) competitively blocks IL-21-dependent T-cell proliferation and does not signal through the IL-21R

Like many other cytokines, IL-21 possesses a very high affinity for its receptor. The strategy for creating an IL-21 antagonist was adapted from our previous experience with the IL-15 antagonist [16, 23, 24]. Based on the sequence homology between IL-21, IL-2 and IL-15, we hypothesized that mutation of glutamine residues at the C-terminus of the 4-helix structures of IL-21 might result in an antagonist similar to the IL-15 antagonist [16]. By PCR-assisted site-directed mutagenesis, we designed primers to target selected glutamine codons of human IL-21 (Q113 and Q118) for mutation into aspartic acid, as described previously [16]. This mutated protein was fused with the Fc part of murine immunoglobulin IgG2a in order to confer prolonged circulating half-life and cytocidal properties. Proliferation of naive ($CD45RA^+$) human $CD4^+$ T lymphocytes induced by IL-21 (25 ng/ml) was completely blocked by addition of the IL-21 antagonist (Figure 1A). In contrast, the IL-2-dependent proliferation of T cells was not inhibited by the IL-21 antagonist (Figure 1A). The IL-21 antagonist also acted on murine $CD4^+$ T cells by inhibiting their IL-21-dependent proliferation very similar to the inhibtion induced by a manufactured antibody against the IL-21 receptor (IL-21R/Fc from R&D Systems) (Fig 1, B).

According to flow-cytometric analysis, the IL-21 antagonist bound to IL-21R expressed on human T lymphocytes (Fig. 1, C). The specificity of the IL-21 antagonist for the IL-21 binding sites was established by an experiment in which the binding of the IL-21 antagonist to the target cells was blocked by addition of a molar excess of rhIL-21 (Fig. 1, C left panel), but not by a molar excess of rhIL-15 (Fig. 1, C right panel), as shown by the shift of the red histogram to the black histogram.

Signaling through IL-21R induces phosphorylation of STAT1 and STAT3 which are crucial to IL-21 activity on targeted cells. Phosphorylation of STAT1 and STAT3 enables their homodimerization, converting them into an active form. To determine whether the IL-21 antagonist would specifically antagonize T-cell response to IL-21 through cytokine signaling, we analyzed STAT1 and STAT3 phosphorylation. Human $CD4^+CD45RA^+$ T cells, sorted as described in Materials and Methods, were cultured with IL-2 (25ng/ml) for 2 weeks in order to obtain sufficient amounts of cells. Before the experiment, cells were washed twice with PBS and cultured overnight in culture

medium without cytokines. The day after, cells were washed twice with PBS, counted and plated at 3 x 10^6 cell/ml in culture medium with 1% FCS at 37°C for 1 h. After that resting period, stimuli were added for 7 to 15 min and cells were processed for intracellular protein isolation.

Western blots of cells isolated from control cultures did not reveal any basal STAT1 or STAT3 phosphorylation (Fig. 1, D), whereas cells isolated from IL-21 cultures showed high levels of both STAT1 and STAT3 phosphorylation at 7 and 15 min, similar to the amounts obtained after IL-2 activation. To determine whether the IL-21 antagonist would activate STAT1 and STAT3 in the same cell culture, we stimulated T cells with the IL-21 antagonist alone or in the presence of IL-21, and under this condition T cells did not show undetectable or very low level of STAT phosphorylation. This demonstrates that the IL-21 antagonist does not induce phosphorylation of STAT in T cells and is also able to inhibit IL-21 signaling.

3.2 IL-21 antagonist (IL-21dm) exacerbates DTH response in mice

To ascertain whether treatment with IL-21 antagonist block T-cell-dependent response to antigen *in vivo*, delayed-type hypersensitivity (DTH) responses were assessed. After initial immunization with TNBC, mice were treated with either rmIL-21, IL-21 antagonist (IL21dm) or mIgG2a, referred to as control, and the injections started a few hours before the challenge with TNBC. As shown in Figure 2A, mice treated with control mouse IgG developed an inflammation of the challenged ear. Treatment with rmIL-21 did not attenuate the DTH response and, interestingly, treatment with the IL-21 antagonist exacerbated the inflammatory response to TNBC agent at 24 and 48 hours. This increase in inflammation of the challenged ear was associated with an influx of inflammatory cells consisting of mononuclear cells (Fig. 2, B). A significant thickening of dermis and epidermis was observed in mice treated with the IL-21 antagonist, accompanied by cell infiltrates (Fig. 2, B). DTH is primarily mediated by alloantigen-specific $CD4^+$ Th1 cells. To test the effect of the IL-21 antagonist on a Th2 model of contact hypersensitivity, we used FITC hapten known to induce a Th2-type response. After FITC immunization and challenge, ear swelling was observed in mice treated with control IgG. Once again, treatment with rmIL-21 did not attenuate the DTH response while treatment with the IL-21 antagonist neither exacerbated nor attenuated the inflammatory response to FITC (Fig. 2, C). Histopathological analyses of skin specimens from FITC-challenged mouse ears over a time period of 72 h after challenge

also revealed a significant infiltration of mononuclear cells in the challenged ears of mice treated with control IgG, rmIL-21 or IL-21 antagonist (Fig. 2, D). The number of $CD4^+$ T cells in the infiltrates were similar under all treatment conditions (Fig. 2, D) but interestingly tissue infiltration by $CD8^+$ T cells was decreased in mice treated with IL-21 antagonist without edema decrease.

3.4 Treatment with IL-21 antagonist (IL-21dm) decreased the incidence and severity of EAE in mice

After initial immunization and subsequent challenge with MOG peptide, an increased number (40 %) of C57B1/6 mice treated with IgG2a developed a severe progressive form of encephalitis (Fig. 3). Those that were affected exhibited decreased mobility of the limbs, eventually resulting in restricted mobility and paralysis matched by a poor clinical score (Fig. 3). Treatment with 5 μg of IL-21 antagonist for 29 consecutive days, starting with the day of challenge with MOG, resulted in a marked decrease in the incidence of encephalitis, with 69% of the treated mice being free of clinical signs by the end of the observation period compared to only 45% of control IgG2a-treated mice being free of clinical signs ($p < 0.01$; Fig. 3). Moreover, the severity of disease was attenuated in mice treated with IL-21 antagonist in comparison to the controls. Interestingly, by the end of treatment on day 29 both progression and severity of disease were significantly less marked in the 12 animals (12 out of 38) that had developed the disease after treatment with IL-21 antagonist than in the controls (21 out of 38) affected by the disease (disease severity in IL-21 antagonist-treated animals: 2.45 ± 0.37 vs. IgG2a-treated animals: 4.39 ± 0.49; $p<0.01$).

3.5 Treatment with IL-21 antagonist (IL-21dm) did not modify the phenotype of Th17 and Treg cells in animals with established disease

The finding that *in vivo* the addition of IL-21 antagonist reduced the development of EAE prompted us to address the question as to how targeting IL-21R affected the T-cell phenotype during EAE disease. To this end we analyzed the effects of treatment with IL-21 mutant protein on Th17 and T regulatory cells both of which are involved in the pathogenesis of the disease. Mice immunized and challenged with MOG and treated for 15 days with either IL-21 antagonist (5μg/day) or control IgG2a (5μg/day) were sacrificed and $CD3^+$ T cells isolated from spleen and lymph nodes. According to FACS

analysis, there was no significant difference in IL-17 production by $CD3^+$ T cells in spleen and lymph nodes 15 days post-immunization in mice treated with IL-21 antagonist as compared to those treated with IgG2a (Fig. 4, A). In addition, for regulatory T cells, as assessed by the expression of Foxp3 in $CD4^+$ $CD25^+$ T cells, IL-21 antagonist did not lead to a significant expression of $Foxp3^+$ $CD4^+$ $CD25^+$ T cells of both spleen and lymph nodes (Fig. 4, B). The absence of modulation of $CD4^+$ $CD25^+$ T regulatory cells may be related to the absence of IL-10 expression and any difference in production of TGF-β (Fig. 4, C and D).

3.6 Effect of IL-21 antagonist on T-cell responses to MOG ex vivo and in vitro

Since IL-21 is a well-known factor in $CD4^+$ T-cell activation and survival, the inhibitory effects of the IL-21 antagonist on T-cell responses to MOG were analyzed both *ex vivo* and *in vitro*. The first set of experiments consisted of isolating peripheral lymph node cells from C57Bl/6 mice immunized and challenged with MOG and treated with either IL-21 antagonist (5μg/day) or control IgG2a (5μg/day) for 15 days (peak of the disease). The purified $CD3^+$ T cells were cultured in the presence of increasing doses of MOG. As shown in Figure 5A, T cells from animals treated with IgG2a proliferated well in response to MOG, but in contrast, the proliferation of $CD3^+$ T cells from animals treated with IL-21 antagonist was significantly reduced. To further assess the effect of the IL-21 antagonist on T-cell proliferation, we labeled isolated $CD3^+$ T cells with CFSE and confirmed that treatment with the IL-21 antagonist decreases the proliferation of $CD3^+$ T cells (11% versus 20% as compared to IgG2a) (Fig. 5, B). The production of IFN-γ is markedly lower in mice treated with IL-21 antagonist than with IgG2a (Fig. 5, C). The production of other cytokines such as IL-17, IL-4, IL-6, TNF-α, and IL-2 was also determined, and as shown in Figure 5D, the expression of IL-17, IFN-γ, TNF-α and IL-2 was much higher in T cells of mice treated with IgG2a than in those that were administered IL-21 antagonist. Of note, $CD3^+$ T cells from animals treated with IgG2a or IL-21 antagonist produced decidedly similar levels of IL-17, IFN-γ, TNF-α and IL-2 when stimulated with α-CD3 together with α-CD28 beads (data not shown). The overall reduced severity of disease in the animals treated with IL-21 antagonist (see Figure 3) was accompanied by a reduced overall expression of the inflammatory cytokines IFN-γ, IL-17, TNF-α and IL-2 in T cells from the above animals.

In a second set of experiments, MOG-specific T-cell proliferation was also assessed. Spleen cells from C57Bl/6 mice were isolated and cultured for 24 h in 96-well plates with 10 ng/ml of MOG. After 24 h, spleen cells were irradiated and used as feeder cells. Spleen and peripheral lymph node cells from 2d2 mice were isolated, and $CD4^+$ T cells were purified and cultured for 72 h in the presence of rIL-21, or IL-21 antagonist. Proliferation index and cytokine production were analyzed. As seen in Figure 6A, MOG-specific T cells cultured in the presence of rIL-21 proliferated better in response to MOG than cells without MOG (control) or cells without rIL-21 (T cells). In contrast, the proliferation of MOG-specific T cells cultured in the presence of the manufactured IL-21R/Fc or IL-21 antagonist (IL-21dm), was significantly reduced. *In vitro*, however, inhibition of IL-21 in this blocking experiment, significantly increased the secretion of IL-17, with a slight extent in the production of IFN-γ (Fig. 6, B). As IL-21 inhibits the production of IFN-γ in T cells, it is therefore not surprising that its antagonist will have the opposite effect. Furthermore, these data demonstrate the same effect on IL-17 production. To assess the effect of the IL-21 antagonist on T-cell proliferation and survival, we first labeled isolated $CD4^+$ T cells from 2d2 mice with CFSE and confirmed that, in vitro, the IL-21 antagonist alone does not modify the proliferation of these cells (Fig. 6, C). To determine the effects of IL-21 antagonist on apoptosis, we analyzed the expression of apoptotic markers on isolated $CD4^+$ T cells from 2d2 mice. After 7 days of culture with IL-21, cell viability was above 80% (Fig. 6, D). In the presence of IL-21 antagonist at increasing doses, viability was maintained at 80% as confirmed by the absence of annexin V or PI double staining (Fig. 6, D).

In summary, T-cell response to MOG after treatment of MOG-immunized mice with IL-21 antagonist was significantly reduced both *ex vivo* and *in vitro*. It is therefore obvious that treatment with IL-21 antagonist inhibits the development and progression of EAE and blunts specific proliferating T cells.

4. Discussion

We have developed a novel antagonist to the pro-inflammatory cytokine IL-21, IL-21 antagonist. It inhibited specifically the transducing pathways of IL-21R and reduced the severity of EAE but had an exacerbating effect in a mouse model of Th1-mediated local inflammation. The pleiotrophic actions of IL-21, its production by lymphocytes and the widespread distribution of its receptor on immune and non-immune cells hint at its potential involvement in numerous pathologies, including allergy, autoimmunity and cancer. IL-21 is currently undergoing clinical evaluation as an option for treating solid tumors. In the setting of autoimmune disease, however, a procedure for tempering the effect of IL-21 may pave the way to an improved clinical outcome, especially in diseases that are driven by amplified autoantibody responses like systemic lupus erythematosus, scleroderma, rheumatoid arthritis, or multiple sclerosis. Thus, while administration of IL-21 may be beneficial in one type of pathology - but where blockade of IL-21 signaling would be extremely harmful - it would have a reverse effect in another pathology.

In our study, administration of IL-21 before induction of EAE enhanced inflammation in the CNS as well as severity of EAE. In addition, purified autoreactive T cells from mice treated with IL-21 induced a more severe form of EAE than did control T cells. When IL-21 was administered after the induction of EAE no such effects were observed [25]. We therefore investigated the implications of blocking the IL-21/IL-21R pathway in this model. We found that treatment with IL-21 antagonist not only prevented the development of encephalitis but was also remarkably effective in slowing progression of disease in its chronic phase. Furthermore, we have provided evidence that treatment with IL-21 antagonist decreases the proliferation of antigen-specific T cells and the secretion of IFN-γ, IL-17 and TNF-α proteins *in vivo*. Besides, the fraction of regulatory T cells and Th17 cells involved in the pathogenesis of the disease were not modified by IL-21 antagonist. IFN-γ plays a complex role in the pathology of EAE and MS, as Th1 cell infiltrates promote neuro-inflammation in EAE [26]. IL-21 has also been shown to inhibit the production of IFN-γ by naive Th cells [27], but our data demonstrate that by blocking IL-21 the production of IFN-γ is decreased, pro-inflammatory activities being thus prevented. Similarly, IL-17 plays a major encephalopathogenic role in EAE development since T cells that produce IL-17, i.e. Th17 cells, induce EAE upon adoptive transfer [28] and since in IL-17-deficient mice models EAE induction is delayed [29]. The production of IL-17 by MOG-specific T cells in the presence of the IL-21 antagonist is intriguing considering its beneficial effect on both EAE induction and

clinical score. We believe that in this in vitro system the production of IL-17 is not representative of actual process in vivo and conflicts with the data shown in figure 5C.

In a delayed-type hypersensitivity mouse model we also examined the extent to which the IL-21/IL-21R pathway is involved in the development of another T-cell-dependent pathology. In these experiments, IL-21 did not exacerbate the disease and administration of its antagonist, the IL-21 antagonist, resulted in a marked clinical aggravation of the disease. Blockade of IL-21 was associated with an increased swelling of the ear, increased edema and mononuclear cell infiltration. Thus the IL-21/IL-21R interaction defines a new pathway that contributes to the inflammatory response in contact hypersensitivity. DTH is driven by pathogenic T-cell response and this pathogenicity has been associated with the production of inflammatory cytokines by Th1 cells, including IFN-γ [27]. As IL-21 maintains Th2 cells [27], we also tested a DTH model involving a Th2 response triggered by FITC [22]. The IL-21 antagonist did not reduce the inflammatory process or cell infiltration although the number of $CD8^+$ T cells was reduced. It has been reported that IL-21 alone or in synergy with other potent cytokines induces $CD8^+$ T cell survival and expansion [30-32]. Therefore, administration of the IL-21 antagonist in vivo, during an inflammatory process, might inhibit $CD8^+$ T-cell proliferation and infiltration in inflamed tissues and on the contrary favor the increase in $CD4^+$ T cells or other inflammatory cells. It has also been shown that IL-21 can down-regulate IgE production, implying that IL-21 might lessen the severity of allergy and asthma. Indeed, in a mouse model of allergic rhinitis induced by ovalbumin, administration of IL-21 during the initial antigen challenge significantly reduced allergic symptoms, with diminished levels of both antigen-specific serum IgE and Th2 cytokines (IL-4, IL-5, and IL-13) [33]. In analogy to the local effects of IL-21 in this allergic rhinitis model, systemic administration of IL-21 blocked antigen-induced anaphylaxis in a mouse model of food allergy [34].

In our hands, IL-21 was involved in both cell-mediated and humoral responses and exhibited specific immunosuppressive properties on T cells *in vitro* [35]. *In vivo*, administration of IL-21 increases the severity of experimental autoimmune encephalomyelitis, it is implicated in Crohn's disease and expands T-lymphocyte populations responsible for autoimmune diabetes [15, 25, 36], suggesting that blockade of the IL-21 pathway might be beneficial in these pathologies. In, a model of lupus-prone mice and another mouse model of collagen-induced arthritis the administration of a soluble IL-21R/Fc proved efficient in reducing disease progression [19, 37]. However, the increased inflammation observed in our experiments

suggests that to counteract the immunosuppressive properties of IL-21 might be detrimental in certain pathologies in that administration of IL-21 assists innate and adaptive immunity in tumor clearance [31, 38, 39]. Consequently, in view of the possible detrimental effect of suppressing IL-21 signaling, great caution should be exercised in extrapolating beneficial results from one disease model to another.

Biomedical research pays considerable attention to cytokines and anti-cytokines as they are part of a new arsenal of treatments for a great number of human diseases that involve a dysfunction of the immune system. On the one hand, cytokines are key factors in the dysregulation of pathological processes, but on the other hand they also exhibit therapeutic properties, like anti-tumoral effects in the case of IL-21. To date, these cytokines or their antagonists are either marketed drugs or have undergone advanced testing for an impressive array of indications including cancer, autoimmune diseases, inflammation, allergic asthma and transplantation. The present new data shed new light on the basic mechanisms of IL-21 activity and may give rise to novel strategies and therapies with a view to modulating TCGF activities and inducing non-antigen-dependent tolerance in autoimmune diseases and inflammatory processes. The challenge in the field is to understand how the balance of cytokines and their inhibition is maintained within the normal immune system as well as to properly regulate it in the treatment of inflammatory diseases.

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[1]Footnote:

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Figure Legends

Figure 1: IL-21 antagonist (IL-21dm) competitively inhibits cell proliferation triggered by IL-21 and binds specifically to the IL-21R

A. Naive ($CD4^+$ $CD45RA^+$) human T cells obtained as described in Materials and Methods were cultured with IL-2 (25 ng/ml) or IL-21 (25 ng/ml) in the presence of purified IL-21 antagonist (IL-21dm), IgG2a protein being used as negative control. Cells were cultured for 72 h in plates coated with autologous irradiated feeder cells and pulsed with ^{3}H-thymidine for the final 12 h. Radioactivity incorporated into proliferating cells was measured with a Betaplate^{TM}, Wallac, Perkin Elmer Life Sciences, Inc. (Boston, MA). Values are mean ± SEM of 3 separate experiments. Statistical analyses were performed to determine the differences between cells treated by IL-2 versus IL-21 or by the IL-21 antagonist versus IL-21 alone (*p <0.05). Data were analyzed using the ANOVA test, p <0.05 being considered significant (Statview 5.1).

B. Naive $CD4^+$ murine T cells were cultured with various doses of IL-21 (0 to 100 ng/ml) in the presence of the manufactured IL-21R/Fc or the purified IL-21 antagonist at various doses. Cells were cultured for 72 h in plates coated with autologous irradiated feeder cells and pulsed with ^{3}H-thymidine for the final 12 h. Statistical analyses were performed to determine the differences between cells treated with IL-21 versus those treated with IL-21 plus the IL-21R/Fc or IL-21 plus the IL-21 antagonist (**p <0.001). Data were analyzed using the ANOVA test, p <0.001 being considered significant (Statview 5.1).

C. Binding specificity to the IL-21R on human $CD4^+$ T cells. T cells obtained as previously described were activated with PMA for 24h. Then the cells were cultured with a molar excess of rhIL-21 (2.5 μg/ml) (Figure 1C, left panel) or rhIL-15 (2.5 μg/ml) (Figure 1C, right panel) for 1 h, then washed with PBS and stained with IL-21 antagonist followed by staining with FITC-conjugated goat anti-mouse IgG2a. The binding of IL-21 antagonist to its specific receptor (red lane) was blocked by a molar excess of rhIL-21 (black lane, left panel), but not by a molar excess of rhIL-15 (black lane, right panel). FITC-conjugated goat anti-mouse IgG was used as control (dotted line). Histograms are representative of three separate experiments performed.

D. IL-21 antagonist does not induce signal transduction in $CD4^+$ T cells. Isolated naive ($CD4^+$ $CD45RA^+$) T cells (4 x 10^6 cells/ 500 μl) were stimulated with IL-2 (25 ng/ml), IL-21 (25 ng/ml) or IL-21dm (1μg/ml) for the indicated time. Total cell lysates were analyzed by Western blot, as described in Materials and Methods, with Abs to Tyr^{701}-phosphorylated

STAT1 (PY-STAT1), Tyr^{703}-phosphorylated STAT3 (PY-STAT3), and Ser^{473}-phosphorylated Akt (PY-Akt). Equal loading was ensured by anti-STAT3 mAb. These results are representative of three individual experiments performed.

Figure 2: Exacerbation of DTH response with IL-21 antagonist (IL-21dm) treatment

IL-21 antagonist increased the inflammatory response in a DTH model using wild-type C57Bl/6 mice.

A. Mice treated with PBS, control mouse-IgG2a, and rmIL-21 exhibited similar responses to TNBC challenge, while ear swelling of mice treated with IL-21 antagonist was significantly increased 48 h after challenge. Values are mean ± SEM of 3 separate experiments. Statistical analyses were performed to determine the differences between mice treated with IL-21 antagonist versus control mouse-IgG or rmIL-21 (*$p < 0.05$. Data were analyzed using the ANOVA test, $p < 0.05$ being considered significant (Statview 5.1).

B. Histological staining for the presence of mononuclear cells after 72 h of TNBC challenge in specimens from mice treated with: PBS, control mouse-IgG2a, rmIL-21 and IL-21 antagonist (IL-21dm). (Cryostat sections, hematoxylin counterstain, x 100 magnifications). These are representative sections from 3 mice from each treatment group.

C. Mice treated with PBS, control mouse-IgG2a, rmIL-21 or treated with IL-21 antagonist exhibited similar responses to FITC 48 h after challenge. Values are mean ± SEM of 3 separate experiments. Statistical analyses were performed to determine the differences between mice treated with IL-21 antagonist versus control mouse-IgG (ns). Data were analyzed using the ANOVA test, $p < 0.05$ and $p < 0.0001$ being considered significant (Statview 5.1).

D. Immunohistological staining for the presence of $CD4^+$ and $CD8^+$ T cells 72 h after FITC challenge. The arrows pinpoint $CD4^+$ or $CD8^+$ T cells in specimens from mice treated with: PBS, control mouse-IgG2a, rmIL-21 and IL-21 antagonist. (Cryostat sections, x 100 magnifications). These are representative sections from 3 mice from each treatment group.

Figure 3: Treatment of EAE with IL-21 antagonist (IL-21dm) decreases onset of disease and inflammatory response in an EAE model

Treatment with IL-21 antagonist decreased incidence and severity of EAE. Wild-type C57Bl/6 mice were immunized with MOG and challenged after three days by intraperitoneal re-administration of MOG. On the day of challenge, mice were randomly divided into two groups and treated with either IL-21 antagonist (5 µg/mouse/day) (■) or with control IgG2a (5

µg/mouse/day) (○) for a total of 24 days. Severity of disease (maximum score per animal = 5) was monitored during treatment and for 30 more days after discontinuation of treatment. Treatment with IL-21 antagonist tempered development and progression of disease throughout the treatment period (*, $p < 0.05$). The results shown, that summarize three separate experiments, were obtained from a total of 38 animals for each of two groups, one of which treated with IL-21 antagonist and the other with control substance. Mean scores of disease severity +/- SEM are specified.

Figure 4. Absence of modulation of TH17 and T reg cells with the IL-21 antagonist (dm)

A. C57Bl/6 females were immunized with MOG and treated with IL-21 antagonist or vehicle (IgG2a). Fifteen days after immunization and treatment, spleen cells and lymph node cells were isolated and percentages of CD4+-IL-17+ cells were determined by FACS analysis. Bars represent mean ± SEM of 3 mice/group.

B. C57Bl/6 females were immunized with MOG and treated with IL-21 antagonist or vehicle (IgG2a). At day 15 after immunization and treatment, spleen cells and lymph node cells were isolated and percentages of CD4+ CD25+ FoxP3+ cells determined by FACS analysis. Bars represent mean ± SEM of 3 mice/group.

C-D. CD4+ murine T cells isolated from spleen and lymph nodes of C57Bl/6 females treated as above, were cultured for 48 h and the protein production of IL-10 (C) and TGF-β (D) was determined by multiplex (Multiplex bead-based Luminex®, Invitrogen™) and ELISA, respectively.

Figure 5: Reduced T-cell activation after blockade of the IL-21 pathway

A. C57Bl/6 females were immunized with MOG and treated with IL-21 antagonist or vehicle (IgG2a). After 15 days of immunization and treatment, spleen and lymph nodes of animals treated with IL-21 antagonist and IgG2a (n=4/group) were removed and processed as described in Materials and Methods. $CD3^+$ T cells were isolated from spleen and lymph nodes of animals treated with IL-21 antagonist or IgG2a and cultured in DMEM medium, either left unstimulated or stimulated with MOG (0 to 50 ng/ml) for 72 h. To note that MOG was added to the feeder cells for 24 h before irradiation of feeder cells and subsequent addition of isolated $CD3^+$ T cells. Cells were pulsed with ^{3}H-thymidine for the final 8 h. Radioactivity incorporated into proliferating cells was then measured. Bars represent mean ± SEM of 3 mice/group. Of note, there were no great differences in numbers of total lymphocytes

recovered in the treated mice, and there was no significant difference in percentage of CD4/CD8 cells between the two groups of treated mice and controls.

B. Isolated $CD3^+$ T cells (from the same group of mice as above) were stained with CFSE as described in Materials and Methods and cultured for 6 days in DMEM medium with MOG (10 ng/ml) and IgG2a (5 μg/ml) or the IL-21 antagonist (5 μg/ml) in plates coated with autologous irradiated feeder cells. First panel, α-CD3 plus α-CD28 beads where added as positive control. CFSE-stained cells were collected and stained with Cy-chrome-conjugated anti-CD4. Data represent percent of proliferation of CFSE-labeled $CD4^+$ T cells. The frequency of T-cell proliferation was determined as follows: the total number of cells in each generation of proliferation was calculated and the number of precursors that generated the daughter cells was determined by using the following formula: $y/2^n$ (y = number of cells in each peak, n = number of cell divisions) [21].

C. Treatment with IL-21 antagonist reduced IFN-γ protein production. Supernatant from the above cultured $CD3^+$ T cells (Fig 5A) was collected and analyzed by ELISA for production of IFN-γ. Bars represent mean ± SEM of 3 mice/ group.

D. Decreased pro-inflammatory cytokine production by IL-21 antagonist. Isolated $CD3^+$ T cells from spleen and lymph nodes from EAE mice treated with IL-21 antagonist or IgG2a (n=3/group) for 15 days were left unstimulated or stimulated for 72 h with MOG (10ng/ml). Supernatants were analyzed by multiplex for cytokine production according to manufacturer's instructions (Multiplex bead-based Luminex®, Invitrogen™). Statistical analyses were performed to determine the differences between IgG2a-treated mice and mice treated with IL-21 antagonist (**p <0.001). Data were analyzed using the ANOVA test, *p <0.05 and **p <0.0001 being considered significant (Statview 5.1).

Figure 6: Specific T-cell proliferation inhibited by the IL-21 antagonist (IL-21dm)

A. $CD4^+$ T lymphocytes from 2d2 mice, isolated as described in Materials and Methods, were used for this purpose. First feeder cells were isolated from the spleen of wild-type C57BL/6 mice and cultured at $25x10^5$ cells/well in U-bottom 96-well plates for 24 h with 10 ng/ml of MOG. After 24 h, these feeder cells were irradiated in the plate. Second, T cells from 2d2 mice (C57BL/6 background) were isolated as above and added to the feeder cells at a concentration of $25x10^5$ cells/well. Cells were cultured in enriched DMEM medium (as above) and various concentrations of rhIL-2, rhIL-21 and the IL-21 antagonists. Cells were incubated at 37^oC for 72 h before addition of 1 μCi of (^3H) TdR (for an additional 12 h. Cells

were then harvested, (^{3}H) TdR incorporation per well was measured and the means assessed for each triplicate. These results are the mean (± SEM) of three separate experiments.

B. Cytokine production. In the same culture conditions, supernatant was harvested after 72 h of culture and processed for testing of cytokine production by multiplex analysis. Expression of the inflammatory cytokine IFN-γ and IL-17 was increased in the presence of the IL-21 inhibitors. These results are the mean (± SEM) of three separate experiments.

C. IL-21 antagonist maintains the proliferation and survival of $CD4^{+}$ T cells. $CD4^{+}$ T lymphocytes from 2d2 mice, isolated as described in Materials and Methods, were stained with CFSE and cultured as above (Figure 6A) with MOG-feeder cells at a concentration of $25x10^{5}$ cells/well. Cells were cultured for 6 days in enriched DMEM medium with and without the IL-21 antagonist (5 μg/ml). CFSE-stained cells were collected and stained with Cy-chrome-conjugated anti-CD4. The frequency of T-cell proliferation was determined as shown in figure 5B.

D. To assess apoptotic cell death, purified 2d2 $CD4^{+}$ T cells were cultured for 7 days with IL-2 (25 ng/ml) and/or IL-21 antagonist (2, 5 or 20 μg/ml). At day 7, cells were harvested and stained with FITC-conjugated annexin V and PE-conjugated PI in accordance with supplier's instructions (BD PharMingen™). The percentages of double-negative subpopulations corresponding to viable cells are indicated in the quadrant.

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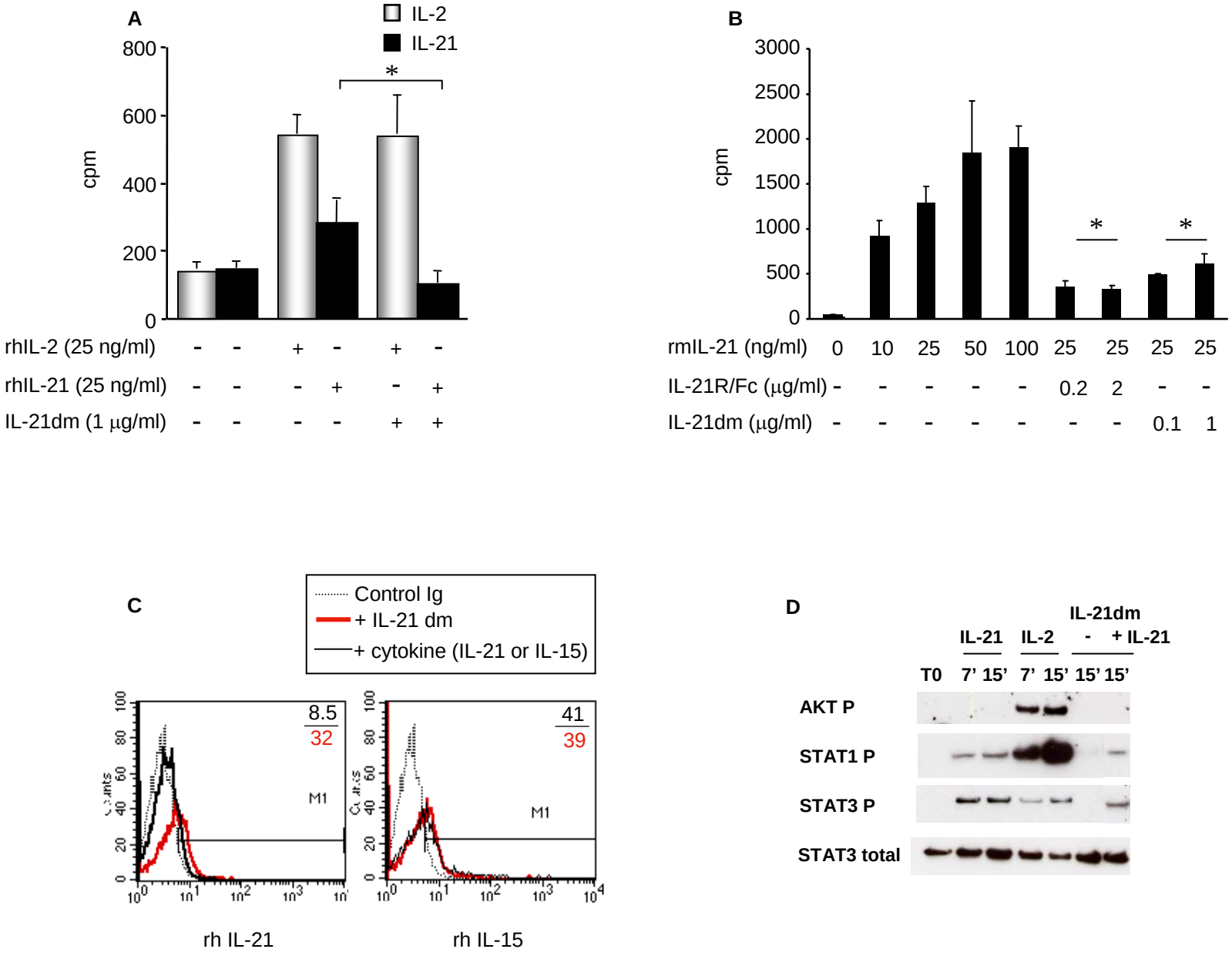
Figure 1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400
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IL-21dm (1 μg/ml) - - - - + +
B
cpm
3000
2500
2000
1500
1000
500
0
*
*
rmIL-21 (ng/ml) 0 10 25 50 100 25 25 25 25
IL-21R/Fc (μg/ml) - - - - - 0.2 2 - -
IL-21dm (μg/ml) - - - - - - - 0.1 1
C
Control Ig
+ IL-21 dm
+ cytokine (IL-21 or IL-15)
8.5
32
M1
41
39
M1
rh IL-21
rh IL-15
D
IL-21dm
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- + IL-21
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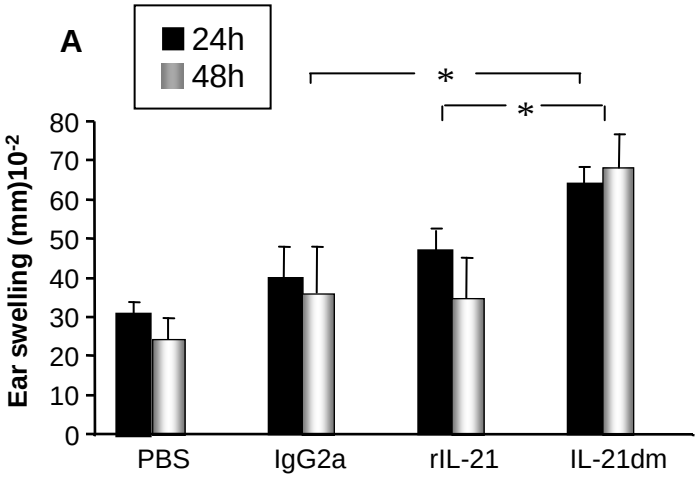
AKT P
STAT1 P
STAT3 P
STAT3 total

Figure 2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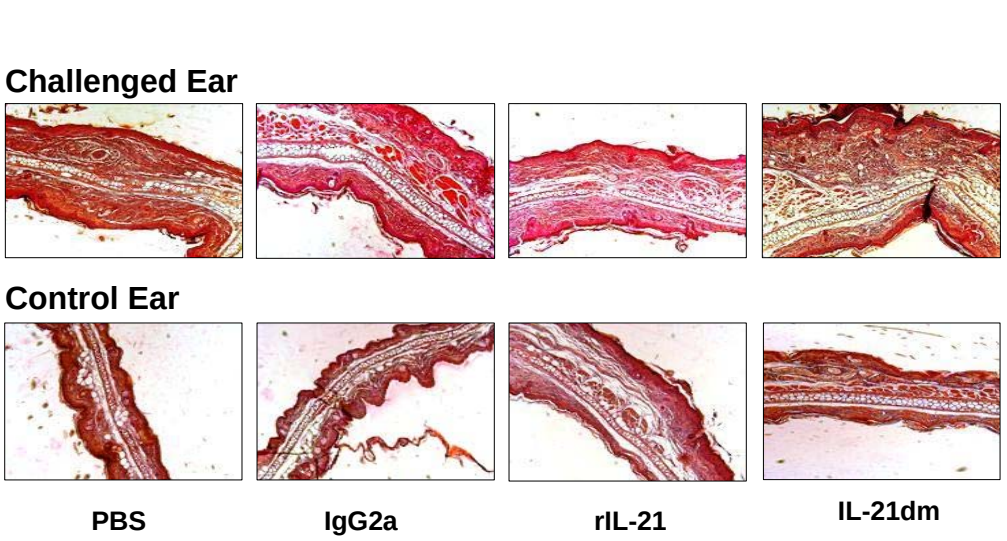
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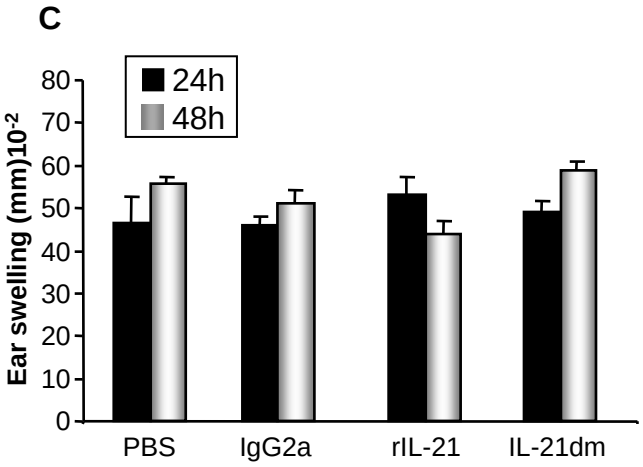
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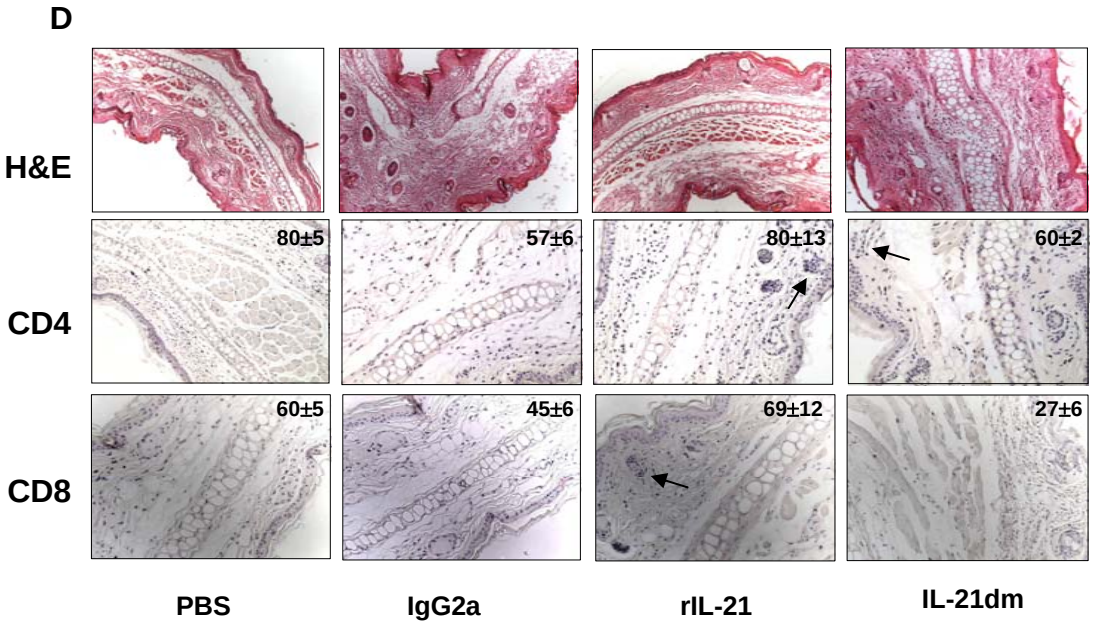
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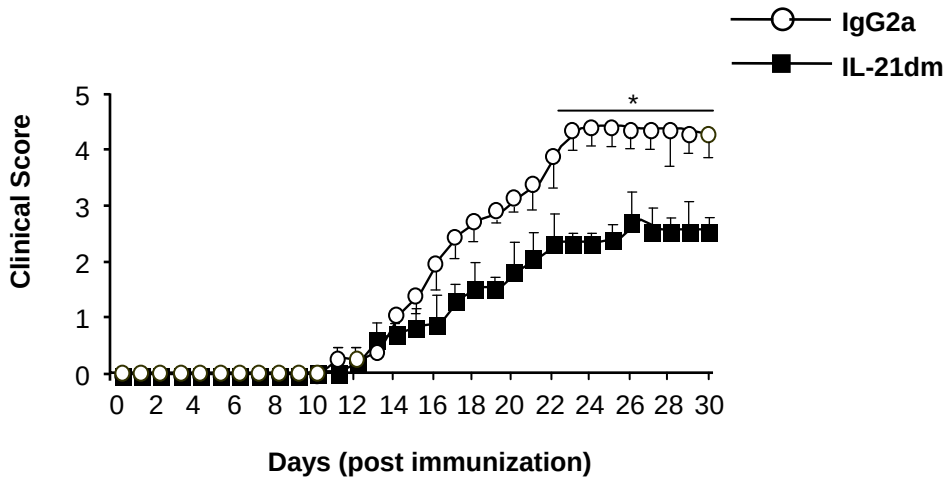
IL-21dm

Figure 3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IL-21dm
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Days (post immunization)
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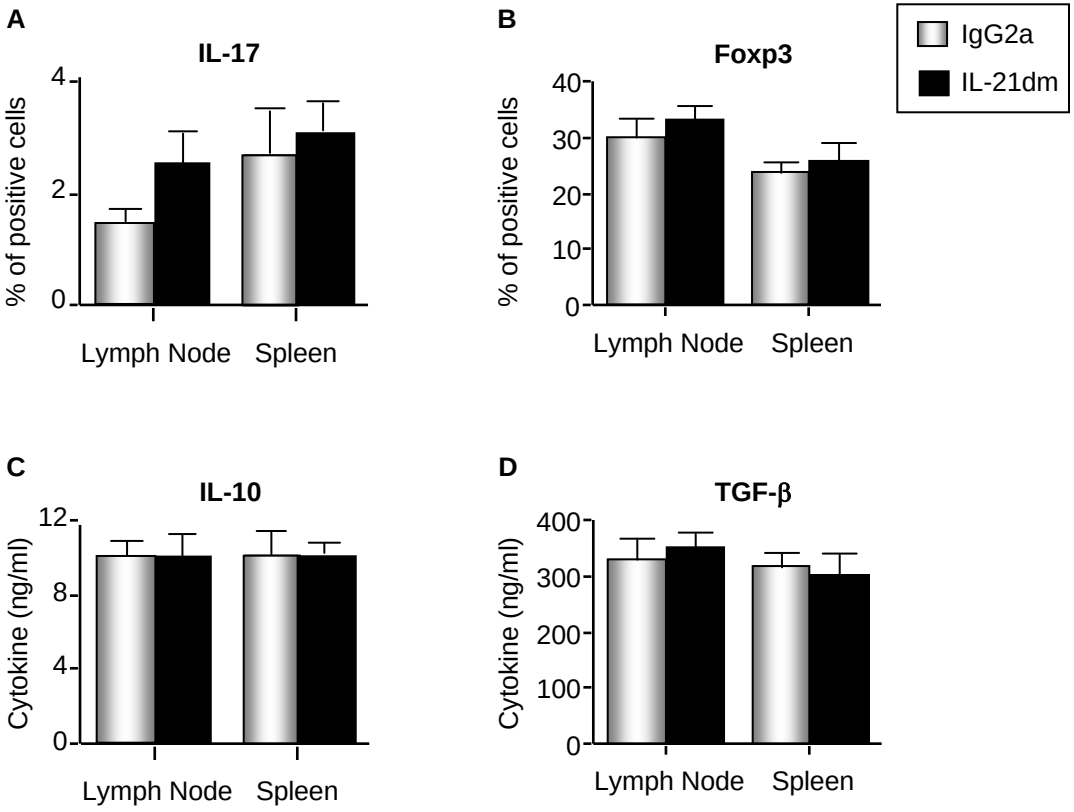
1
2
3
4
5
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30

Figure 4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Lymph Node
Spleen
B
Foxp3
% of positive cells
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20
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0
Lymph Node
Spleen
IgG2a
IL-21dm
C
IL-10
Cytokine (ng/ml)
12
8
4
0
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D
TGF-β
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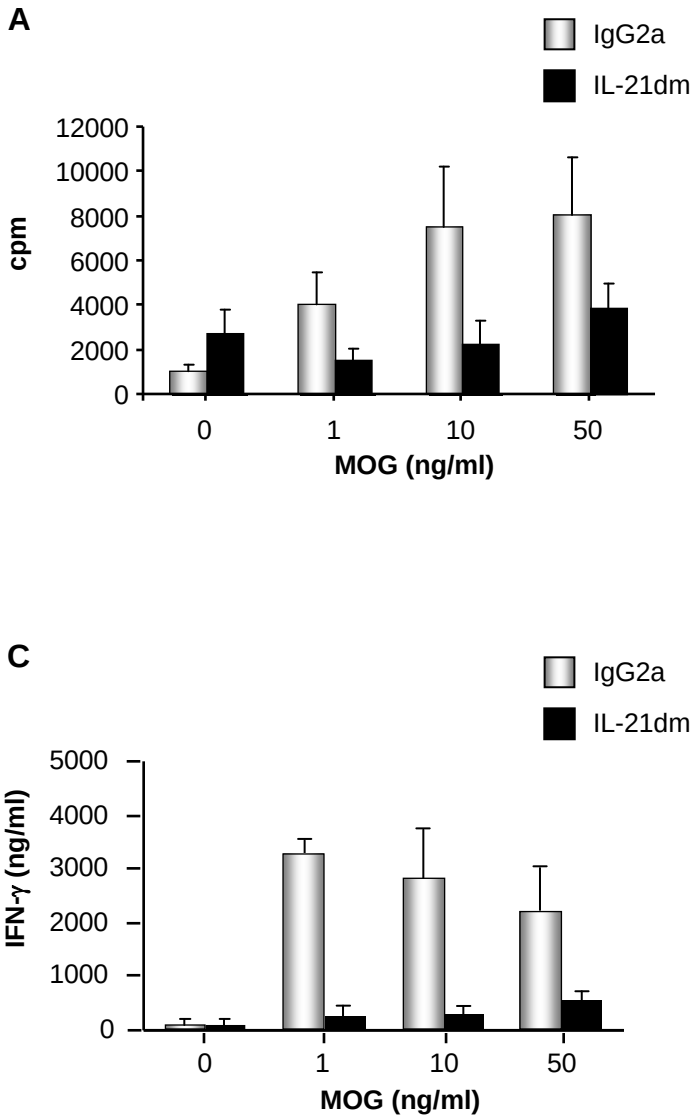
Lymph Node
Spleen

Figure 5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IL-21dm
cpm
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10000
8000
6000
4000
2000
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0
1
10
50
MOG (ng/ml)
B
α-CD3 + α-CD28
57%
IgG2a
20%
IL-21dm
11%
CD4+
CFSE
C
IgG2a
IL-21dm
IFN-γ (ng/ml)
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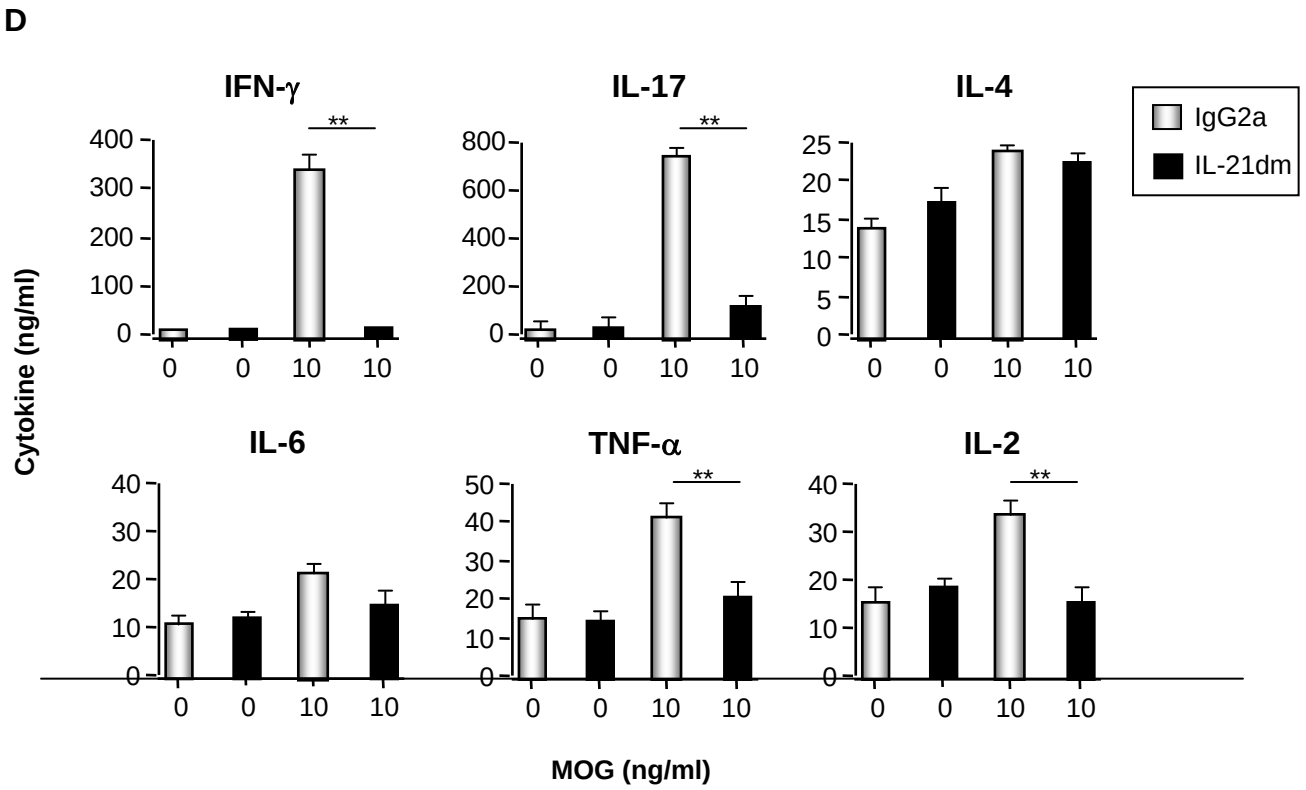
4000
3000
2000
1000
0
0
1
10
50
MOG (ng/ml)

Figure 5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MOG (ng/ml)
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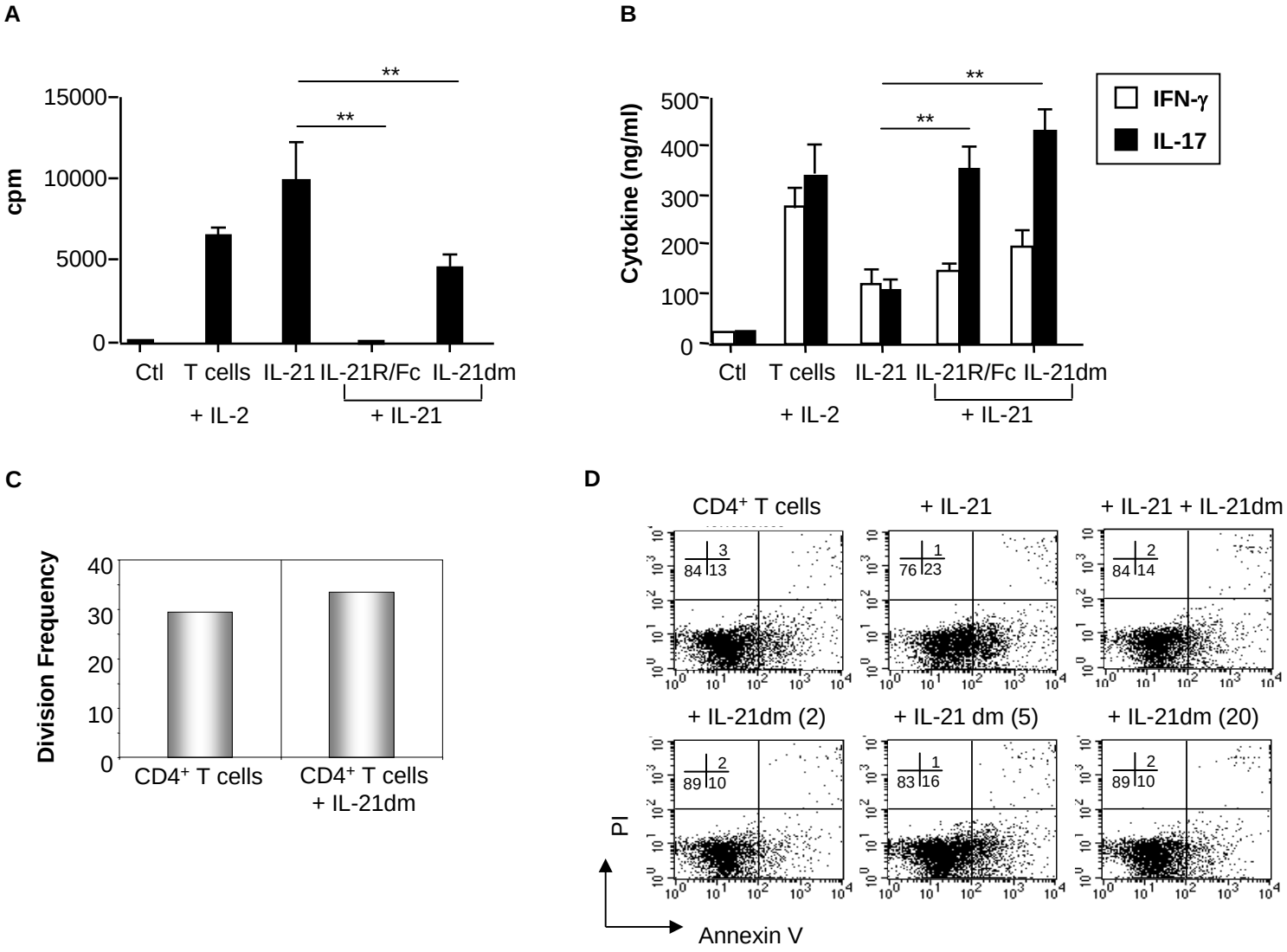
10

Figure 6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10000
5000
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Ctl
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+ IL-2
IL-21
IL-21R/Fc
IL-21dm
+ IL-21
B
Cytokine (ng/ml)
500
400
300
200
100
0
IFN-γ
IL-17
C
Division Frequency
40
30
20
10
0
CD4+ T cells
CD4+ T cells + IL-21dm
D
CD4+ T cells
+ IL-21
+ IL-21 + IL-21dm
+ IL-21dm (2)
+ IL-21 dm (5)
+ IL-21dm (20)
PI
Annexin V

II. C)
Immunomodulatory effects of therapeutic molecules in MS, EAE and in other autoimmune disease

Glatiramer acetate increases IL-1 receptor antagonist but decreases T cell-induced IL-1beta in human monocytes and multiple sclerosis

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INTRODUCTION

Glatiramer acetate (GA) is one approved therapy for RR-MS and has shown to decrease the number of CNS lesions and the frequency of relapses. Recently it has been reported that GA exerts immunomodulatory activity on monocytes/macrophages and dendritic cells. In this study they showed that monocytes from GA treated patients with MS secrete less IL-12 and TNF in response to LPS stimulation compared with monocytes from healthy controls and untreated patients with MS. In addition DCs and monocytes isolated from GA-treated patients produce more anti-inflammatory IL-10 and less pro-inflammatory IL-12. Also GA promotes the development of anti-inflammatory type II monocytes in EAE, with Treg induction and increase of IL-10 and TGF β. IL-1β is a pleiotropic cytokine which is controlled by it natural secreted IL-1 receptor antagonist (IL-1Ra), IL-1Ra -/- mice are highly susceptible to EAE. In this section we were interested to study the effect of GA on the IL-1 system. We showed in this study that GA treatment increased sIL-1Ra blood levels in MS patient and also in EAE mice; however IL-1β levels remain undetectable. These results were confirmed *in vitro.* This study opens more our eyes on a mechanism that is likely to participate in the therapeutic effects of GA in MS.

OBJECTIVES

In this project we investigated the effect of GA on IL-1 system *in vivo* and *in vitro.*

Glatiramer acetate increases IL-1 receptor antagonist but decreases T cell-induced IL-1β in human monocytes and multiple sclerosis

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Mechanisms of action as well as cellular targets of glatiramer acetate (GA) in multiple sclerosis (MS) are still not entirely understood. IL-1β is present in CNS-infiltrating macrophages and microglial cells and is an important mediator of inflammation in experimental autoimmune encephalitis (EAE), the MS animal model. A natural inhibitor of IL-1β, the secreted form of IL-1 receptor antagonist (sIL-1Ra) improves EAE disease course. In this study we examined the effects of GA on the IL-1 system. In vivo, GA treatment enhanced sIL-1Ra blood levels in both EAE mice and patients with MS, whereas IL-1β levels remained undetectable. In vitro, GA per se induced the transcription and production of sIL-1Ra in isolated human monocytes. Furthermore, in T cell contact-activated monocytes, a mechanism relevant to chronic inflammation, GA strongly diminished the expression of IL-1β and enhanced that of sIL-1Ra. This contrasts with the effect of GA in monocytes activated upon acute inflammatory conditions. Indeed, in LPS-activated monocytes, IL-1β and sIL-1Ra production were increased in the presence of GA. These results demonstrate that, in chronic inflammatory conditions, GA enhances circulating sIL-1Ra levels and directly affects monocytes by triggering a bias toward a less inflammatory profile, increasing sIL-1Ra while diminishing IL-1β production. This study sheds light on a mechanism that is likely to participate in the therapeutic effects of GA in MS.

experimental autoimmune encephalitis | cellular contact | inflammation | autoimmune disease

Glatiramer acetate (GA; copolymer-1; Copaxone) is composed of a mixture of synthetic peptides of 50 to 90 aa randomly composed of L-glutamic acid (E), L-lysine (K), L-alanine (A), and L-tyrosine (Y). Initially developed to mimic the myelin basic protein, a major component of the myelin sheath, and to induce experimental autoimmune encephalitis (EAE), GA unexpectedly inhibited EAE in both rodents and monkeys (1). In subsequent clinical trials, GA reduced relapse rate and progression of disability in patients with relapsing–remitting multiple sclerosis (MS; RRMS) leading to its approval in 1995 (2).

A number of investigations in MS and EAE addressed the immunological basis of GA clinical effects. However, the mechanisms of GA action are still elusive. Initial investigations attributed most GA activity to a preferential Th2-polarization of myelin-specific T cells, thus focusing on its effects on the adaptive immune response (3). However, recent reports indicated that GA treatment also exerts immunomodulatory activity on cells of the monocytic lineage, i.e., monocytes/macrophages and dendritic cells (4–9). For instance, monocytes from GA-treated patients with MS secrete less IL-12 and TNF in response to LPS stimulation compared with monocytes from healthy controls and untreated patients with MS (4). Accordingly, dendritic cells and monocytes isolated from GA-treated patients produce more anti-inflammatory IL-10 and less pro-inflammatory IL-12 (5, 9). Furthermore, GA promotes the development of anti-inflammatory type II monocytes in EAE, accompanied by induction of regulatory T cells and increased secretion of both IL-10 and TGF-β (10).

IL-1β is a pleiotropic pro-inflammatory cytokine whose production is tightly controlled at several levels (11). Indeed, as recently reviewed, there are several roadblocks to the release of IL-1β beginning with the transcription of the *IL1B* gene and ending with the exit of the active cytokine from the cell. In the extracellular space, IL-1β activity is mainly ruled by the secreted IL-1 receptor antagonist (sIL-1Ra), which binds type I IL-1 receptor without triggering signals (12). As it potently inhibits the various effects of IL-1, sIL-1Ra is considered an important regulator of the inflammatory and overall immune response mediated by IL-1 (13). Because of its extreme efficacy as a pro-inflammatory mediator, if these intracellular and extracellular roadblocks are not enough to limit IL-1β activity, it may also be reduced by the preferential binding to the cell surface or soluble form of type II IL-1 receptor, preventing it from triggering the signal-transducing type I receptor (11). Finally, the facilitation of IL-1β processing by the caspase 1 inflammasome through ATP activation of the $P2X_7$ receptor can also be viewed as a potential roadblock to the activity of IL-1β (11).

IL-1β is mainly produced upon activation of cells of the monocytic lineage. In chronic/sterile immuno-inflammatory diseases, the factors triggering pro-inflammatory cytokine production are still elusive. T cells may exert a pathological effect through direct cellular contact with monocytes/macrophages, inducing massive up-regulation of IL-1β and TNF (14). Besides triggering pro-inflammatory cytokine production, contact-mediated activation of monocytes induces the production and/or shedding of cytokine inhibitors such as sIL-1Ra and soluble receptors of IL-1 and TNF (15). The importance of T cell contact-mediated activation of monocytic cells in MS was further demonstrated in vitro in co-cultures of T cells and microglial cells (16, 17).

In MS, IL-1β is mainly expressed by microglial cells and infiltrating monocyte/macrophages throughout the white matter and in acute lesions (18). This assertion was further confirmed in EAE studies. Indeed, dark agouti rats treated with sIL-1Ra during the induction of EAE, or after adoptive transfer with myelin antigen-primed lymph node cells, develop milder signs of

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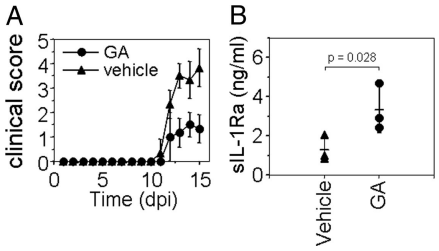

Fig. 1. sIL-1Ra levels are elevated in sera of EAE mice treated with GA. (*A*) GA ameliorates EAE. C57BL/6 mice were injected s.c. daily with GA (150 μg) or vehicle (PBS solution) 7 d before immunization with 10 μg myelin oligodendrocyte glycoprotein 35–55 peptide (dpi, day post-immunization). (*B*) EAE mice treated (GA) or not (vehicle) were killed at disease peak and their serum analyzed for IL-1β and sIL-1Ra content. IL-1β was not detected (not shown).

the disease (19). sIL-1Ra delivered by non-replicative HSV-1 vectors in EAE C57BL/6 mice delays disease onset and decreases disease severity (20). In addition, IL-1α/β double deficient (IL-1$^{-/-}$) mice exhibit significant resistance to EAE induction with reduction in disease severity, whereas IL-1Ra$^{-/-}$ mice are highly susceptible to EAE induction in the absence of pertussis toxin administration (21). These observations demonstrate that the IL-1/IL-1Ra system is crucial for autoantigen-specific T cell induction in mice and that sIL-1Ra efficiently blocks IL-1β effects and ameliorates EAE disease course (19–22).

In this study we addressed the question of the effects of GA on IL-1 system in vivo and in vitro. The results show that GA-treatment increases the circulating levels of sIL-1Ra in both EAE mice and patients with MS. This is reflected in vitro by the direct effect of GA on human blood monocytes. Indeed, GA induces the production of the cytokine inhibitor sIL-1Ra and diminishes the production of IL-1β in conditions related to chronic inflammation, i.e., in monocytes activated by direct contact with stimulated T cells.

Results

sIL-1Ra Serum Levels Are Elevated in GA-Treated EAE Mice. To assess whether GA-treatment affected sIL-1Ra levels in the MS animal model, EAE was induced in mice treated either with GA or PBS solution (i.e., vehicle). As shown in Fig. 1*A*, EAE severity was reduced in GA-treated mice, as previously demonstrated (10). At peak disease, mouse sera were analyzed for levels of sIL-1Ra and IL-1β. IL-1β was not detectable in any of the sera (not shown). However, sIL-1Ra was significantly elevated in mice treated with GA (3,336 ± 1,190 pg/mL sIL-1Ra, mean ± SD) compared with animals that received vehicle as a control (1,296 ± 657 pg/mL sIL-1Ra; Fig. 1*B*). This demonstrates that GA-treatment enhanced sIL-1Ra concentration in EAE mouse serum.

sIL-1Ra Levels Are Elevated in Sera of Patients with MS Treated with GA. sIL-1Ra circulating levels in MS have been shown to vary as a function of clinical status and treatment, so we examined whether GA-treatment would affect sIL-1Ra levels in patients with MS. IL-1β and sIL-1Ra levels were measured in sera of patients with RRMS treated with GA or untreated, and in healthy controls (Table 1). IL-1β was not detectable in any of the sera. As shown in Fig. 2, sIL-1Ra was significantly increased in serum of patients treated with GA (434 ± 265 pg/mL sIL-1Ra) whereas there was no significant difference between untreated patients (218 ± 60 pg/mL sIL-1Ra) and healthy controls (188 ± 65 pg/ml sIL-1Ra). This demonstrates that GA treatment enhances sIL-1Ra in the serum of patients with MS.

GA Differentially Regulates IL-1β and sIL-1Ra Production in Human Monocytes. To assess whether GA per se would affect the IL-1 system in human monocytes, freshly isolated human monocytes were activated by increasing doses of GA. The production of sIL-1Ra was enhanced by GA in a dose-dependent manner, reaching a plateau at 25 μg/mL (Fig. 3*A*). The latter dose was used for the in vitro experiments described later. Noticeably, GA did not induce IL-1β production, demonstrating that GA triggers an anti-inflammatory bias in human monocyte cytokine production.

To confirm that GA affected the IL-1 system, we assessed its effect on human monocytes activated upon chronic/sterile and acute/infectious inflammatory conditions as mimicked by direct cellular contact with stimulated T cells and LPS, respectively. Studies of cell-cell interactions such as those occurring in T cell contact activation of human monocytes are usually complicated by the simultaneous presence of at least 2 viable cell types. To obviate this problem, and possible interferences caused by the

Table 1. Clinical characteristics of patients with MS and healthy controls

Clinical category	Sex	Age (y)	Disease duration (y)	EDSS	GA treatment duration (mo)
Healthy controls					
1	F	31	—	—	—
2	F	34	—	—	—
3	F	36	—	—	—
4	M	29	—	—	—
5	M	24	—	—	—
6	F	24	—	—	—
7	F	30	—	—	—
8	F	35	—	—	—
9	F	44	—	—	—
10	M	40	—	—	—
Mean ± SD	—	32.7 ± 6.4	—	—	—
Untreated RRMS					
1	F	45	17	3.0	—
2	F	31	10	1.5	—
3	M	15	1	1.5	—
4	F	23	1	2.0	—
5	F	41	6	7.0	—
6	F	36	3	2.0	—
7	F	34	5	2.0	—
8	M	27	3	1.5	—
9	F	31	10	1.0	—
10	F	46	6	2.5	—
11	M	41	6	4.0	—
Mean ± SD	—	33.6 ± 9.6	6.2 ± 4.7	2.5 ± 1.7	—
GA-treated RRMS					
1	M	39	6	2.5	56
2	F	33	7	2.0	18
3	M	26	8	1.5	60
4	F	45	15	1.5	27
5	F	32	11	2.0	13
6	F	27	4	0	44
7	M	23	2	1.0	29
8	M	39	9	2.0	36
9	F	25	5	1.0	19
Mean ± SD	—	32.1 ± 7.6	7.4 ± 3.9	1.5 ± 0.8	33.6 ± 16.8

EDSS: Expanded Disability Status Score; RRMS: relapsing-remitting multiple sclerosis; GA: glatiramer acetate. Data expressed at time of sampling.

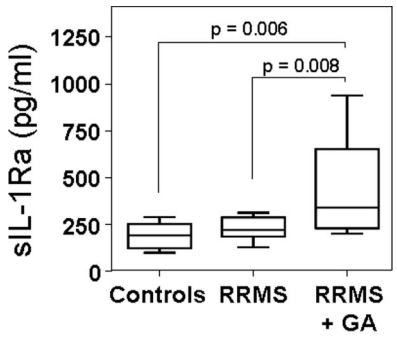

Fig. 2. sIL-1Ra levels are elevated in sera of patients with MS treated with GA. The levels of sIL-1Ra and IL-1β were measured in sera of patients with RRMS treated with GA or not treated, and age-matched healthy controls, as described in Table 1. IL-1β was under the detection limit (15 pg/mL) in all individuals. There was no significant difference between healthy controls and untreated patients with RRMS. Results are presented as a box plot (GraphPad Prism 4).

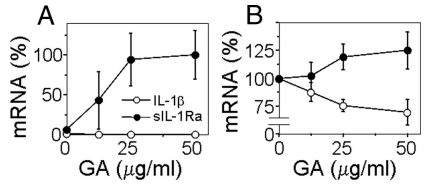

Fig. 4. GA affects sIL-1Ra and IL-1β mRNA in both CE_{sHUT}-activated and resting monocytes. (*A*) Monocytes (2×10^6 cells/well/3 mL) were preincubated for 1 h with the indicated dose of GA and then cultured for 3 h in the presence of CE_{sHUT} (6 μg/mL). Total mRNA was isolated and analyzed by duplex real-time quantitative PCR (see *Materials and Methods*) for the presence of IL-1β (open circles) and sIL-1Ra (filled circles) transcripts. Results are presented as percentage of mRNA expression level, 100% being transcript expression measured after 3 h of monocyte activation by CE_{sHUT} in the absence of GA; mean $\pm$ SD of 3 different experiments. (*B*) Monocytes (2×10^6 cells/well/3 mL) were activated for 18 h with the indicated dose of GA. Total mRNA was isolated and analyzed by duplex real-time quantitative PCR (see *Materials and Methods*) for the presence of IL-1β (open circles) and sIL-1Ra (filled circles) transcripts. Results are presented as percentage of mRNA expression level, 100% being the transcript level at 50 μg/mL GA; mean $\pm$ SD of 3 different experiments, i.e., monocytes prepared from 3 different blood donors.

fact that target cells are potentially phagocytic, isolated membranes from stimulated HUT-78 cells were solubilized with CHAPS and used as a stimulus, referred to as CE_{sHUT} (23). As shown in Fig. 3*B*, GA enhanced the production of sIL-1Ra in monocytes activated by CE_{sHUT} and LPS in a similar manner, and GA-induced sIL-1Ra production was additive to that triggered by CE_{sHUT} or LPS. In contrast, the production of IL-1β induced by CE_{sHUT} was inhibited by GA, whereas LPS-induced production of IL-1β was enhanced in the presence of GA (Fig. 3*C*). These observations suggest that GA displays opposite effects on signaling events downstream of LPS and CE_{sHUT}.

GA Affects the Expression of Cytokine Transcripts. To assess whether GA affected the production of cytokines at the transcriptional level, monocytes were incubated for 1 h with increasing doses of GA and then activated by CE_{sHUT} or not activated. As shown in Fig. 4*A*, GA, in the absence of other stimuli, induced the expression of sIL-1Ra transcript in a dose-dependent manner, whereas that of IL-1β was not induced. When monocytes were activated by CE_{sHUT}, GA diminished IL-1β mRNA expression by 30% whereas it enhanced sIL-1Ra mRNA expression by 25% in monocytes activated by CE_{sHUT} (Fig. 4*B*), corroborating the effects of GA on protein production (Fig. 3). Together, these data suggest that GA displays opposite activity toward IL-1 system members in monocytes/macrophages by directly inducing sIL-1Ra expression and production and by modulating both IL-1β and sIL-1Ra expression/production induced by CE_{sHUT}.

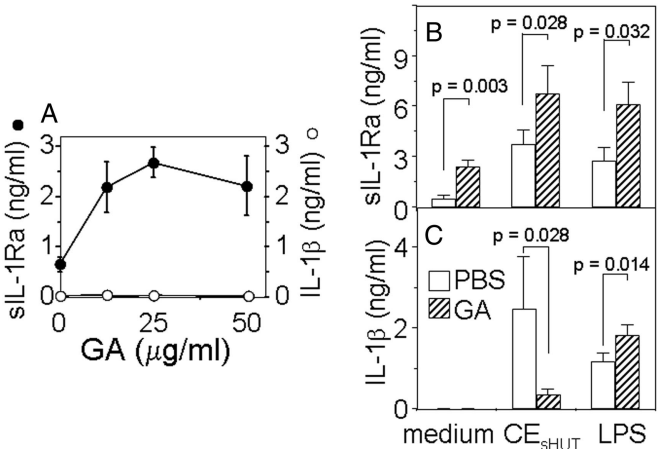

Fig. 3. GA differentially regulates IL-1β and sIL-1Ra production in human monocytes. (*A*) Monocytes (5×10^4 cells/200 μL/well; 96-well plates) were activated with the indicated dose of GA. After 48 h, supernatants were harvested and the production of IL-1β (open circles) and sIL-1Ra (filled circles) were measured in triplicate wells and represented as mean $\pm$ SD. Results are representative of 3 different experiments. (*B*) Monocytes (5×10^4 cells/200 μL/well; 96-well plates) were preincubated for 1 h with or without 25 μg/mL GA and then cultured for 48 h in the presence or absence of CE_{sHUT} (1 μg/mL) or LPS (100 ng/mL). sIL-1Ra was measured in culture supernatants (mean $\pm$ SD, $n = 3$ different experiments). (*C*) Monocytes (5×10^4 cells/200 μL/well; 96-well plates) were preincubated for 1 h with or without 25 μg/mL GA and then cultured for 48 h in the presence or absence of CE_{sHUT} (6 μg/mL) or LPS (100 ng/mL). IL-1β was measured in culture supernatants (mean $\pm$ SD, $n = 3$ different experiments, i.e., monocytes prepared from 3 different blood donors).

Discussion

The present study sheds light on mechanisms by which GA might exert beneficial effects in MS. GA treatment enhances sIL-1Ra blood levels in patients with MS and in EAE mice. This is likely to be the consequence of the direct triggering effect of GA on monocytic production of sIL-1Ra. In addition, GA diminishes monocytic IL-1β production induced by direct contact with stimulated T cells. Thus, through different mechanisms, GA dampens IL-1β activity, which correlates with disease severity.

Recent insights derived from studies on the mechanism of action of GA show a pivotal role of monocytes in the modulation of the immune system and highlight the importance of these cells as a target for pharmacologic intervention in autoimmune diseases (4–10, 24). These results suggest that GA might be useful in autoimmune diseases other than MS, as suggested by its beneficial effect in animal models of autoimmune diseases such as uveoretinitis (25) and inflammatory bowel disease (26), and graft rejection (27), whereas its efficacy has not been demonstrated in animal models of systemic lupus erythematosus (28) and collagen-induced arthritis (29).

The premise that GA enhances sIL-1Ra levels in treated patients with MS is reminiscent of observations made with another immunomodulator used in MS. Indeed, IFNβ also increases circulating serum levels of sIL-1Ra in patients with MS (30). Interestingly, with both immunomodulators, the circulating levels of sIL-1Ra are doubled in treated patients compared with untreated individuals. Together these observations suggest that the enhancement of sIL-1Ra might be relevant to therapeutic effects of both GA and IFNβ. Indeed, sIL-1Ra is transported and expressed into the CNS, where it could inhibit the pro-

inflammatory activities of IL-1β, whose expression is increased in MS lesions (18, 31). The efficiency of sIL-1Ra treatment was demonstrated in EAE animals, in which it results in delayed and milder disease (19, 22). Besides, polymorphisms encoded within the IL-1 gene cluster were associated with MS (32). In particular, mild/moderate disease has been correlated to allele 2 of the IL-1Ra gene (*IL1RN*) variable number of tandem repeats genotype, which favors the production of sIL-1Ra (12, 33). In addition, families displaying high innate IL-1β/sIL-1Ra ratio are at increased risk to have a relative who develops MS (34). Together, these studies reinforce the potential clinical benefit of GA to selectively induce sIL-1Ra secretion by monocytes in MS, as demonstrated in the present study. Of note, direct treatment with the commercially available form of sIL-1Ra (Anakinra) may represent an alternative treatment for MS, although its short lifespan once injected in humans may limit its efficacy (13). Nevertheless, as demonstrated here, the enhancement of intrinsic production of sIL-1Ra might be a mediator of the beneficial effects of GA in MS.

Most studies have focused on the effects of GA treatment on the adaptive immune system, in particular on T cells. Recent data favor the view that primary immune modulation of APC directs T cell immune modulation as a secondary step. Indeed, in mice lacking mature B and T cells, the GA treatment effect on monocytes is unbowed as indicated by an anti-inflammatory "type II" cytokine shift (10). This indicates that GA does not require T cells or T cell products to alter monocytic cytokine production. Furthermore, adoptive transfer of highly purified, GA-induced type II monocytes into mice with EAE triggers T cell immune modulation and ameliorates the disease course of recipient mice. However, MHC II-deficient type II monocytes were unable to mediate this effect on T cells or disease severity (10). Taken together, these observations indicate that in vivo GA treatment exerts a direct effect on APC, which rules T cell immune modulation as the effector arm of GA clinical benefit in CNS autoimmune disease. The present study confirm the direct effect of GA on cells of the monocytic lineage by demonstrating that it down-regulates T cell contact-induced IL-1β production and directly triggers the production of sIL-1Ra. Thus, GA directly affects both the antigen presentation and cytokine production of monocytic cells.

Direct cellular contact with stimulated T cells is a major pathway for the production of IL-1β and TNF in monocytes/macrophages under sterile conditions (17, 35, 36). Indeed, contact-mediated activation of monocytes/macrophages by stimulated T lymphocytes is as potent as optimal doses of LPS to inducing IL-1β and TNF production in monocytes (37, 38). This model was recently used to assess the potency of kinase inhibitors in acute and chronic inflammatory conditions (39). It is thus likely that this mechanism is highly relevant to the pathogenesis and persistence of chronic/sterile inflammation in diseases such as MS. The effect of GA on cytokine production induced by contact with stimulated T cells in human microglial cells was previously demonstrated (16). Stimulated T cells that were pretreated with GA induced lower levels of TNF, IL-1β, and IL-6 in human microglial cells and phorbol 12-myristate 13-acetate (PMA)/IFNγ-treated U937 monocytic cells. However, in the latter study, GA was absent during microglial cell activation, implying that GA rather inhibited the ability of T cells to activate cytokine production by cells of the monocytic lineage. The present study demonstrates that GA influences cytokine production by acting directly on human monocytes. Together, these studies suggest that GA affects the activation stage of both T cells and monocytes/macrophages to diminish contact-induced pro-inflammatory cytokine production.

GA displays opposite effects on monocytes activated by LPS and T cell contact. Indeed, in contrast with CE_{sHUT}-activated monocytes, the production of the pro-inflammatory cytokine IL-1β was up-regulated when cells were activated by LPS. This result is in agreement with a previous study showing that GA enhances the production of IL-1β in the human monocytic cell line THP-1 when activated by LPS (40). Thus, GA displays opposite effects on cytokine production by monocytes activated upon acute/infectious (i.e., LPS) and chronic/sterile (i.e., CE_{sHUT}) inflammatory conditions. Therefore, the use of LPS as an in vitro stimulus should be used with caution to mimic inflammatory conditions when chronic/sterile inflammatory diseases are investigated (39).

In conclusion, this study demonstrates that GA directly affects monocytes/macrophages by triggering the production of the anti-inflammatory cytokine sIL-1Ra. As sIL-1Ra can be both transported through the blood–brain barrier and induced within the CNS, it might exert immunomodulatory effects in both systemic and CNS compartments. In the latter, GA may also dampen the production and activity of IL-1β. Finally, the present data strengthen recent demonstrations that, in addition to the modulation of the adaptive immune system, GA significantly affects the innate immune system.

Materials and Methods

Patients. Patients and healthy volunteers were recruited at the University Hospital of Geneva in accordance with institutional guidelines, and approval of the local ethical committee was obtained. Blood was drawn from 10 healthy controls, 11 untreated patients with RRMS, and 9 GA-treated patients with RRMS (Table 1). Sex, age, clinical score, and disease duration were matched between groups. All enrolled patients had definite RRMS according to revised McDonald criteria (41). At the time of blood sampling, GA-treated patients received 20 mg of GA s.c. daily for at least 1 year, with mean treatment duration of 33.6 ± 16.8 months (Table 1). None of the patients were receiving an immunomodulatory or immunosuppressive drug in addition to GA. Patients from the untreated group did not receive any immunosuppressive or immunomodulatory drug for at least 6 months preceding the study.

EAE Induction and GA Treatment. EAE was induced in 6 C57BL/6 mice using 10 μg myelin oligodendrocyte glycoprotein 35–55 peptide in complete Freund adjuvant. After immunization and 48 h later, mice received an i.v. injection of 200 ng pertussis toxin. Mice were scored as follows: 0, no symptoms; 1, decreased tail tone; 2, mild monoparesis or paraparesis; 3, severe paraparesis; 4, paraplegia and/or quadriparesis; and 5, moribund condition or death. Mice received daily s.c. injections of 150 μg GA suspended in PBS solution ($n = 3$) or PBS solution alone ($n = 3$) starting 7 days before EAE induction as described elsewhere (42). All experiments were carried out in accordance with guidelines prescribed by the Institutional Animal Care and Use Committee at the University of California, San Francisco.

Materials. FCS, streptomycin, penicillin, L-glutamine, RPMI-1640, and PBS solution free of Ca^{2+} and Mg^{2+} were purchased from Gibco; purified phytohemagglutinin from EY Laboratories; Lymphoprep from Axis-Shield; PMA, polymyxin B sulfate, and mouse anti-β-tubulin antibody from Sigma; and GA from Sanofi-Aventis. Other reagents were of analytical grade or better.

Monocytes. Peripheral blood monocytes were isolated from buffy coats of blood of healthy volunteers as previously described (43). To avoid activation by endotoxin, polymyxin B sulfate (2 μg/mL) was added in all solutions during isolation procedure.

T Cell Stimulation and Membrane Isolation. HUT-78, a human T cell line, was obtained from the American Type Culture Collection. Cells were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 50 μg/mL streptomycin, 50 IU/mL penicillin, and 2 mM L-glutamine in 5% CO_2-air humidified atmosphere at 37 °C. HUT-78 cells (2×10^6 cells/mL) were stimulated for 6 h by phytohemagglutinin (1 μg/mL) and PMA (5 ng/mL). Plasma membranes of stimulated HUT-78 cells were prepared as previously described and solubilized in 8 mM CHAPS (23, 44). CHAPS extract of membranes of stimulated HUT-78 cells was referred as to CE_{sHUT}. Previous studies demonstrated that fixed, stimulated HUT-78 cells, plasma membranes of the latter

cells, and CE_{sHUT} display similar ability to induce cytokine production in human monocytes (15). To activate monocytes, CE_{sHUT} was used at either 1 μg/mL or 6 μg/mL proteins as previously determined (38, 44).

Cytokine Production. Monocytes (5×10^4 cells/well/200 μL) were activated with the indicated stimulus in RPMI medium 1640 supplemented with 10% heat-inactivated FCS, 50 μg/mL streptomycin, 50 U/mL penicillin, 2 mM L-glutamine, and 5 μg/mL polymyxin B sulfate in 96-well plates and cultured for 48 h. The production of sIL-1Ra and IL-1β was measured in culture supernatants and patients' serum by commercially available enzyme immunoassay: IL-1β (Immunotech) and sIL-1Ra (Quantikine; R&D Systems). IL-1β and sIL-1Ra concentrations in serum of patients with RRMS and healthy controls were determined by triplicate measurements of the same sample.

mRNA Quantification. Monocytes (2×10^6 cells/well/3 mL) were cultured in 6-well plates with the indicated stimulus for 3 h or 18 h. Total RNA was isolated and analyzed by quantitative real-time PCR as previously described (23).

Statistics. When required, significance of differences between groups was evaluated using the Student *t* test.

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Section II.C.2

Interferon-beta induces brain-derived neurotrophic factor in peripheral blood mononuclear cells of multiple sclerosis patients

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INTRODUCTION

IFN-β is a type I interferon, which is used in the treatment of RR-MS and can reduce the frequency of clinical exacerbations via it anti-inflammatory effects given that MS consist inflammatory mechanisms and neural damages, both effective immunomodulator and neuroprotective therapies are required. Conflicting results arise from the studies assessing INF-β induced brain-derived neurotrophic factor (BDNF) a key regulator of neuronal development that may promote axonal regeneration. Some groups support that INF-β induce BDNF and others reported no effect on INF-β, in this project we analysed expression of BDNF in PBMC from RR-MS patients under treatment or not of INF-β. We showed higher levels of BDNF in PBMC of INF-β -treated MS patients but not in the serum compared with control, and then we proposed that INF-β has a neuroprotective effect but not directly by increasing BDNF in the PBMC, these cells treated with INF-β have the capacity to release BDNF in specific tissue such as CNS of MS patients.

OBJECTIVES

In this project we assessed the level of BDNF within PBMC and in the serum from INF-β treated RR-MS patients compared to healthy controls.

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Short communication

Interferon-β induces brain-derived neurotrophic factor in peripheral blood mononuclear cells of multiple sclerosis patients

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Abstract

Interferon-β (IFN-β) achieves its beneficial effect on multiple sclerosis (MS) *via* anti-inflammatory properties. In this study, we assessed the expression of the brain-derived neurotrophic factor (BDNF) in peripheral blood mononuclear cells (PBMC) from relapsing–remitting multiple sclerosis (RRMS) patients treated or not with IFN-β. Intracellular BDNF was measured by Western blot and ELISA and compared with serum BDNF. We found higher levels of BDNF in PBMC of IFN-β-treated versus non-treated patients, whereas serum levels of BDNF were similar. We hypothesize that the increased intracellular BDNF secondary to IFN-β is not released in the periphery. This release is probably not tissue specific but in MS patients, BDNF could be specifically delivered by PBMC at the site of re-activation, i.e. within the central nervous system.

Keywords: Multiple sclerosis; Interferon-β; Neurotrophin; Brain-derived neurotrophic factor; Peripheral blood mononuclear cells; Neuroprotection

1. Introduction

Multiple sclerosis (MS) is an autoimmune disease associated with inflammation of the central nervous system (CNS) leading to demyelination and axonal loss (Hafler et al., 2005).

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family which includes nerve growth factor (NGF). Neurons are the major source of BDNF (Lewin and Barde, 1996) but constitutive as well as stimulated-expression of BDNF was found in human lymphocytes and monocytes (Kerschensteiner et al., 1999). The presence of BDNF in blood cells suggests a neuroprotective role for infiltrating immune cells into the CNS (Gielen et al., 2003; Hohlfeld, 2008; Hohlfeld et al., 2000, 2006; Kerschensteiner et al., 1999; Weinstock-Guttman et al., 2007). BDNF is generated as a 32 kDa precursor that is post-translationally processed to give rise to a mature bioactive BDNF of 14 kDa (Mowla et al., 2001).

Interferon-β (IFN-β) is one of the treatments of choice with well-established clinical benefits for relapsing-remitting (RR) MS. The beneficial effect of IFN-β is attributed to its immunomodulatory effects on inflammatory mediators and T-cell reactivity (Zafranskaya et al., 2007; Zhang et al., 2002). Indeed, IFN-β modulates cytokine levels and inhibits transmigration of autoreactive T cells into the CNS (Theofilopoulos et al., 2005). Given that MS consists of inflammatory mechanisms and neuronal damages, both effective immunomodulator and neuroprotective therapies are required. Increasing BDNF levels could be one of the neuroprotective mechanisms of IFN-β.

Glatiramer acetate (GA), another immunomodulator used in MS therapy has been shown to induce BDNF in GA-reactive T cells (Aharoni et al., 2000; Chen et al., 2003; Ziemssen, 2004; Ziemssen et al., 2002, 2005). These findings suggest that GA-reactive T cells provide important source of BDNF and may participate in neuroprotective mechanisms. Conflicting results arise from the studies assessing IFN-β-induced BDNF. Some of them reported no effect of IFN-β (Caggiula et al., 2006; Petereit et al., 2003; Sarchielli et al., 2007), while others demonstrated IFN-β-dependent increase in BDNF levels (Hamamcioglu and Reder, 2007). These studies have examined secreted BDNF

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either in the serum or in the supernatant from stimulated or non-stimulated immune cells, but not directly within immune cells.

The purpose of this study was to assess the levels of BDNF within peripheral blood mononuclear cells from IFN-β1a-treated RRMS patients, untreated RRMS patients and healthy controls (HC) and compare with serum BDNF of the same groups.

2. Materials and methods

2.1. Patients and healthy donors

Thirty MS patients fulfilling 2005-revised Mc Donald Criteria (Polman et al., 2005) as well as 15 age- and gender-matched healthy individuals were recruited at the University Hospital of Geneva in accordance with institutional guidelines. All patients were classified as RRMS in remission according to clinical history. Patients under IFN-β were treated with Rebif® (IFN-β1a), 44 μg 3×/week s.c. for at least a year. Characteristics of patients and HC are described in theTable 1. All individuals had peripheral blood cell counts within the reference range.

2.2. Antibodies and reagents

Polyclonal antibodies against BDNF and GAPDH were from Santa Cruz (Santa Cruz, CA) and Biomedical Laboratories (Golden, CO), respectively. Horseradish-conjugated secondary antibody and ECL detection reagents were obtained from Amersham (Buckinghamshire, UK). Nitrocellulose membranes and the DC protein assay were from Bio-Rad (Hercules, CA). Chemiluminescence Biomax films were obtained from Kodak (Rochester, NY)). Restore™ Western blot stripping buffer was purchased from Pierce (Rockford, IL). Protease inhibitor cocktail tablets were from Roche Diagnostics (Penzberg, Germany).

2.3. Peripheral blood mononuclear cells (PBMCs) isolation

Blood was sampled directly into Ficoll-containing BD Vacutainer CPT tubes (Becton Dickinson, Franklin Lakes, NJ). Peripheral mononuclear cells (PBMC) were separated by centrifugation at 1650 g for 20 min at room temperature. Further, a washing step was performed according to the manufacturer's instruction (method A of BD protocol: centrifugation at 300 g 2× (15′ and 10′)). This technique allows removing possible contamination by platelet. Isolated PBMC were washed and kept frozen at −80 °C until use.

Table 1
Clinical characteristics of MS patients and healthy controls

Clinical category	*n*	Sex (F/M)	Age mean (years±S.D.)	EDSS (±S.D.)	Disease duration (years±S.D.)
HC	15	10/5	34 (±8)	–	–
RRMS	15	10/5	35 (±10)	1.9 (±0.8)	3.2 (±3)
RRMS-IFN-β	15	10/5	36 (±8)	1.9 (±0.9)	6.1 (±2.2)

HC: healthy controls; RRMS: relapsing–remitting multiple sclerosis; RRMS-IFN-β: Interferon-β1a-treated RRMS patients; ±S.D: standard deviation; EDSS: Expanded Disability Status Score.

2.4. Preparation of cell extracts

PBMC were resupended in a lysis buffer (10 mM Tris–HCL (pH 7.4) 1 mM Na-vanadate, 1% SDS) supplemented with a protease inhibitor cocktail (Roche). Proteins were precipitated with 6 volumes of cold acetone overnight at −20 °C. The pellet was then washed twice with cold acetone and resuspended in the lysis buffer. Total protein content was determined using the Bio-Rad DC protein assay (Bio-Rad).

2.5. Gel electrophoresis and Western blot analysis

Samples were loaded on a 12% SDS-PAGE (20 μg protein per lane). Proteins were transferred onto nitrocellulose membrane (Bio-Rad). The membrane was incubated in a blocking buffer (20 mM Tris, 150 mM NaCl (pH 7.6), 0.1% Tween-20=TBS-T) and 5% non-fat milk. The membrane was probed with a polyclonal anti-BDNF (1 μg/ml) (Santa Cruz). After 3 washes with TBS-T buffer, the membrane was incubated with a donkey anti-rabbit-HRP (Amersham) in the blocking buffer. The blot was finally washed 3 times, and immunoreactive BDNF was detected by ECL kit (Amersham). In order to re-probe the blot, the membrane was stripped and incubated with another antibody. Band intensities were quantified using NIH Image J.

2.6. ELISA analysis

BDNF was further determined in serum and cell lysates using BDNF E_{max}® Immunoassay System according to the manufacturer's instructions (Promega, Madison, WI, USA).

2.7. Induction of BDNF in vitro

PBMC from healthy donor were cultured in complete RPMI medium in the presence or absence of IFN-β1a (R&D Systems, Abingdon, UK) for 24 h. Supernatants were collected and the cells processed for Western blot as described above.

2.8. Statistical analysis

Statistical analyses were performed using Student's *t*-test. Intergroup differences were considered significant when $p<0.05$.

3. Results

3.1. BDNF detected by Western blot is increased in PBMC of IFN-β treated patients

A representative immunoblot of the 14-kDa mature form of BDNF protein from three non-treated and three IFN-β-treated RRMS is shown in Fig. 1A. Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as loading control. BDNF was analysed in PBMC from 15 patients of each group of RRMS patients and 15 healthy controls. There were interindividual differences in BDNF expression, but generally lower levels were found in untreated patients. When normalized to corresponding

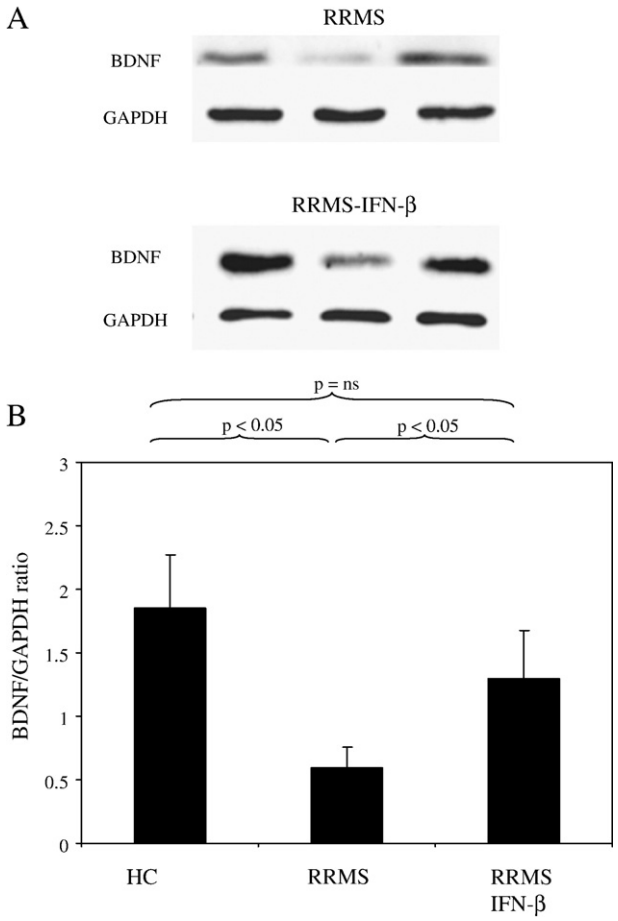

Fig. 1. (A) Representative Western blot analysis of the bioactive 14 kDa BDNF isolated from PBMC of 3 untreated RRMS patients and 3 IFN-β-treated RRMS patients. (B) BDNF to GAPDH ratio. After BDNF immunoblotting, the membrane was stripped and re-probed with a polyclonal anti-GAPDH antibody. Band intensities were quantified by NIH Image. HC ($n=15$) and IFN-β-treated patients ($n=15$) expressed significantly higher levels of BDNF compared to untreated RRMS patients ($n=15$) (mean±SD; $p<0.05$).

GAPDH, a decrease of the mature form of BDNF was observed in non-treated RRMS patients *vs* healthy controls (BDNF/GAPDH ratio (mean±SD)=0.7±0.3 *vs* 1.8±0.8, $p=0.002$). A higher level of BDNF was also found in IFN-β-treated RRMS patients in comparison with untreated patients (BDNF/GAPDH OD ratio (mean±SD)=1.4±0.7 versus 0.7±0.3, $p=0.04$) (Fig. 1B).

3.2. BDNF detected by ELISA was increased in PBMC of IFN-β treated patients

To confirm the previous observation, we further measured BDNF in PBMC cell extracts from the same groups of patients and controls by standard ELISA (Fig. 2). A higher level of BDNF was found in PBMC lysate of IFN-β-treated patients in comparison with untreated RRMS ((mean pg/mg protein±SD)=230.9±51 versus 177.8±41, $p=0.004$). A decrease of BDNF in PBMC cell extract was observed in RRMS *vs* HC but the difference was not statistically significant ((mean pg/mg protein±SD)=177.8±41 versus 205.9±64, $p=$ns).

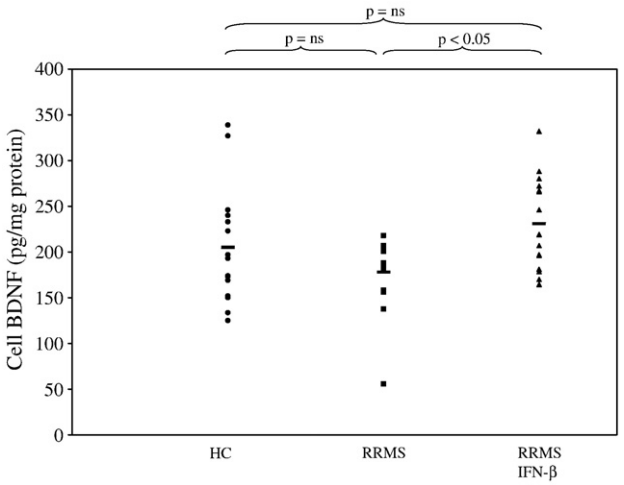

Fig. 2. ELISA analysis of BDNF in PBMC cell lysates. Cell extracts corresponding to 100 μg protein were analyzed by BDNF immunoassay. BDNF content is expressed as pg per mg of protein. Difference between untreated and treated RRMS patients is significant ($p<0.05$). HC showed higher levels of BDNF than untreated RRMS patients but statistical difference was not reached ($p=$ns).

3.3. No difference of serum levels of BDNF is detected between patients and controls

BDNF was measured in the serum of HC, IFN-β-treated and untreated patients (Fig. 3). BDNF levels in the serum were not different between the 3 groups (19.5±7.2 ng/ml (HC) *vs* 20.4±6.4 ng/ml (RRMS) *vs* 16.3±6.7 ng/ml (RRMS/IFN-β), $p=$ns).

3.4. In vitro induction of BDNF on PBMC by IFN-β

Dose-dependent induction of BDNF by IFN-β1a demonstrated by Western blot (Fig. 4). PBMC were incubated with progressive concentrations of IFN-β1a for 24 h. GAPDH was used as loading control. Densitometry analysis of the blots

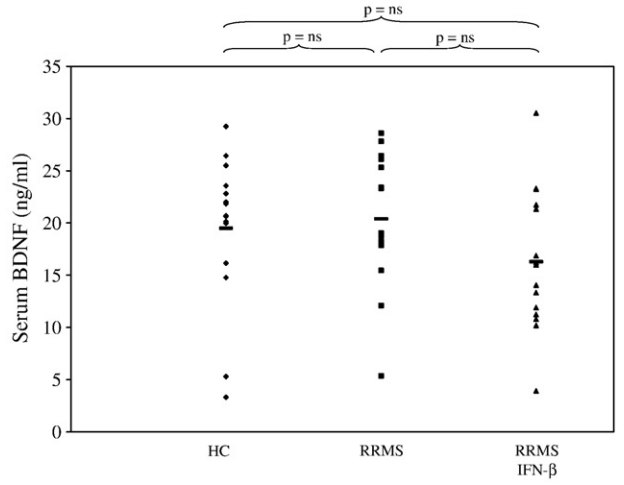

Fig. 3. Determination of BDNF in the serum by ELISA. ELISA for BDNF was performed on sera from the three groups (HC, untreated RRMS and IFN-β-treated RRMS patients). BDNF levels in the serum were similar in all groups.

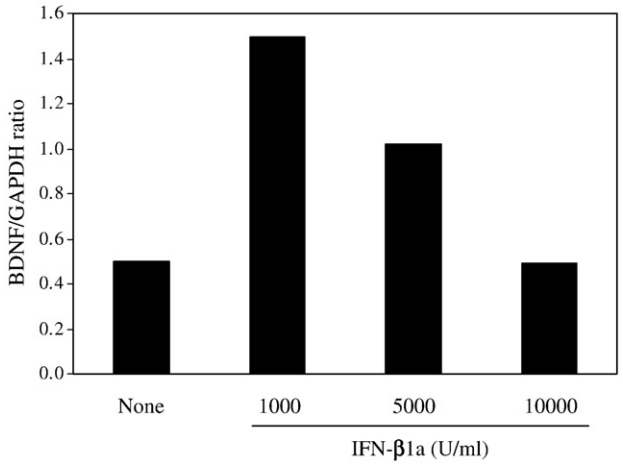

Fig. 4. *In vitro* dose-dependent induction of BDNF by IFN-β1a within PBMC. PBMC were incubated with progressive concentrations of IFN-β1a for 24 h. Analysis was performed by western blot and GAPDH used as loading control. Densitometry analysis of the blots normalized to corresponding GAPDH showed a 3-fold increase with a maximum effect at 1000 U/ml of IFN- β1a.

normalized to corresponding GAPDH showed a 3-fold increase with a maximum effect at 1000 U/ml of IFN-β1a.

3.5. Comparison of the sensitivity of the two assays (Western blot and ELISA)

The Western blot has been done with progressive concentration of human recombinant BDNF to determine its sensitivity. With the same protocol (primary/secondary antibodies) as the one described in the Materials and methods section, we were able to detect a minimal concentration of 50 pg of human BDNF. The ELISA used in the manuscript (Promega) has a detection cut-off of 20 pg to detect BDNF. Thus, the ratio of sensitivity of the two assays to detect BDNF is 2.5 in favor the ELISA.

4. Discussion

In this study, we addressed the question whether IFN-β therapy could induce the synthesis of the neurotrophin BDNF in immune cells of MS patients. To this purpose, expression of the bioactive form of BDNF protein (14 kDa) was analyzed within PBMC from HC, RRMS and IFN-β-treated RRMS patients ($n=15$ for each group). We found a decrease of the bioactive form of BDNF in RRMS patients compared to HC, whereas the BDNF was found to increase to HC values in the IFN-β-treated RRMS group.

The same samples were tested by ELISA which confirmed the increase of BDNF in PBMC from IFN-β-treated RRMS patients compared to non-treated patients. A decrease of BDNF in non-treated patients compared to HC was noted also the difference didn't reach significance. Noteworthy, the ELISA may detect both the 14 kDa bioactive and the 32 kDa precursor form of BDNF with no distinction between these two forms whereas the antibody used for the Western blot allowed a specific detection of the bioactive form. This may explain this apparent discrepancy between the two assays. Thus, IFN-β may have a specific effect on the bioactive form rather than the precursor form of BDNF.

No BDNF difference was found in the serum when each group was compared. This result is in line with several reports showing that BDNF detected in the serum or the supernatant of stimulated PBMC is not modified in IFN-β-treated patients (Caggiula et al., 2006; Petereit et al., 2003; Sarchielli et al., 2007). To our knowledge, no studies have assessed the levels of BDNF *ex-vivo* within non-stimulated PBMC in IFN-β-treated RRMS patients. Thus, the increase of BDNF detected within PBMC of IFN-β-treated RRMS patients can be seen as a correction of BDNF to HC values. Further, the increase of BDNF within PBMC treated by IFN-β was demonstrated *in vitro*.

Our study is based on BDNF expression within PBMC and do not differentiate which PBMC population mainly produces this neurotrophic factor. BDNF is known to be predominantly produced by T cells but can also be expressed by B cells. In addition, leukocytes of the innate immune system, such as monocytes, could also produce BDNF. Thus, further studies should assess whether IFN-β may influence BDNF production through an antigen-specific effect.

It is not possible, based on these data, to demonstrate that IFN-β has a neuroprotective effect secondary to the increase of BDNF. Nevertheless, it can be hypothesized that the absence of release of BDNF in the serum may be associated with the capacity of PBMC from IFN-β-treated RRMS patients to release BDNF directly in specific targets such as the damaged CNS of patients.

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Section II.C.3

Inhibition of naive Th1 CD4+ T cells by glatiramer acetate in multiple sclerosis

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INTRODUCTION

Glatiramer acetate is one approved therapy for RR-MS. It mechanism of action is mediated by a Th2 bystander deviation, and a decreased activation of APC. Our data indicate that GA treatment downregulates an accelerated Th1 differentiation of CD4 T cell, and to affect T-cells differentiation. Thus, GA act also in an antigen-independent manner.

OBJECTIVES

In this section we investigated whether GA treatment may have an effect in Th1 differentiation at different maturation stage. More specifically we analyzed the effect of GA on IL-2 and TNF secretion by naïve, memory and effectors CD4 and CD8 T cells in MS patients.

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Inhibition of naive Th1 CD4$^+$ T cells by glatiramer acetate in multiple sclerosis

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Abstract

We investigated whether glatiramer acetate (GA) treatment may affect Th1 differentiation at various T-cell maturation stages. Specifically, we analyzed the effect of *in vivo* GA treatment on intracellular synthesis of IL-2 and TNF-α by naive, memory and effector CD4$^+$ and CD8$^+$ T cells by five-colour flow cytometry. Our data indicate that GA treatment downregulates/normalizes an accelerated Th1 differentiation of CD4$^+$ T cells in RRMS patients at all stages of T-cell maturation. Most notably, we conclude that, by altering naive, unprimed CD4$^+$ T cells, GA treatment appears to affect T-cell differentiation, at least in part, in an antigen-independent manner.

Keywords: Multiple sclerosis; Glatiramer acetate; T-cell differentiation; CD4$^+$ T cell; CD8$^+$ T cell

1. Introduction

Multiple sclerosis (MS) is characterized by the development of multifocal demyelinating lesions disseminated in time and space throughout the central nervous system (CNS). Relapsing–remitting multiple sclerosis (RRMS) is the most common MS subtype that includes about 85% of patients at initial presentation and is characterized by alternating worsening (relapse) and recovery (remission). MS is a chronic inflammatory disease thought to be mediated by CD4$^+$ myelin-reactive Th1 cells although other immune factors may contribute to the pathogenesis (Steinman, 1996; Hemmer et al., 2002, 2003; O'Connor et al., 2001; Sospedra and Martin, 2005). Glatiramer acetate (GA) (Copaxone®) is one approved therapy for RRMS and has shown to decrease the number of CNS lesions and the frequency of relapses. GA is a copolymer of 40–100 residues which is randomly composed of the four amino acids glutamate, lysine, alanine and tyrosine in a defined molar ratio. The mechanism of action of GA is thought to be mediated mainly by GA-specific Th2 cells, producers of IL-4, which dampen the activity of autoreactive T cells (Duda et al., 2000; Neuhaus et al., 2001, 2000; Farina et al., 2005). Further, GA might act as an altered peptide ligand modifying the T-cell response to several myelin antigens. Other mechanisms of GA include a decreased activation of antigen presenting cells secreting an anti-inflammatory cytokine profile (Karandikar et al., 2002; Weber et al., 2004; Vieira et al., 2003). In addition, a possible neuroprotective effect of GA has been suggested by the fact that GA-activated T cells can produce brain-derived neurotrophic factor (BDNF), a key regulator of neuronal development that may promote axonal regeneration (Aharoni et al., 2003; Ziemssen et al., 2002; Azoulay et al., 2005).

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Table 1
Clinical characteristics of MS patients and healthy controls

Clinical category	*n*	Sex (F/M)	Age mean (years±S.D.)	EDSS (±S.D.)	Disease duration (years±S.D.)
HC	13	9/4	34 (±8)	–	–
RRMS	11	8/3	34 (±10)	2.5 (±1.7)	6 (±5)
RRMS-GA	7	4/3	32 (±8)	1.5 (±0.8)	8 (±4)

HC: healthy controls; RRMS: relapsing–remitting multiple sclerosis; RRMS-GA: glatiramer acetate-treated RRMS patients; S.D.: standard deviation; EDSS: Expanded Disability Status Score.

We previously reported that the frequency of $CD4^+$ T cells primed for TNF-α synthesis is increased in all stages of MS, including RRMS, and normalized to values of HC in GA-treated patients (Fellay et al., 2001). In addition, a significant decrease in the frequency of $CD4^+$ T cells primed for secretion of IL-2 and TNF-α was shown in GA-treated patients, reaching values of HC (Fellay et al., 2001). In the present study, we investigated the impact of GA on cytokine responses by naive, memory and effector $CD4^+$ T cells. We analysed $CD4^+$ T-cell subsets according to van Lier and colleagues (Hamann et al., 1997; Baars et al., 1995) who demonstrated the presence of phenotypically and functionally distinct subsets of both $CD8^+$ and $CD4^+$ primed T cells by analyzing the cell surface co-expression of CD27 and CD45RA molecules.

In this report, we demonstrate that GA treatment decreases the priming of memory and effector $CD4^+$ T cells for pro-inflammatory cytokine synthesis. Interestingly, GA treatment was found to inhibit the priming of the naive $CD4^+$ T cells for TNF-α and IL-2 synthesis which suggests that GA may act, at least in part, in an antigen-independent manner.

2. Materials and methods

2.1. Patients and healthy donors

Eighteen MS patients fulfilling Poser criteria (Poser et al., 1983) as well as 13 age- and gender-matched healthy individuals were recruited at the University Hospital of Geneva in accordance with institutional guidelines. All patients were classified as RRMS in remission according to clinical history. Seven patients treated by GA were under therapy for at least 1 year, whereas the 11 non-treated patients had not received any immunomodulatory therapy for at least 1 year. Characteristics of patients and HC are described in Table 1. All individuals had peripheral blood counts within the reference range.

2.2. Antibodies and reagents

Labelled monoclonal antibodies and reagents used in this study were: human CD3 biotinylated (Diaclone, Besançon, France); CD8-APC-Cy7, CD45RA-APC and Streptavidin-PE-TR (Caltag, Burlingame, CA, USA); CD27-FITC, TNFα-PE and IL-2-PE (BD PharMingen,

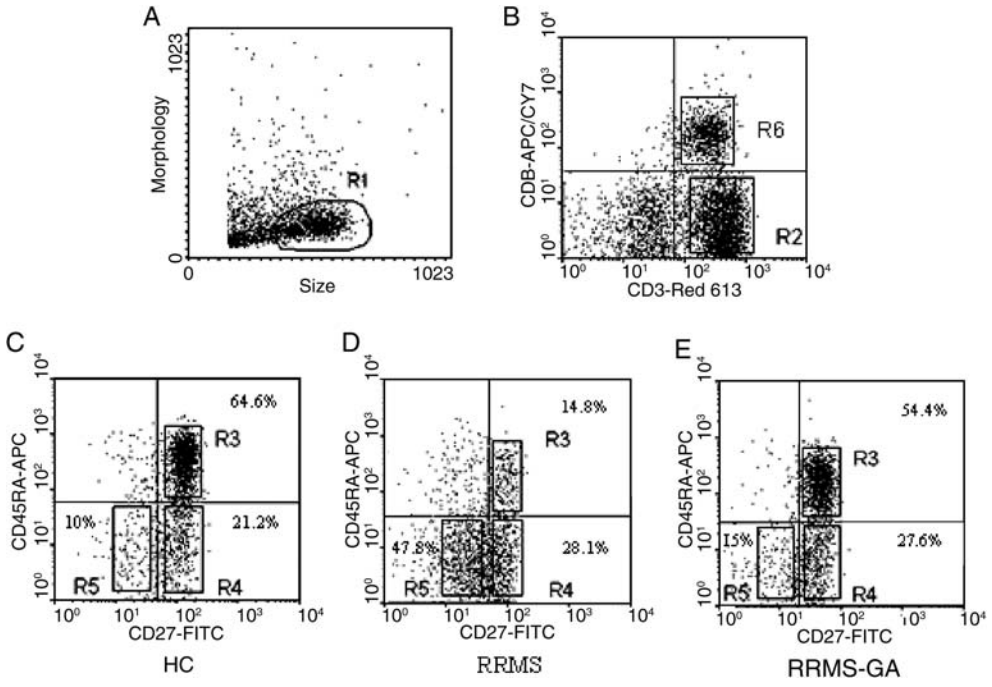

Fig. 1. Representative FACS analysis of $CD4^+$ T-cell subsets. Mononuclear cells were isolated from healthy control (HC), RRMS patient without treatment (non-treated) or a RRMS patient receiving GA as immunotherapy (GA-treated). Plotting CD3 *vs* $CD8^+$ allowed to gate on $CD4^+$ cells in region R2 as $CD3^+CD8^-$ (A, B). On the basis of expression of CD45RA and CD27 markers, the $CD4^+$ T cells were further distributed into the different subsets: R3 = $CD45RA^+CD27^+$ (naive), R4 = $CD45RA^-CD27^+$ (memory) and R5 = $CD45RA^-CD27^-$ (effector). Percentage of each subset is displayed in the corresponding quadrant (C–E). $CD8^+$ T cells were gated in R6 (B).

San Diego, CA, USA); ionomycin (Alexis Biochemicals, San Diego, CA, USA); phorbol 12-myristate 13-acetate (PMA) and brefeldin A (Sigma, St. Louis, MI, USA); RPMI 1640 medium, L-glutamine and penicillin–streptomycin (Invitrogen, Praisley, Scotland); fetal calf serum (FCS) (Sera-Tech, Aidenbach).

2.3. Peripheral blood mononuclear cells (PBMCs) isolation and stimulation

PBMCs were isolated from ethylenediaminetetraacetic (EDTA)-anticoagulated blood by standard Ficoll–Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) density gradient centrifugation. After 3 washes with Hanks' balanced salt solution (HBSS), PBMCs (10^6 cells/ml) were resuspended in RPMI medium (Invitrogen Life Technologies) containing 10% heat-inactivated fetal calf serum (FCS), 1% penicillin–streptomycin and 2% glutamine (Invitrogen Life Technologies). The cells were stimulated for 16 h with 20 ng/ml phorbol 12-myristate 13-acetate (PMA) (Sigma) and 1 μg/ml ionomycin (P/I) (Sigma). The protein transport inhibitor brefeldin A (10 μg/ml) was added for the last 14 h of incubation (O'Neil-Andersen and Lawrence 2002).

2.4. Five-colour staining and flow cytometry analysis

PBMCs from HC, non-treated or GA-treated RRMS patients were analysed by fluorescence-activated cell sorter (FACS). PBMCs (2.5×10^5/well) were first labelled for surface markers: CD3, CD8, CD45RA and CD27 for 30 min in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 0.1% azide (NaN_3) (labelling buffer). Cells were fixed in 1% paraformaldehyde (PFA) in the labelling buffer for 15 min at room temperature (RT). The cells were further permeabilized with 0.05% saponin in the labelling buffer for 15 min at RT and stained for intracellular cytokine IL-2 or TNF-α. Five-colour-stained $CD4^+$ and $CD8^+$ T cells were gated and analysed in a FACSvantage SE (BD Biosciences). FACS analysis was performed within 24 h after blood sampling. For each sample, 5×10^4 cells were acquired and results were analysed with WinMDI 2.8 software.

A representative FACS analysis of $CD4^+$ T-cell subsets is shown in Fig. 1. PBMCs were gated according to forward scatter/side scatter (FSC/SSC) criteria (R1) and analyzed for CD3/CD8 co-staining. The stimulation of PBMC with phorbol 12-myristate 13-acetate (PMA) downregulates the surface membrane CD4 by internalisation (Moller et al., 1990; Petersen et al., 1992). Therefore, in accordance with several previous studies (Picker et al., 1995; Ledru et al., 1998; Fellay et al., 2001), the percentage of $CD4^+$ T cells was determined as the difference between the percentage of $CD3^+$ T cells and the percentage of $CD8^+$ T cells (in R2 region). Co-expression of CD45RA and CD27 markers was then analyzed on R3-gated cells. The following $CD4^+$ T-cell subsets were identified: $CD45RA^+CD27^+$ (naive) in R3, $CD45RA^-CD27^+$ (memory) in R4 and $CD45RA^-CD27^-$ (effectors) in R5. The percentage of each population is displayed in the corresponding areas.

2.5. Statistical analyses

Statistical analyses were performed using PRISM software (GraphPad, San Diego, CA). The statistical significance

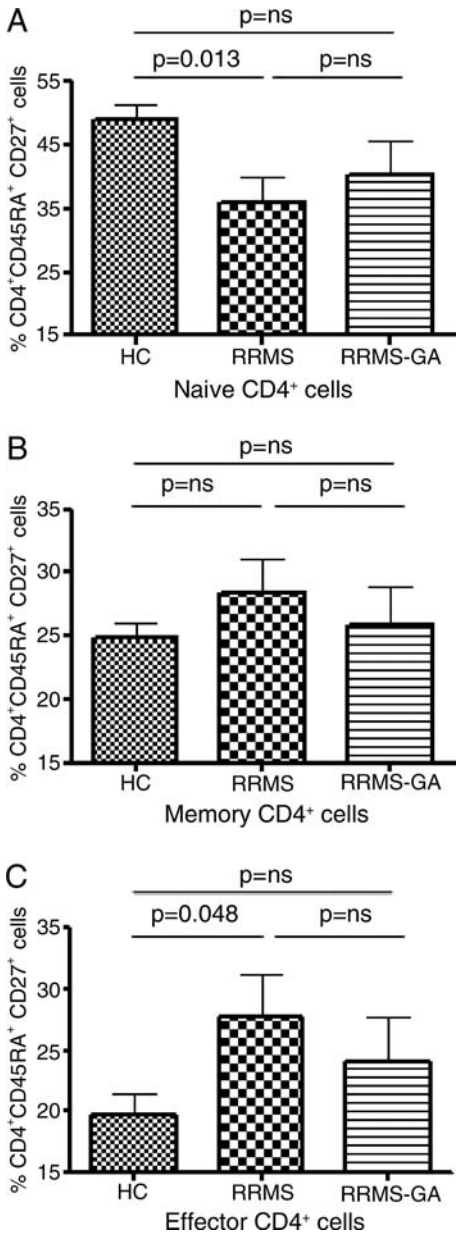

Fig. 2. Frequencies of naive, effector and memory $CD4^+$ T cells from healthy controls (HC), non-treated RRMS (RRMS) and GA-treated (RRMS-GA) patients. Data are means±SEM from HC ($n=13$), RRMS ($n=11$) and RRMS-GA ($n=7$). The number of naive cells was significantly decreased in RRMS ($p=0.013$) whereas effector cells were increased ($p=0.048$) in RRMS, when compared to HC. No difference was found between RRMS-GA and HC in the memory T-cell subset ($p=$ns).

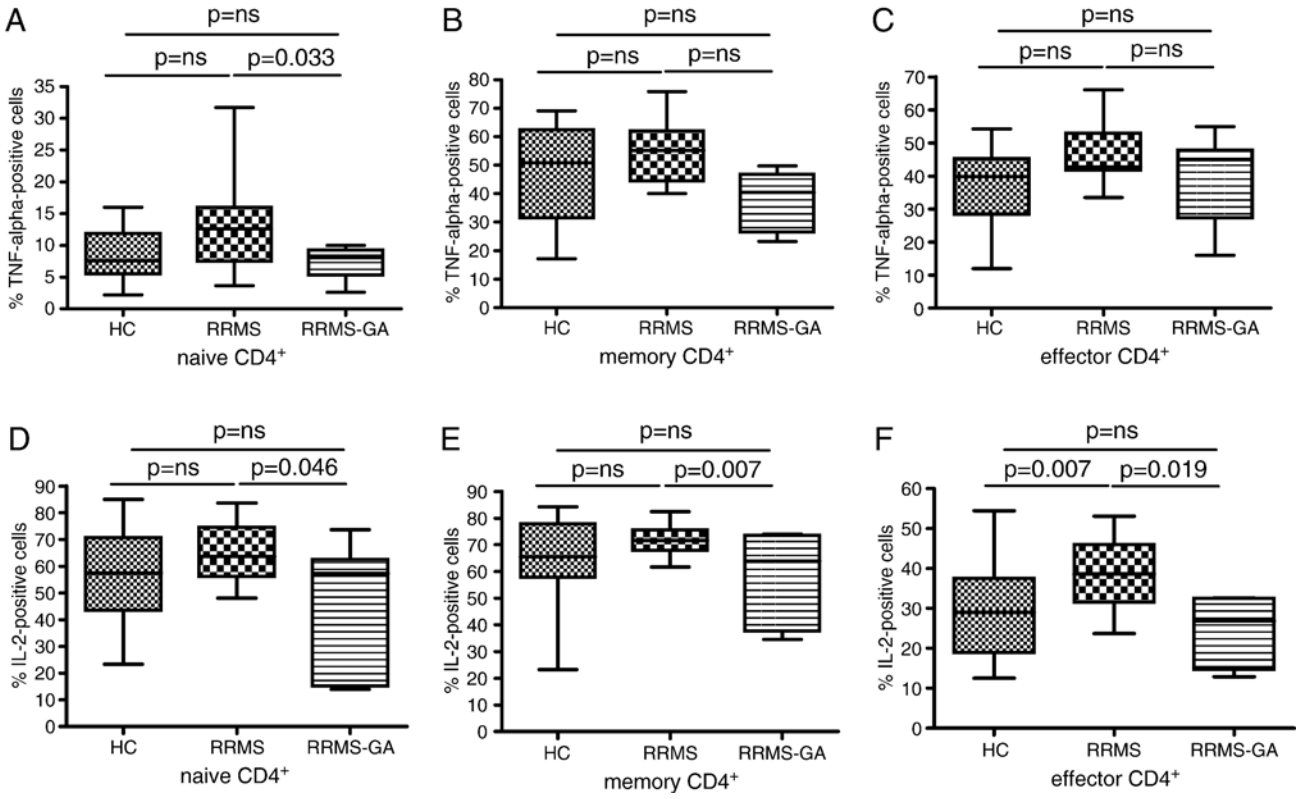

Fig. 3. Intracellular TNF-α and IL-2 production in naive, memory and effector $CD4^+$ T cells. PBMCs derived from HC ($n=13$), RRMS ($n=11$) and RRMS-GA ($n=7$) were stimulated with PMA/ionomycin in the presence of brefeldin A and intracellular cytokine was analysed with specific antibody according to the five-colour technique (see details in the Materials and methods section). Compared to RRMS, RRMS-GA patients showed a significant decrease of TNF-α in naive ($p=0.033$) (A) and effector ($p=0.006$) (C) subsets while IL-2 was lowered by GA in the three subpopulations: naive ($p=0.046$) (D), memory ($p=0.007$) (E) and effector ($p=0.05$) (F). No cytokine difference was found when HC were compared to RRMS except for IL-2 in the effector subset ($p=0.007$) (F).

(p value of <0.05) was determined by the non-parametric Mann Whitney test.

3. Results

3.1. $CD4^+$ T-cell subsets in HC, untreated RRMS and GA-treated RRMS patients

The percentages of naive, memory and effector $CD4^+$ T-cell subsets were determined in HC, RRMS and RRMS-GA patients (Fig. 2). A significant decrease of naive $CD4^+$ T cells (36±3.8% (mean±SEM) *vs* 48.9±2.5%; $p=0.013$) as well as an increase of effector $CD4^+$ T cell (27.7±3.5% *vs* 19.7±1.8%; $p=0.048$) was observed in non-treated RRMS patients compared to HC (Fig. 2A, C). In contrast, compared to HC GA-treated RRMS patients showed similar proportions of naive (40.4±5.3% *vs* 48.9±2.5%; $p=$ns), memory (25.8±3.1% *vs* 24.8±1.1%; $p=$ns) and effector (24±3.7% *vs* 19.7±1.8%; $p=$ns) $CD4^+$ T cells. Interestingly, GA-treated RRMS patients showed an increase in naive $CD4^+$ T cells (40.4±5.3% *vs* 36±3.8%) and a decrease in effector $CD4^+$ T cells (24±3.7% *vs* 27.7±3.5%) compared to RRMS patients, although the difference was not statistically significant ($p=$ns).

Table 2
Frequencies of TNF-α and IL-2 expressing cells in naive, memory and effector $CD4^+$ T cells

	% TNF-α^+ cells			% IL-2^+ cells		
	Naive	Memory	Effector	Naive	Memory	Effector
HC	8.4±3.9	45.2±8.2	47.8±16.4	58.5±16.6	59.5±16.9	29.5±12
RRMS	13.1±7.3	47.1±9	55.1±11.1	65.8±10.5	71.6±6.2	38.5±9.1
RRMS-GA	7.2±2.6	37.9±9.9	39.6±13.4	48.9±23	59.5±16.9	27.0±7.6

PBMCs were analyzed by flow cytometry as indicated in Fig. 1, after gating on $CD3^+CD8^-$ cells. Data are means±S.D. (HC, $n=13$; RRMS, $n=11$; RRMS-GA, $n=7$). Statistical differences are described in Fig. 3. HC: healthy controls; RRMS: relapsing–remitting multiple sclerosis; RRMS-GA: glatiramer acetate-treated RRMS patients.

Table 3
Frequencies of TNF-α and IL-2 expressing cells in naive, memory and effector $CD8^+$ T cells

	% $TNF\text{-}\alpha^+$ cells			% $IL\text{-}2^+$ cells		
	Naive	Memory	Effector	Naive	Memory	Effector
HC	4.7±3	36.2±10.2	37.8±14.1	22.3±11.5	34.5±13	6.3±3.3
RRMS	7.6±6.6	42±14.2	44.5±14.6	27.1±10.5	41.1±11.8	9.9±5.2
RRMS-GA	3±1.5	31.5±17.7	29.6±10.7	18.4±11.9	31.7±12.5	5.5±3.3

PBMCs were analyzed by flow cytometry as indicated in Fig. 1, after gating on $CD8^+$ cells. Data are means±S.D. (HC, $n=13$; RRMS, $n=11$; RRMS-GA, $n=7$). No statistical difference was reached when comparing the different groups and the T-cell subtypes. HC: healthy controls; RRMS: relapsing–remitting multiple sclerosis; RRMS-GA: glatiramer acetate-treated RRMS patients.

3.2. GA treatment downregulates the synthesis of pro-inflammatory cytokines by memory, effector and naive $CD4^+$ T cells

We evaluated the influence of GA treatment on cytokine synthesis by $CD4^+$ T cells at different stages of T-cell maturation comparing GA-treated with untreated RRMS patients. As shown in Fig. 3 and in Table 2, the frequency of TNF-α- and IL-2-producing naive, memory and effector $CD4^+$ T cells was decreased in GA-treated *vs* untreated patients. Indeed, compared to the RRMS group, a significant decrease of naive ((mean±SEM) 13.1±2.2% *vs* 7±2.6%; $p=0.033$) and effector (55.1±3.6% *vs* 37.9±3.8%; $p=0.006$) $CD4^+$ T cells producing TNF-α was observed in the RRMS-GA group (Fig. 3A, C). A decrease of memory $CD4^+$ T cells producing TNF-α (47.1±2.8 *vs* 39.5±5.1) was also found in GA-treated patients, although the difference was not statistically significant (Fig. 3B). Furthermore, GA treatment induced a significant decrease in the frequency of naive (65.8±3.2% *vs* 44±9.3%; $p=0.046$), memory (71.6±1.9% *vs* 55.9±6.8%; $p=0.007$) and effector (38.5±2.8% *vs* 25.2±3.2%; $p=0.019$) $CD4^+$ T cells primed for IL-2 synthesis when compared to non-treated RRMS (Fig. 3D–F).

3.3. Selective effect of GA on $CD4^+$ T cells

To address whether the inhibitory effect on pro-inflammatory T-cell differentiation would be restricted to the $CD4^+$ T-cell compartment we next evaluated TNF-α and IL-2 synthesis in naive, memory and effector $CD8^+$ T cells of the same subjects. Data shown in Table 3 confirm our previous observation that in RRMS patients, the $CD8^+$ T-cell subset is not significantly altered in its capacity to produce the pro-inflammatory cytokines TNF-α and IL-2 (Fellay et al., 2001). Accordingly, we found that the frequency of TNF-α or IL-2 producing naive, memory and effector $CD8^+$ T cells are comparable between RRMS patients and HC.

Finally, we observed that GA treatment had no significant effect on the $CD8^+$ T-cell subsets when compared to non-treated RRMS and HC.

4. Discussion

Inflammatory T cells, particularly $CD4^+$ T cells, are found in large numbers in MS lesions (Martino and Hartung, 1999; Martino et al., 2002). The balance between pro- and anti-inflammatory T cells and T-cell derived cytokines is a key factor regulating MS activity. The imbalance favouring pro-inflammatory cytokine release may exacerbate MS severity, whereas anti-inflammatory cytokines may enhance recovery from relapses and reduce the extent of MS lesions (Hafler, 2004). Observations in experimental animal models of MS suggest that anti-inflammatory Th2 cells may inhibit activation of Th1 cells within the CNS by bystander suppression (Racke et al., 1994) and it has been shown that activation of $CD4^+$ T cells correlates with MS activity (Khoury et al., 2000). GA is thought to mainly mediate its clinical effect by the induction of GA-reactive Th2 cells deviation and inhibition of proliferation and Th1 differentiation of myelin-reactive T cells (Gran et al., 2000).

In this study, we evaluated whether GA treatment may affect the production of two pro-inflammatory cytokines, TNF-α and IL-2, by naive, memory and effector T cells. First, we observed that GA therapy does not significantly affect cytokine release by naive, memory and effector $CD8^+$ T cells, confirming our previous report that the $CD8^+$ T-cell compartment appears to be not affected by GA treatment (Fellay et al., 2001). With regard to the $CD4^+$ T-cell subset, we observed that the percentage of naive $CD4^+$ T cells was significantly decreased in non-treated RRMS patients compared to age-matched HC. This finding correlates to previous observations showing an impairment of T-cell homeostasis associated with MS (Sospedra and Martin, 2005). In addition, this observation is in agreement with the description of the predominant role of naive T cells in generating myelin-reactive T cells in MS (Muraro et al., 2000) and with another study showing that in MS, myelin basic protein-specific T cells were mostly isolated from the memory T-cell subset (Burns et al., 1999).

Specifically, alterations in the naive peripheral compartment have been described in RRMS patients on the basis of a decreased expression of T-cell receptor excision circles (TRECs), a molecular marker of newly produced T cells (Hug et al., 2003; Duszczyszyn et al., 2006). Conversely, we found the frequency of effector $CD4^+$ T cells in non-treated RRMS patients to be increased compared to HC. This imbalance between naive and effector cells may reflect a general immune activation leading to an activated, pro-inflammatory phenotype.

Pro-inflammatory cytokines produced by activated T cells have been suggested to be involved in the pathogenesis of MS (Clerici et al., 2001). In particular, TNF-α has been associated with enhanced MS activity and demyelination (Navikas and Link, 1996; Matusevicius et al., 1996; Bitsch et al., 2000; Chofflon and Fellay, 1998), although some homeostatic role has also been suggested (Martino et al., 2002; Liu et al., 1998). TNF-α is predominantly released by T cells but also by macrophages and microglia (Bitsch et al., 2000; Hartung et al., 2004). In comparison with non-treated RRMS patients, we found that GA-treated patients revealed a significant decrease in the frequency of effector and memory $CD4^+$ T cells primed for TNF-α synthesis. Interestingly, this was also observed for the naive $CD4^+$ T-cell subset, suggesting that the effect of GA on T-cell priming is not dependent on prior T-cell antigen recognition.

IL-2 is a growth factor required for T-cell activation, proliferation, and survival. High affinity IL-2 receptor (IL-2R) was found to be upregulated in MS patients and an increased frequency of IL-2-responsive T cells specific for myelin antigens has been reported (Zhang et al., 1994). The IL-2 receptor is likely to be an important component of inflammation in MS and has accordingly been targeted in novel MS therapies (Rose et al., 2004). Correlation of IL-2 and soluble IL-2R with activity of MS has been established (Sharief and Thompson, 1993). Our study shows that IL-2 was mostly expressed by naive and memory $CD4^+$ T cells in both MS patients and healthy donors. In all three $CD4^+$ T-cell subsets RRMS patients showed a significantly enhanced synthesis of IL-2 when compared to HC. Strikingly, GA treatment again decreased the frequency of IL-2-synthesizing $CD4^+$ T cells, whether expressing markers of naive, memory or effectors. Thus, GA treatment appears to downregulate the priming of all three $CD4^+$ T-cell subsets for the pro-inflammatory cytokines TNF-α and IL-2. Furthermore, the comparison to corresponding maturation stages of $CD8^+$ T cells confirms the selective functional alteration of $CD4^+$ T cells in MS and the beneficial immunomodulatory effects of GA on this subset.

GA treatment is thought to primarily induce GA-reactive Th2-like regulatory cells (Neuhaus et al., 2001, 2000). Our study indicates that GA treatment exerts an immunomodulatory effect on both activated/differentiated and naive $CD4^+$ T cells independent of their antigen-specificity. Thus, the beneficial effect of GA is detected not only on GA-specific T-cell clones but also on polyclonal $CD4^+$ T cells at different levels of maturation and on non-primed T cells, as it has been suggested by *in vitro* studies on PBMC of non-treated MS patients (Wiesemann et al., 2001).

Interestingly, the immunomodulatory effect of GA was recently shown to influence the innate immune system (Weber et al., 2004; Vieira et al., 2003). The fact that naive T cells are influenced by GA treatment could be also seen as a secondary effect of GA on APC. Indeed, following GA treatment, APC could directly influence effector and memory T cells but could also have an effect on the priming of naive T cells. It thus can be hypothesized that the inhibitory impact of GA on Th1 priming of $CD4^+$ T cells may occur secondary to APC immunomodulation by GA.

In conclusion, our results give new insights on the mechanism of action of GA *in vivo* and confirm recent studies demonstrating that GA has a broad immunological effect not restricted to an antigen-specific immunomodulation of the acquired immune system. In addition, this report underscores the therapeutic potential of GA for treatment of autoimmune diseases other than MS.

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Section II.C.4

Glatiramer acetate treatment does not modify the clinical course of (NZB x BXSB)F1 lupus model

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INTRODUCTION

In this section we studied the potential effect of GA in systemic lupus erythematosus (SLE) animal model. SLE is an autoimmune disorder characterized by the involvement of multiple organ systems with alternating clinical exacerbations and remissions. Tissues damage and organ dysfunctions are caused by deposit of immune complexes and auto-Abs (drived by autoreactive T and B cells. (NZB x BXSB)F1, male mice bearing Y-linked autoimmune acceleration, is a lupus-prone mouse model which is associated with a monocyteosis accelerating disease progression. In this study, and after treatment with GA before disease onset until death, we showed that GA exerted no beneficial effect on the median survival after up to 7 months of treatment

OBJECTIVES

To study the effect of GA in the progression of SLE disease in the (NZB x BXSB)F1 murine model.

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Glatiramer acetate treatment does not modify the clinical course of (NZB × BXSB)F1 lupus murine model

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Keywords: Copaxone®, experimental autoimmune encephalitis, (NZB x BXSB)F1 lupus-prone animal model, systemic lupus erythematosus, Y-chromosome-associated accelerator

Abstract

Glatiramer acetate (GA, copolymer-1, Copaxone®), a therapy approved for treatment of multiple sclerosis (MS), prevents and reverses experimental autoimmune encephalomyelitis, the animal model of MS. In central nervous system autoimmune disease, GA is thought to act through modulation of antigen-presenting cells, such as monocytes, mediating an antigen-independent T_h2 shift and development of FoxP3+ regulatory T cells. Recent reports indicate that GA may also be effective in models of other autoimmune diseases such as uveoretinitis, inflammatory bowel disease and graft rejection. To date, the potential effect of GA in lupus animal models has not been described. (NZB × BXSB)F1, male mice bearing *Y-linked autoimmune acceleration* , is a lupus-prone mouse model which is associated with a monocytosis accelerating disease progression. These mice were treated with GA before disease onset until death and both mortality rate and biological parameters were assessed to investigate whether GA may be beneficial in this spontaneous model of systemic lupus erythematosus. GA exerted no beneficial effect on the median survival after up to 7 months of treatment. Humoral and cellular parameters used as markers for lupus progression, such as anti-chromatin, anti-double-stranded DNA and anti-erythrocytes antibodies, hematocrit and monocytosis, were similarly unchanged. Our study demonstrates that GA has no significant effect on the progression of the (NZB × BXSB)F1 lupus-prone animal model. These results reinforce the hypothesis that GA may exert its beneficial effect in some specific autoimmune diseases only.

Introduction

Glatiramer acetate (GA) (Copaxone®) is a copolymer of 40–100 residues which is randomly composed of the four amino acids glutamate, lysine, alanine and tyrosine in a defined molar ratio (1). Initially developed to mimic a major component of the myelin sheath, MBP, and induce experimental autoimmune encephalomyelitis (EAE), GA unexpectedly inhibited EAE in both rodents and monkeys (2–5). In subsequent clinical trials, GA reduced relapse rate and progression of disability in patients with relapsing–remitting multiple sclerosis (MS) leading to its approval in 1995.

The mechanism by which GA is beneficial in central nervous system (CNS) autoimmune disease (reviewed in refs 6, 7) is thought to be mediated through a preferential T_h2 deviation of myelin-specific T cells (8–10). Other effect of GA involves modulation of CD8+ T cells (11) and antigen-presenting cells (APC) (12). Recently, GA has been shown to induce monocytes to direct differentiation of regulatory T cells (Treg) in an antigen-independent manner (13). This observation favors the hypothesis that this drug may be effective in autoimmune diseases other than MS.

Systemic lupus erythematosus (SLE) is an autoimmune disorder characterized by the involvement of multiple organ systems with alternating clinical exacerbations and remissions. Circulating immune complexes and auto-antibodies cause tissue damage and organ dysfunction with manifestations involving the skin, serosal surfaces, CNS and kidneys (14).

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These manifestations are believed to be the result of interactions between autoreactive lymphocytes that arise from both hereditary immunoregulatory defects and environmental factors, including chemicals and UV radiation. Autoreactive T and B cells drive the production of auto-antibodies and the formation of immune complexes, which ultimately lead to tissue damage and organ failure in SLE (15). It is well established that in SLE, several genetic factors independently contribute to the overall susceptibility and progression of the disease (16–18).

Despite many common characteristics, the mouse models of lupus exhibit unique histological and serological manifestations as well as unique disease accelerators. Male BXSB mice carry the Y-chromosome-associated accelerator of autoimmunity termed *Y-linked autoimmune acceleration* (*Yaa*). The *Yaa* mutation has been shown to be responsible for the acceleration of the lupus-like autoimmune syndrome in BXSB mice and in their F_1 hybrids with NZB or NZW mice (19, 20). More recently, the *Yaa* mutation was shown to be a consequence of a translocation from the telomeric end of the X chromosome onto the Y chromosome (21, 22). Hybrid (NZB × NZW) F1 *Yaa*-bearing mice spontaneously develop a generalized autoimmune disorder resembling human SLE (23). Monocytosis is a unique cellular abnormality associated with the *Yaa* mutation (24). In PBMCs from an 8-month-old male BXSB mouse, monocytes reach >50% of leucocytes and is thought to be a major trigger of the accelerated development of lupus disease in this animal model (25, 26). Thus, in this project, the lupus (NZB × BXSB)F1 model was chosen for its accelerated disease associated with specific monocytosis, in regard of the recent demonstration of the potential antigen-independent effect of GA on monocytes and T cell function (13).

Methods

Mice

Female NZB and male BXSB mice bearing the *Yaa* mutation were obtained from local colonies and the hybrids (NZB × BXSB)F1 male used in this study were obtained by local breeding. Mice were bled (from retro-orbital sinus puncture) at 1 and 2 months post-initiation of GA injection. C57BL/6 mice were used as negative controls for monocytosis assessment. The blood was allowed to clot at room temperature, and the serum was stored at −20°C until use.

GA treatment

GA was injected daily subcutaneous (s.c.) (150 μg in 100 μl PBS–mannitol) as previously described (13). Randomized control littermates were injected with vehicle treatment consisting of PBS and mannitol at equal concentration contained in the GA preparation. GA and vehicle treatment were injected from the age of 3 months until death. At 10 months of age, mice still alive were sacrificed and kidney histology was performed.

Serological assays

Serum levels of IgG auto-antibodies against chromatin and double-stranded DNA (dsDNA) were determined by ELISA (27, 28). Chromatin prepared from chick erythrocytes was directly coated to ELISA plates, while dsDNA was coated to ELISA plates precoated with poly-L-lysine (Sigma–Aldrich, St Louis, MO, USA). Then, the plates were incubated with 1:100 diluted serum samples, and the assay was developed with alkaline phosphatase-labeled goat anti-mouse IgG, IgG1 and IgG2a. Results are expressed in U ml^{-1} in reference to a standard curve derived from a serum pool of MRL-*Fas*^{lpr} mice.

Flow cytometric analysis of monocytosis

Flow cytometry was performed using two-color staining of PBMC and analyzed with a FACSCalibur (Becton Dickinson, Mountain View, CA, USA). The following mAbs were used: anti-CD11b (M1/70) and anti-F4/80. Mice displaying percentages of monocyte levels above the mean ± 3 SD of controls (C57BL/6) were considered as positive for monocytosis (25).

Determination of hematocrit

Blood samples were collected into heparinized microhematocrit tubes and centrifuged at 12 000 r.p.m. for 5 min in a microfuge (Sigma-201 M, Auer Bittmann Soulie AG, Geneva, Switzerland). Percentage of packed RBC volume was directly measured after centrifugation.

Detection of Coombs antibodies

A flow cytometric assay was used to detect anti-erythrocyte antibodies. After washing three times with 1% BSA–PBS, a similar number of RBC [according to their hematocrit (Hct)] was incubated with biotinylated rat anti-mouse kappa-chain mAb (H139.52.1.5), followed by PE-conjugated streptavidin (eBioscience, San Diego, CA, USA), and analyzed by FACS. The results are expressed in ng ml^{-1} in reference to a standard curve obtained with known concentration of 34.3C IgG2a anti-RBC.

Histopathology

Kidney samples were collected when mice were moribund or at the end of the experiment (10 months of age). Histological sections were stained with periodic acid–Schiff reagent. The extent of glomerulonephritis (GN) was graded on a 0–4 scale based on the intensity and extent of histopathological changes as described previously (29). Histopathology was performed in a blinded way by expert mice lupus pathologist. GN with grade 3 or 4 was considered a significant contributor to clinical disease and/or death.

Statistical analyses

Statistical analyses were performed using PRISM software (GraphPad, San Diego, CA, USA). The statistical significance ($P < 0.05$) was determined by the non-parametric Mann–Whitney test.

Results

GA treatment does not modify mortality of (NZB × BXSB)F1 lupus mice

Mice received daily s.c. injection of GA or vehicle treatment from the age of 3 months until death. GN is a pathological

hallmark feature of murine SLE. To examine the effect of GA treatment on lupus nephritis and pathology, histological examination of the kidney was performed. Disease development started in both groups within the first month of injection. Classical progression of SLE as well as mortality was present in both GA- and vehicle-treated groups with no difference. In the GA group, median time to death was 6.0 ± 2.4 months, while in the placebo group it was 7.0 ± 2.6 months (P = ns). The Fig. 1(A) details the mortality rate of the GA- (n = 15) and the vehicle-treated (n = 17) groups. To avoid possible bias like death of mice of other cause than GN, the mortality curve includes only mice that developed typical GN with a score of 3+ or more (Fig. 1B).

GA treatment does not influence anti-erythrocytes antibody production and Hct

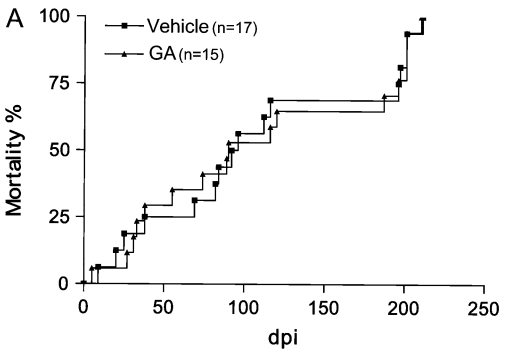
Auto-antibodies are essential factors for several clinical manifestations associated with SLE (14). The hybrid mice used in this study develop auto-antibodies directed against erythrocyte (RBC) which induce anemia that can be detected during the three to four first months of life. The production of Coombs antibodies was assessed in (NZB × BXSB)F1 male mice. No significant difference of anti-RBC antibodies was found at the age of 4 months (i.e. 1 month post-treatment injection) between GA- (n = 18) and vehicle-treated (n = 19) mice (anti-RBC ng ml^{-1} ± SEM; 0.3 ± 0.04 versus 0.45 ± 0.06; P = ns) (Fig. 2A). The anti-RBC antibodies were increased in the GA- and vehicle-treated groups when compared with 2-month-old (NZB × BXSB)F1 mice (anti-RBC ng ml^{-1} ± SEM; 0.15 ± 0.02; $P < 0.05$). Hct was measured in parallel to Coombs test. Hct values tested at the age of 4 months (i.e. 1 month post-treatment injection) were not different between GA- (n = 18) and vehicle-treated (n = 19) mice (Hct% ± SEM; 41.9 ± 1.2 versus 39.7 ± 1.1) but were significantly decreased as compared with those of 2-month-old mice ($P < 0.05$) (Fig. 2B). Anti-RBC antibodies and Hct were also measured at the age of 5 months (i.e. 2 months post-treatment injection) with no significant differences between GA- and vehicle-treated groups (data not shown).

Total IgG and subclasses IgG1 and IgG2a anti-chromatin and anti-dsDNA production are not altered by GA treatment

NZB mice are genetically predisposed to develop auto-antibodies that provoke tissue lesions as a result of their deposition as immune complexes in renal glomeruli and vessels (30, 31). To investigate whether GA treatment influences the production of auto-antibodies, total IgG, IgG1 and IgG2a subclasses against chromatin were tested in the serum of 4-month-old mice (i.e. 1 month post-treatment) by ELISA in GA- (n = 19) and vehicle-treated groups (n = 18). Compared with the vehicle-treated group, total IgG anti-chromatin titers were decreased in the GA group although a significant difference was not reached (median ± SEM; 19.1 ± 4.5 versus 37.0 ± 10.9 IgG U ml^{-1}) (Fig. 3A). Total IgG anti-chromatin from both GA- and vehicle-treated groups was significantly increased when compared with the control consisting of 2-month-old mice (before disease development) ($P < 0.05$). To further analyze IgG subtypes, anti-chromatin IgG1 and IgG2a were tested. IgG1 anti-chromatin titers did not show any significant difference when GA was compared with the vehicle-treated group and when both groups were compared with controls [GA versus vehicle treated versus control; 97.1 ± 20.0 versus 92.3 ± 30.6 versus 53.4 ± 6.9 U ml^{-1} (Fig. 3B)]. IgG2a anti-chromatin was significantly increased in both the GA and vehicle groups when compared with the control group (IgG2a from GA versus vehicle versus control; 12.1 ± 3.6 versus 21.2 ± 5.5 versus 2.3 ± 0.6 U ml^{-1}; GA or vehicle versus control, $P < 0.05$; GA versus vehicle, P = ns) (Fig. 3C). IgG, IgG1 and IgG2a against chromatin were also tested in the same groups at the age of 5 months (i.e. 2 months post-treatment initiation) with similar results (data not shown). Similar results were obtained when testing the anti-dsDNA IgG, IgG1 and IgG2a (Fig. 3D–F).

Monocytosis observed in (NZB × BXSB)F1 is not modified by GA treatment

Monocytosis is a unique cellular abnormality associated with the *Yaa* mutation (24). Monocytes reach a frequency of 50% of PBMC in 6- to 8-month-old BXSB male mice and have been associated with the rapid progression of SLE in this



B

	Glomerulonephritis grading	
	+++	++++
GA-treated (n=15)	9	6
Vehicle-treated (n=17)	14	3

Fig. 1. Mortality rate of GA- or vehicle-treated (NZB × BXSB)F1. Mice were injected at the age of 3 months with GA 150 μg s.c. or vehicle only. Injections were performed once a day until death (dpi = day post-injection). (A) No difference of mortality is observed when GA-treated group is compared with vehicle-treated group (P = ns). (B) Only mice that developed GN histology-proven graded 3 or 4 were included in the Kaplan–Meier survival curve (n = number of mice with GN histological grading 3+ or 4+).

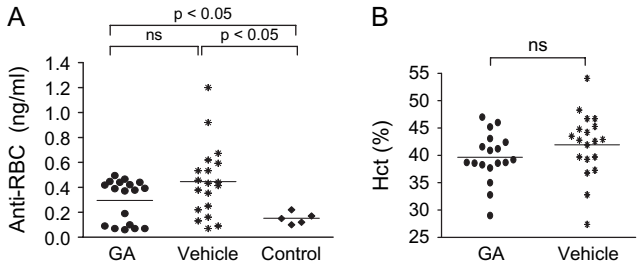


Fig. 2. Assessment of anti-erythrocyte (anti-RBC) antibodies and Hct 1 month post-treatment onset. (A) Anti-RBC antibodies were tested by the Coombs method following standard procedure. Negative control consisted in 2-month-old (NZB × BXSB)F1 mice (i.e. before disease onset). Anti-erythrocyte antibodies were significantly increased 1 month after treatment initiation in both GA- and vehicle-treated groups when compared with negative control ($P < 0.05$). When comparing GA- to vehicle-treated group, no difference was found (P = ns). Similar results were observed at month 2 post-injections (data not shown). (B) Hct was tested in both GA- and vehicle-treated groups after 1 month of treatment. Mice in both treated groups presented with Hct under 40% but no significant difference was observed between the groups.

animal model. The level of monocytosis was analyzed by FACS in both treated groups at the age of 5 and 6 months (i.e. 2 and 3 months post-treatment initiation; GA $n = 6$, vehicle treated $n = 7$). Controls consisted of 2-month-old mice of the same strain (i.e. before disease onset). The median value of monocytes expressed in % of total PBMC for GA- versus vehicle-treated mice was not different at both the second (median ± SEM; 35.4 ± 23.8% versus 35.5 ± 17.3%) and the third month post-treatment initiation (median ± SEM; 35.0 ± 10.9% versus 47.2 ± 16.8%) (Fig. 4A and B). When compared with the control group (median monocytes ± SEM; 9.1 ± 0.8%), both monocytosis of GA- and vehicle-treated groups were considerably increased at the two time points ($P < 0.05$). Monocytosis was defined as described in a previous report (25) (dotted line, Fig. 4A and B).

Discussion

Over the last three decades, despite intensive investigation of the disease and its mechanisms, there has been a lack of major improvements in the treatment of SLE. Genetic predisposition, environmental factors and complicated interactions within the immune system all contribute to the complexity of this autoimmune disease. With the characterization of several animal models, however, investigators were able to primarily focus on the mechanisms of action underlying the immunological pathophysiology. Results suggest that the autoimmune process in lupus-prone animals may be initiated by small numbers of B cells that have a low threshold for activation against self-antigens (15, 25). Therefore, it should be therapeutically beneficial to block the initial autoreactive responses as well as the activation of the downstream cascades. A unique cellular abnormality associated with the *Yaa* mutation in the lupus-prone mouse strain used in this study is an impressive monocytosis (24). The frequency of monocytes can reach over 50% of all leukocytes in 6- to 8-month-old BXSB male mice. Although the real significance of monocytosis in lupus disease remains to be determined, the development of a progressive monocytosis in this strain is associated with the rapidity of SLE development suggesting that T cells, B cells and auto-antibodies are not the unique trigger of disease progression.

Recent insights derived from studies on the mechanism of action of GA show a pivotal role of monocytes in the modulation of the immune system and highlight the importance of these cells as a target for pharmacologic intervention in autoimmune diseases (7, 12, 13). These results suggest that GA might be useful for autoimmune diseases other than MS. The pathogenesis of SLE is different from MS, although some similarities can be found. First, both diseases are considered to be triggered by a deregulation of the immune system and no definite putative antigen has been found in either situation. Both SLE and MS pathogenesis are in part driven by T cells and APC (32–34) and up to 20% of SLE have CNS involvement. In addition, anti-nuclear antibodies are characteristic for SLE and can be found in up to 30% of MS patients. Preliminary data show that rituximab, a monoclonal chimeric antibody directed against CD20 and depleting B cells, can be beneficial in both SLE and MS (35–37). A major difference between the two diseases it that SLE, more than MS, is driven by auto-antibodies and immune complexes that activate the complement pathway as confirmed by kidney histopathology. Contrary to MS, specific auto-antibodies such as anti-dsDNA can be detected in the serum of SLE patients. In MS, the pathogenesis is mostly directed by autoreactive T_h1 T cells (34), although antibody- and complement-mediated demyelination are considered as possible co-pathogenic factors in >50% of all MS patients (38).

The animal model of SLE and MS also differs in regard to their pathogenesis although both models are driven by a strong T_h1 autoimmune response. The pathogenesis of lupus-prone (NZB × BXSB)F1 mice is mainly mediated by auto-antibodies and an intrinsic B cell defect (39), whereas chronic EAE induced in C57BL/6 mice is mostly mediated by generation of encephalitogenic T_h1 and T_h17 T cells, with only a minor role for auto-antibodies associated with demyelination. NZB mice are genetically predisposed to develop Coombs-positive hemolytic anemia, splenomegaly and auto-antibodies (31). To determine the effect of GA on the progression of lupus mice's disease, we used the mouse hybrid (NZB × BXSB)F1 characterized by the strength and rapidity to develop clinical signals of SLE. In these mice, the disease onset can be present at 3 months, 50% of the population generally die within the first 5 months (40).

The hypothesis of our study was that GA, through the modulation of APC, such as monocytes and subsequently alteration of the T cell phenotype may inhibit the progression of the disease. Our data demonstrate that GA treatment initiated at disease onset (3 months of age) and continuously administered until death exerted no effect on mortality as the primary clinical readout. Further biological examinations, including standard controls of disease progression such as rise of anti-erythrocytes antibodies (Coombs test) and Hct, showed no significant difference either. Additional measurements of humoral and cellular markers of disease progression were performed. Anti-chromatin and anti-dsDNA IgG can provoke tissue lesions due to their deposition as a form of immune complex in renal glomeruli and vasculitis (14).

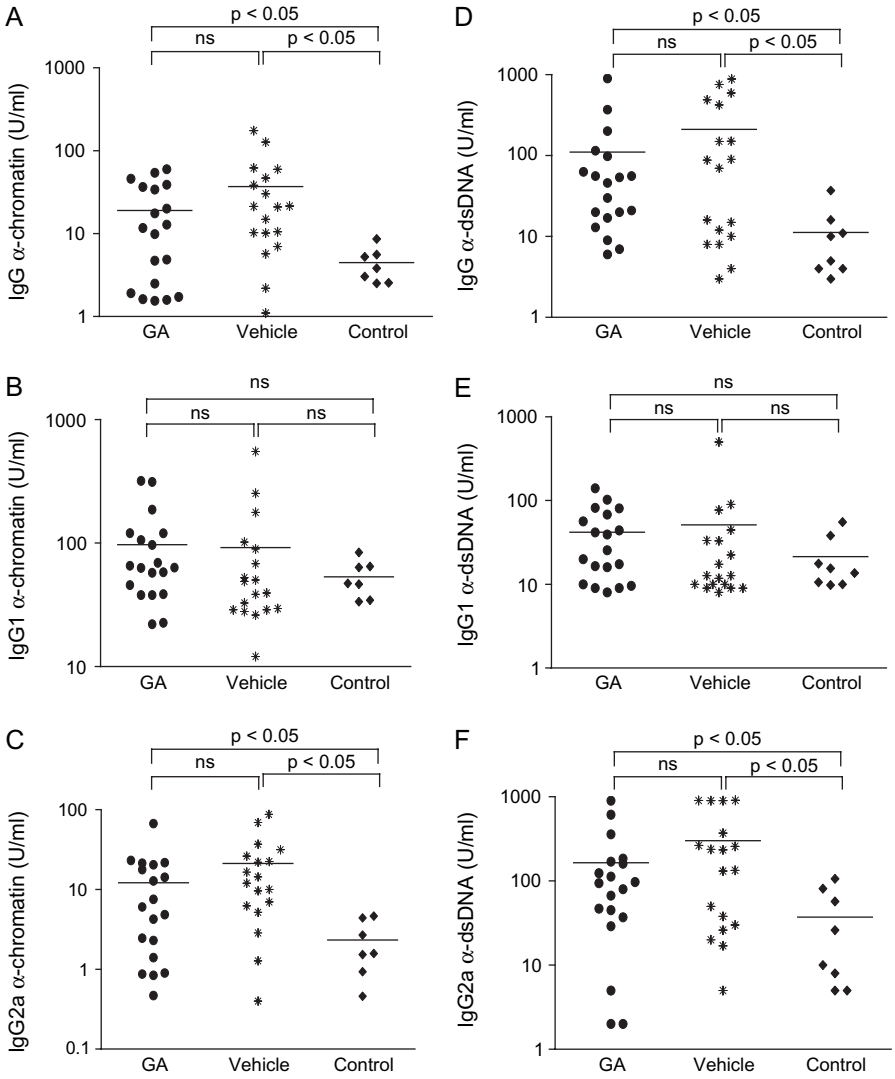


Fig. 3. Anti-chromatin and anti-dsDNA IgG subclasses examination 1 month after treatment onset. (A) Total anti-chromatin IgG is increased in the GA-treated group although significance is not reached when compared with vehicle-treated group (P = ns). Difference is significant when both treated groups are compared with control group ($P < 0.05$). (B) Anti-chromatin IgG1 subclass is similar in both treated and control groups. (C) Anti-chromatin IgG2a subclass is increased in both GA- and vehicle-treated groups but inter-group comparison is not different. Similar results were obtained when IgG, IgG1 and IgG2a against dsDNA (D–F) are tested in the serum 1 month after treatment onset. IgG, IgG1 and IgG2a against chromatin and dsDNA were also tested in the same groups 2 months post-treatment initiation with similar results (data not shown).

The influence of GA in switching and synthesis of IgG subclasses in EAE mice is unknown. In a clinical study on GA-treated MS patients, IgG1, and to a lesser extent IgG2, were found to be increased in the serum. GA is known to promote development of T_h2 cell. Thus, in our study, a GA-dependent modulation of anti-chromatin and anti-dsDNA IgG subclasses from the pathogenic IgG2a (T_h1 dependent) to the less pathogenic IgG1 (T_h2 dependent) subclass was anticipated. Total anti-chromatin and anti-dsDNA IgG, IgG1 and IgG2a subclasses were tested by ELISA 1 and 2 months post-treatment initiation. Total IgG and the predominantly pathogenic IgG2a auto-antibodies (41) increased over time in both GA- and vehicle-treated groups, with no difference between the two groups.

The progressive monocytosis observed over time in (NZB × BXSB)F1 mice was not modified in GA-treated mice as shown on Fig. 4. Recent data showed that GA could promote type II monocytes that induced Treg specific for a variety of antigens. Adoptive transfer studies demonstrated that Treg specific for a foreign antigen could ameliorate EAE, indicating that neither GA specificity nor recognition of self-antigen was required for their therapeutic effect (13). A specific Treg population was not analyzed in our study, but our results show that the frequency of monocytes was not altered by GA during lupus disease progression and that a possible effect of GA on monocytes, such as cytokine production or T cell modulation, did not modify the clinical course of the mice.

Many hypotheses can be raised about the absence of efficacy of GA in these lupus-prone mice. First, the animal model used in this study is one among several spontaneous, transgenic or toxic SLE animal models (42–44). Thus, the failure

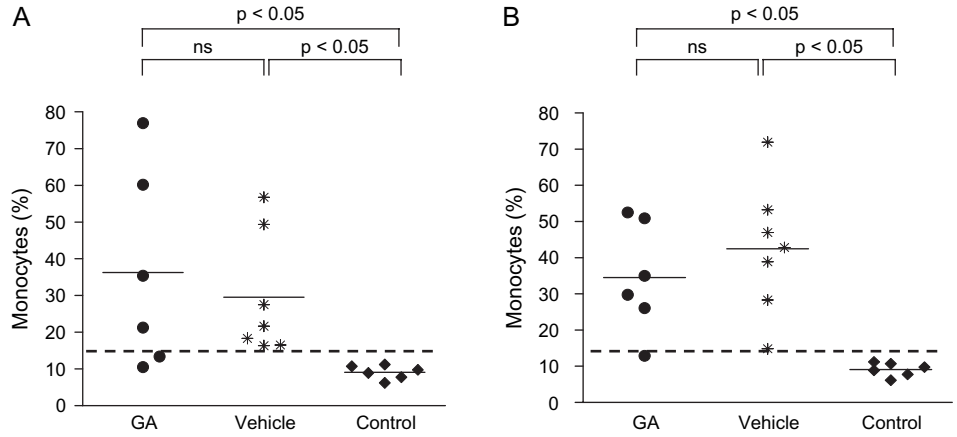


Fig. 4. Monocytosis examination in GA- and vehicle-treated mice after treatment initiation. The median value of monocytes in PBMC for GA- versus vehicle-treated mice is not different for both the second (A) and the third (B) month post-GA treatment (P = ns). Both treated groups demonstrate the presence of monocytosis when compared with control group consisting of 2-month-old (NZB × BXSB)F1 mice ($P < 0.05$). Dotted line = mean value ± 3 SD of monocytes in control group (C57BL/6 mice).

of GA treatment in preventing lupus progression in (NZB × BXSB)F1 mice cannot be taken as a general inefficacy of GA treatment in lupus animal models. Second, GA is known to induce T_h2 T cell that may potentially stimulate B cells to secrete auto-antibodies and favor SLE progression. Third, GA treatment was injected at an identical concentration as performed in EAE models and it cannot be excluded that another dose may provide different results. However, GA has been effective in the treatment of other models of autoimmune diseases including uveoretinitis (45), inflammatory bowel disease (46) and graft rejection (47). In addition to autoimmune diseases, pre-clinical data also suggest that GA may have a beneficial effect for Alzheimer's disease (48, 49). In conclusion, this is to the best of our knowledge the first report assessing the effect of GA in the treatment of a lupus animal model and results suggest, at least in the (NZB × BXSB)F1 lupus mice model, the absence of efficacy.

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Abbreviations

APC	antigen-presenting cell
CNS	central nervous system
dsDNA	double-stranded DNA
EAE	experimental autoimmune encephalomyelitis
GA	glatiramer acetate
GN	glomerulonephritis
Hct	hematocrit
MS	multiple sclerosis
s.c.	subcutaneous
SLE	systemic lupus erythematosus
Treg	regulatory T cell
Yaa	Y-linked autoimmune acceleration

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III) CONCLUSION AND PERSPECTIVES

Conclusion

The main project of this thesis is represented by the study of the immunomodulatory effects of HGF in EAE. The first part of the conclusion will describe the collaborative projects, the second part will address the main project and the third part concerns the perspectives of the main project based on preliminary data.

Collaborative projects:

Six collaborative projects were addressed during my thesis. All these collaborations concern immune mechanisms involved in MS, EAE or closely-related animal model of autoimmunity.

In our first collaboration, we showed that continuous PTX treatment in mice induce protection from active EAE, via the induction of Treg cells and increase serum level of TGF-β and IL-10. Besides its therapeutic implication, this finding indicates that some microbial products may not only be involved in the pathogenesis of CNS autoimmune disease but also in its regulation. (Martin *et al.* In submission).

In a subsequent project we assessed the effects of human IL-21 antagonist in terms of prevention and treatment of EAE. We showed that mice treated with IL-21 antagonist were protected against EAE. We showed also that IL-21 antagonist had a dual effect in that it enhanced both inflammation and T-cell response in a murine model of Th1 contact hypersensitivity. These findings confirm conclusively that IL-21 plays a part in innate and adaptive immune response. Targeting of IL-21 could become a new means of inhibiting and preventing autoimmunity, but its mode of action should be carefully evaluated as it may enhance inflammation like it did in the model of contact hypersensitivity. (Djaafar *et al*. In submission).

We were also interested to better understand the immunomodulatory effect of two therapeutic molecules used in MS, GA and IFN-β.

We first examined the effects of GA on the IL-1 system. *In vivo* and *in vitro*, GA treatment enhanced sIL-1Ra and diminished the expression of IL-1β. Indeed, in control experiments using LPS activated monocytes, IL-1β and sIL-1Ra production were increased in the presence of GA. These results demonstrate that, in chronic inflammatory conditions, GA enhances circulating sIL-1Ra levels and directly affects monocytes by triggering a bias

toward a less inflammatory profile, increasing sIL-1Ra while diminishing IL-1β production. This study sheds light on a mechanism that is likely to participate in the therapeutic effects of GA in MS (170).

In another publication, we investigated whether GA treatment may affect Th1 differentiation at various T-cell maturation stages. Specifically, we analyzed the effect of *in vivo* GA treatment on intracellular synthesis of IL-2 and TNF by naïve, memory and effector CD4 T cells. Our data indicate that GA treatment downregulates/normalizes an accelerated Th1 differentiation of CD4 T cells in RR-MS patients at all stages of T cell maturation. Most notably, we conclude that, by altering naïve, unprimed CD4 T cells, GA treatment appears to affect T cell differentiation, at least in part, in an antigen-independent manner (409).

We were also interested to assess the expression of the BDNF in PBMC from RR-MS patients treated or not with IFN-β. We showed higher levels of BDNF in PBMC of IFN-β-treated versus non-treated patients, whereas serum levels of BDNF were similar. We hypothesize that the increased intracellular BDNF secondary to IFN-β is not released in the periphery. This release is probably not tissue specific but in MS patients, BDNF could be specifically delivered by PBMC at the site of re-activation, i.e. within the CNS (165).

Finally, we assessed whether GA may be beneficial in (NZB x BXSB)F1 male bearing Y-linked autoimmune acceleration systemic lupus erythematosus. We showed that GA has no effect on the progression of this spontaneous model, which is known to be associated with a monocytosis accelerating disease progression, anti-chromatin, anti-double-stranded DNA, anti-erythrocytes Abs, and hematocrit. Our study demonstrates that GA has no significant effect on these parameters. These results reinforce the hypothesis that GA may exert its beneficial effect in some specific autoimmune diseases only (410).

Main project:

The following discussion will address the main project, i.e. the immunomodulatory effects of HGF in EAE. Both HGF and its receptor c-Met are expressed during brain development and persist in the adult (358, 411). c-Met is expressed in neurons but also in other brain-resident cells like oligodendrocytes, astrocytes and microglia (376-378). The global immunomodulatory effect of HGF is described to be of anti-inflammatory nature, although some pro-inflammatory effects have been described. On the one hand, HGF was initially described to increase adhesion and migration of inflammatory cells of both the adaptive and the innate immune system (354, 355). On the other hand, more recent reports

revealed several anti-inflammatory effects of HGF, including: i) a Th2/Th3 bystander deviation with increase of TGF-β and IL-10 (358, 392), ii) inhibition of APC function (347, 358), iii) down-regulation of MCP-1 and RANTES chemokines (412), iv) blocking of NF-kappa B function (412). In addition, DCs, the APC type that is known to play a major role in EAE by priming naïve CD4 T cells to induce clinical disease development and CNS inflammation (263), can be tolerized by HGF (358). The study presented in this thesis describes the EAE clinical course inhibition effect of an overexpression of HGF in the CNS. To address this question, we used C57BL/6 mice carrying a HGF transgene under the control of NSE promoter (HGF-Tg mice) leading to selective overexpression of HGF by neurons in the CNS. In this model, there is no difference of HGF serum levels to those of WT littermate controls. Introduction of HGF under the control of the NSE promoter into mice lead to expression of HGF specifically in postnatal neurons of the CNS and subsequent extracellular secretion of HGF in the CNS where it can act on glial and immune cells. Using this experimental setup, the neural as well as the glial system were found to be physiologically normal during development and in the adult (378).

EAE induced either by immunization with MOG_{35-55} or by adoptive transfer of T cells from 2D2 transgenic mice (which express a T cell receptor (TCR) specific for MOG_{35-55}), was inhibited in HGF-Tg mice before peak disease was reached. Notably, the level of inflammatory cells infiltrating the CNS decreased in these mice, with the exception of Treg cell population, which increases. However, no significant influence of HGF was observed in the spleen and also in the lymph node, since the overexpression of HGF was in the CNS and not in the periphery. This inhibition of EAE was confirmed by CNS histology analyses, which show a decrease of inflammatory and demyelination lesions as well as a trend of decrease of axonal loss in spinal cord of HGF Tg mice. The reduction of inflammation in the CNS of HGF Tg mice was associated with a decrease of pro-inflammatory Th1 cytokines including TNF, IFN-γ, IL-12p70 and a strong increase of IL-10 anti-inflammatory cytokine and also IL-4 spinal cord content. *In vitro* functional assay (ASR) which reproduce immune response during EAE with DCs stimulated with MOG_{35-55} peptide treated or not with rHGF and co-cultured with CD4 T cells from 2D2 mice which express TCR-MOG specific, showed that HGF was a potent immunomodulatory factor that inhibits DC function through downregulation of their CD40 co-stimulatory molecule with a decrease of IL-12p70 secretion and also inhibition of T cell proliferation. Furthermore, DCs treated with recombinant HGF *in vitro* induced differentiation of IL-10-producing Treg cells, along with a decrease of IL-17-producing T cells and a downregulation of surface markers of T cell activation. Collectively,

these data indicated that HGF can inhibit the clinical course of EAE through DC tolerization and induction of Treg cell population.

In addition, our results suggested that HGF is a candidate of high interest for the development of new treatments for immune-mediated demyelinating diseases associated with neurodegeneration such as MS by combining potentially neuroprotective and myelin repair properties as well as immunosuppressive effects. Figure 7 illustrates the results of HGF effects in the CNS during EAE.

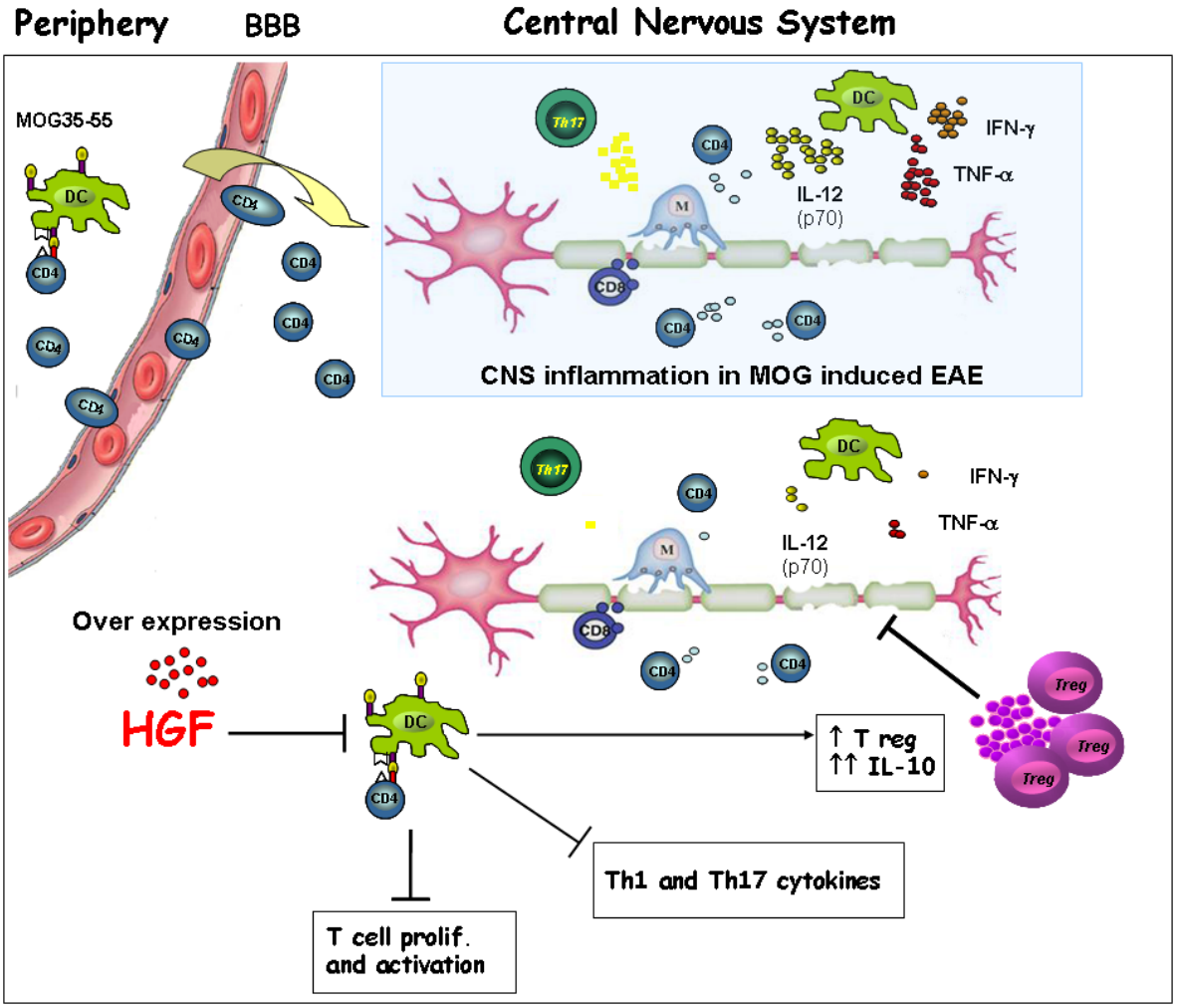

Figure 7: Summary of HGF effects during EAE.

Perspectives

Since not only CNS-resident DCs but also DCs present in secondary lymphoid organs express the HGF receptor (c-Met), we hypothesize that HGF-treated DCs present in the spleen or lymph nodes could inhibit the presentation of myelin antigen by DCs to T cells, and consecutively inhibit EAE severity. To address this question, we propose to use two different

protocols (HGF-containing microspheres and plasmid encoding HGF) to increase HGF levels in the systemic compartment and to observe whether EAE clinical course (induced by MOG immunization or T cell adoptive transfer) can be modified. In a further project we are exploring the signalling pathway of HGF-treated DCs to understand how these cells are tolerized by HGF. In addition, we are assessing how HGF-treated DCs modify the function of CD8 T cells, since only CD4 T cells were examined in our previous project. Finally, we will assess the function of the HGF/c-Met pathway in plasma cells, since these cells also express the c-Met receptor.

Study of a systemic increase of HGF in EAE

The main question that will be addressed here is to examine whether an increase of HGF levels, not in the CNS as previously investigated (413), but in the systemic compartment (periphery) can attenuate the clinical course of EAE. We believe that this question is of high interest since a direct (through immediate release of the HGF protein) or an indirect (through other protein/drugs than HGF) increase in peripheral HGF could be new approaches for MS treatment, whereas CNS increase in HGF through systemic release remains more difficult to obtain, mainly due to difficulties for the 82 kDa molecule to cross the blood brain barrier. Nevertheless, the objective of an artificial increase of peripheral HGF may be hampered by some specific tissues. For example, the liver is the major organ responsible for HGF clearance (402). If HGF is injected i.v., the protein is rapidly sequestered in the liver. In addition, HGF vanishes rapidly from organs; thus, it may be difficult to deliver enough HGF to the target tissue. In this regard, repetitive i.v. injection of HGF will probably not be sufficient to obtain a sustained increase of HGF in the systemic compartment. Thus, to ensure an effective and constant systemic delivery of HGF, we adopted two different protocols described to be effective in increasing HGF in other animal models of inflammatory diseases. The first protocol will use a slow-releasing form of recombinant murine HGF mounted into biodegradable gelatin hydrogel microspheres (403, 404). The second protocol will use systemic administration of plasmid DNA containing the HGF gene with a hydrodynamics-based transfection system (414). Both protocols will be evaluated with two different method of EAE induction, i.e. MOG_{35-55}-induced EAE and adoptive transfer of 2D2 (TCR-MOG Tg) splenocytes.

Study of signalling pathways in HGF-treated DCs

In this part, we plan to better understand intracellular signalling pathways activated in HGF-tolerized DCs. NF-κB pathway is known to be influenced by HGF and may explain part of its immunomodulatory effect (412). This pathway plays an important role in inflammation, including EAE and MS (1). But it is evident that the tolerating effect of HGF on APCs is certainly more complex than a restricted effect on NF-κB signalling. In addition, a major immunomodulatory effect of HGF-treated DCs is the induction of Treg population secreting the anti-inflammatory IL-10 as shown is this work. Here, we tried to explain the link between the strong Treg induction and IL-10 production in HGF-treated DCs, by assessing of the effect of HGF in The "glucocorticoid-induced leucine zipper" protein (GILZ) expression, witch is known to mediate the immunosuppressive effects of the glucocorticoids. GILZ plays a crucial role in controlling protein trafficking and signalling. Its overexpression down-regulates the Th1 and up-regulates the Th2 responses in glucocorticoid hormones treatment (415). GILZ blocks the NF-κB, MAPK, and AP-1 signal transduction pathways in several types of cells (416-419). Interestingly these pathways are involved also in HGF/c-Met signalling. The up-regulation of GILZ is associated with IL-10 increase in macrophages (416). In addition, GILZ modify DC phenotype (420) and induces differentiation of T cells into Treg population (421). Our preliminary data show that the up-regulation of GILZ was induced in HGF-treated DCs in a dose-dependent manner (Figure 8). Hence, GILZ may play an important role in the tolerizing effect of HGF-treated DCs associated with the differentiation of Treg cells to produce IL-10.

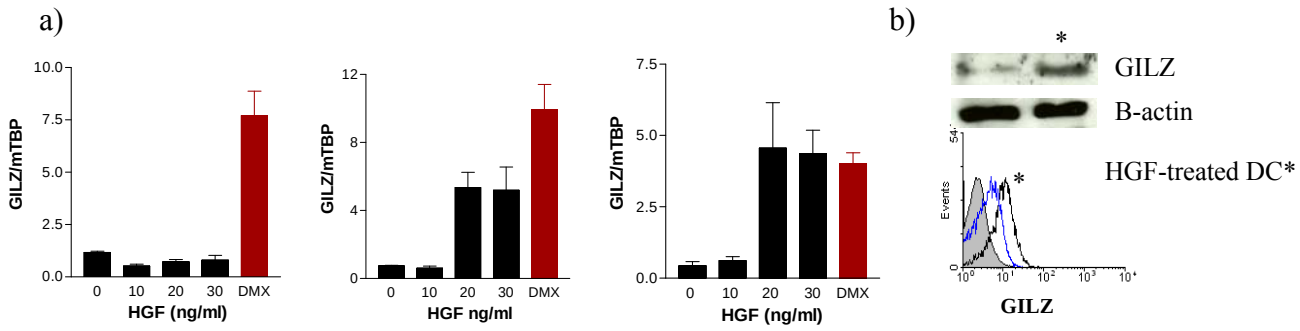

Figure 8: (a) DCs isolated from C57BL/6 and treated with increasing concentration of HGF (0-30 ng/ml); real-time RT-PCR of GILZ performed at different time points (6 to 24h). (b) Expression of GILZ assessed by western blotting and by FACS (HGF at 30 ng/ml) showed that GILZ is up-regulated in HGF-treated DCs. DMX (dexametasone, 10 E-7 Mol) is the control for GILZ induction (preliminary data).

In further experiments, we examined the spinal cord content of GILZ in MOG-induced EAE in HGF-Tg mice versus controls. As preliminary data, we showed elevated mRNA expression of GILZ from inflammatory cells of spinal cord HGF Tg mice compared with WT at peak disease (Figure 9). This increase of GILZ correlates with a decrease of EAE severity and a CNS increase of both Treg cells and IL-10.

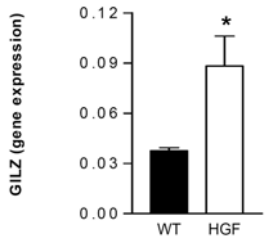

Figure 9: Real-time RT-PCR of GILZ expression from immune cells extracted from the spinal cord of HGF-Tg vs WT mice at peak disease in MOG-induced EAE. A significant increase of GILZ is detected in the spinal cord of HGF-Tg mice.

To confirm that the induction of GILZ plays a major role in the tolerization of DCs by HGF and that GILZ is directly linked to the differentiation of Treg cells and IL-10, we will inhibit GILZ expression in DCs by transfection with small interfering RNA (siRNA) *in vitro* in functional assay (ASR). With these experiments, we will observe whether EAE protection can be reversed by adoptive transfer of HGF-treated splenocytes pre-treated *ex-vivo* with GILZ siRNA.

Role of HGF in the inhibition of CD8 T cell function

We showed in this thesis that an overexpression of HGF in the CNS was associated not only with a decrease of CD4 but also CD8 T cells. It is known that CD8 T cells play an important role in MS pathogenesis (422) and can initiate the disease in some EAE models (189). In addition, neuroectodermal cell, including neurons and oligodendrocytes, express MHC class I and are therefore potential targets for cytotoxic CD8 T cells in MS (423-425). Modulation of MHC class I expression is found in areas of inflammatory activity and MHC class I-mediated immunity plays an important role in MS (426, 427) and EAE pathogenesis (428, 429).The immunomodulatory effect of HGF on CD8 T cells has not been addressed to our knowledge. Here, we started to assess whether HGF may specifically modulate the CD8 T cell response. We performed an *in vitro* model of peptide presentation via MHC class I. We used Pmel-1 TCR Tg mice recognizing the H-2Db-restricted epitope from gp100 ($gp100_{25–33}$) (430), a specific peptide of lymphoma cells. *In vitro* functional assays (ASR) using similar

protocol as described in result section were performed with CD8 T cells instead of CD4 T cells. CD8 T cells were purified from Pmel-1 mice and not from 2D2 mice, and DCs were loaded with gp100 peptide (Figure 10). These preliminary results showed anti-proliferative effect of HGF on CD8 T cells associated with a decrease of CD8 T cell-activation.

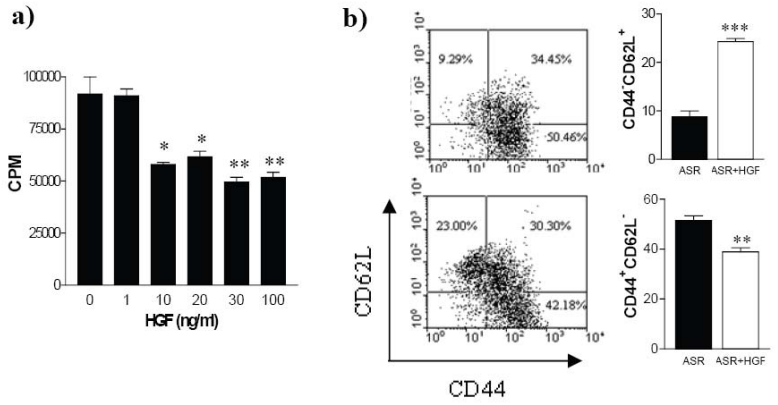

Figure 10: Proliferation assay of CD8 T cells with [^{3}H] thymidine incorporation. (a) HGF-treated DCs co-cultured with CD8 T cell induced a decrease of CD8 T cell proliferation. (b) FACS analysis of cell surface molecules showed a decrease of the level of CD8 T cells activation (decrease of $CD44^+CD62^-$ CD8 T cell population and increase of $CD44^-CD62^+$ CD8 T cell population) (preliminary data).

It will then be interesting to explore the immunomodulatory effect of HGF on CD8 T cells, by assessing whether HGF may modify the cytotoxic capacity of CD8 T cells. By this way we should increase our chance to demonstrate a possible immunomodulatory effect of HGF on CD8 T cell. If we will arrive to demonstrate that HGF modulate CD8 T cell pathogenicity, we will plan in the future further experiments on the role HGF-tolerized CD8 T cell in CNS autoimmunity.

Study of the effect of HGF in plasma cells function

A role of B cells and antibodies in the pathology of MS is strongly suggested (35). In case of inflammation, B cells, antibodies, and complement can cross the brain blood barrier and enter the CNS. Expression of c-Met on plasma-cell is known to play a role in cell migration and adhesion (431). In preliminary data (not shown in results section), we showed a different expression level of c-Met receptor on plasmablasts ($B220^+CD138^+$) and plasma-cells ($B220^-CD138^+$) (Figure 11) from mouse bone marrow. c-Met is more expressed in plasma-cell then in plasmablast, this fluctuation in expression may suggest an important role of HGF in development, survival or function of plasma-cells.

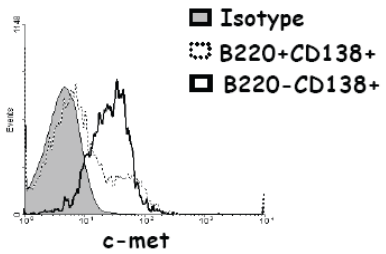

Figure 11: c-Met expression by FACS analysis is highly expressed in bone marrow-derived $B220^{-}$ $CD138^{+}$ plasma-cells and slightly expressed in a sub-population of $B220^{+}CD138^{+}$ plasmablast.

Significance of the perspectives

The follow up experiments that already started should first elucidate whether HGF, a strong neuroprotective factor with potential remyelinating properties and immunomodulatory functions in CNS autoimmunity, may also prevent EAE when administered in the systemic compartment. In parallel, we will develop several experiments to elucidate the mechanism of action of HGF in tolerizing DCs and to explore several other immunomodulatory functions of HGF in autoimmunity. HGF is, to our knowledge, a unique molecule that could potentially have a triple beneficial effect in immune-mediated diseases of the CNS, such as MS, based on neuroprotection, myelin repair properties and immunomodulatory effects. Since there is an urgent need for a compound that not only block inflammation in MS but also actively induces neuroprotection and neurorepair, we strongly believe that pursuing our studies on HGF in CNS autoimmunity is important. Based on the triple beneficial effect of HGF observed in EAE, we expect that this molecule could be further investigated in MS as a new therapeutic approach.

My contribution to the articles included in the thesis as collaborator

1) Repetitive pertussis toxin promotes development of regulatory T cells and prevents CNS autoimmune disease

I participated in this project by performing EAE studies. I injected the mice with the pertussis toxin every week and induced EAE with MOG peptide. I followed up the clinical score until the chronic phase of EAE. Then, I performed experiments including spleen cell proliferation, ELISA of IL-10, and FACS of regulatory T cells. This project was conducted over 18 months.

2) Opposite effects of IL-21 inhibition by a novel receptor antagonist in two murine models of autoimmune disease

My contribution in this project is summarized in the Fig. 3 which corresponds to the EAE part. I immunized mice with MOG peptide and I followed the progression of clinical score.

3) Glatiramer acetate increases IL-1 receptor antagonist but decreases T cell-induced IL-1β in human monocytes and multiple sclerosis

In this project, I treated mice with glatiramer acetate, induced EAE and followed the mice by taking clinical score daily.

4) Interferon-beta induces brain-derived neurotrophic factor in peripheral blood mononuclear cells of multiple sclerosis patients

In this project I performed part of the dosage of BDNF by western blot of PBMC in IFN-β treated and non-treated patients.

5) Inhibition of naive Th1 CD4+ T cells by glatiramer acetate in multiple sclerosis

In this project, I helped to isolate PBMC from RRMS patients and healthy donors. I also performed part of the FACS assays to analyse the different sub populations of T cells.

6) Glatiramer acetate treatment does not modify the clinical course of (NZB x BXSB)F1 lupus murine model

In this project, I treated lupus mice (NZB x BXSB)F1 with glatiramer acetate for 10 months, I helped to bleed the mice during this period, and also participated in Coombs analysis and ELISA.

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