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# Immunomodulation and immunopathogenesis in the autoimmune disease with emphasis on autoimmune neuritis and arthritis

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*To my family*

献 给 我 的 家 人

## ABSTRACT

Experimental autoimmune neuritis (EAN) and arthritis are $CD4^+$ T cell mediated autoimmune animal models for the study of immunomodulation and immunopathogenesis of human Guillain-Barré syndrome (GBS) and rheumatoid arthritis (RA). Inflammatory cell infiltration and cytokine production in the target organs are characteristic features of both diseases, suggesting a role of cytokine production in the pathogenesis.

A significant reduction in the incidence and severity of EAN and a delayed time of onset of EAN were found in IL-12 deficient ($IL\text{-}12^{-/-}$), as compared to wild type mice. The clinical symptoms were associated with a reduced IFN-$\gamma$ and TNF-$\alpha$, while enhanced IL-4 production in the sciatic nerve as well as significantly suppressed levels of anti-P0 peptide IgG2b antibody in serum suggested that IL-12 has a major role in the initiation, enhancement and perpetuation of pathogenic events in EAN by promoting a Th1 cell-mediated immune response and suppressing the Th2 response. These results demonstrate that IL-12 may play a critical role in the pathogenesis of EAN.

Tumor necrosis factor receptor I (TNFR I) is thought to mediate the majority of TNF activities. When administered soluble TNFR I (sTNFR I) to mice immunized with P0 peptide the severity and the duration of EAN were decreased. This was accompanied in vitro by a marked reduction in antigen-specific T cell proliferation and IFN-$\gamma$ synthesis by spleen cells in sTNFR I treated mice. Immunohistochemical analysis revealed a strong decrease in the number of infiltrating macrophages, $CD4^+$ T cells and $CD8^+$ T cells in the sciatic nerve. These data directly demonstrate a pivotal role for TNF in the development of EAN and also suggest that sTNFR I may have a therapeutic potential in human GBS.

CC chemokine receptor 5 deficient ($CCR5^{-/-}$) mice showed a significant reduction in the incidence of collagen-induced arthritis in comparison to wild-type ($CCR5^{+/+}$) mice. However, the severity score once they developed arthritis showed clinical features similar to wild-type mice. There were significantly lower levels of antibodies against CII in $CCR5^{-/-}$ mice compared to wild-type mice, especially IgG2a and IgG2b, and obviously higher levels of IL-10 in $CCR5^{-/-}$ mice. There was overproduction of MIP-1$\beta$ in serum and culture supernatant of spleen cells in CCR5 deficient mice after CII-immunization that might partly have contributed to the severity of arthritis. Our results indicate that CCR5 plays a role in the pathogenesis of arthritis, but its role can probably be substituted by other factors.

Changes of glia and cytokine expression were found in the spinal cord of adjuvant-induced arthritic (AIA) rats. Macroglia and MHC class II immunostaining were enhanced, and the numbers and immunostaining intensity of astrocytes expressing GFAP were increased. Using *in situ* hybridization and immunohistochemical methods, both mRNA and protein levels of IL-1$\beta$, IL-6 and TNF-$\alpha$ were significantly increased in the spinal cord of arthritic rats. Higher levels of cytokine expression were noted in reactive astrocytes and microglia.



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# 摘　要

实验性自身免疫性神经炎(EAN) 和关节炎是 $CD4^+$ T 细胞介导的自身免疫性疾病。它们分别是人的格林巴利综合症和风湿性关节炎的动物模型, 该模型用于研究自身免疫疾病的发病机理。这两种疾病的共同特点是靶器官炎性细胞渗出和某些细胞因子的异常表达, 因此提示这些细胞因子可能起到某些病理作用。

在 IL-12 缺陷($IL\text{-}12^{-/-}$) 鼠, 明显地降低了 EAN 的发病率和疾病的严重程度, 并且推 迟了发病时间。临床症状的减轻与坐骨神经(靶 组 织) 中 IFN-γ 和 TNF-α 的减少, IL-4 的增加有关。在 $IL\text{-}12^{-/-}$ 鼠, 抗神经髓鞘成份 P0 180—199 肽的 IgG2b 抗体明显地被抑制。这些结果表明 IL-12 在 EAN 的始发, 增强, 维持上起着重要的作用, 而这种作用是通过 IL-12 促进 T h 1 细胞介导的免疫反应和抑制了 T h 2 细胞免疫反应产生的。

肿瘤坏死因子(TNF) 的活性主要是通过肿瘤坏死因子受体 I (TNFR I) 介导的。 给予可溶性的 TNFRI ( s TNFRI), 可明显减轻 EAN 的临床症状, 并且缩短了临床过程。这是由于减少了抗原特异性的 T 细胞增殖和脾细胞的 IFN-γ 分泌。免疫组化分析表明, 在给予鼠 s TNFRI 以后，其坐骨神经中巨噬细胞, $CD4^+$ T 细胞和 $CD8^+$ T 细胞的浸润明显减少。这些结果直接证明了 TNF 在 EAN 中的重要作用, 同时也给 s TNFRI 用于人的格林巴利治疗提供了实验依据。

用 II 型胶原 (CII)诱导 CC 化学趋化因子受体 5 (CCR5) 缺陷鼠( $CCR5^{-/-}$ )的关节炎, 其发病率明显降低，然而一旦这些鼠发生了关节炎, 其临床表现与 $CCR5^{+/+}$ 鼠相似。在 $CCR5^{-/-}$ 鼠, 明显低的抗 CII 抗体, 尤其是 IgG2a 和 IgG2b 抗体和高水平的 IL-10, 可能与低的发病率有关。 在 $CCR5^{-/-}$鼠的血清和脾细胞培养上清中, 大量的 MIP-1β 可能与关节炎的严重程度有关。 这些结果表明, CCR5 在关节炎中起着一定的作用，但在疾病的后期它的作用很可能被其他因素替代。

发现有神经胶质细胞的变化和细胞因子的表达在佐剂诱导的关节炎鼠的脊髓中。用原位杂交和免疫组化的方法, IL-1β, IL-6 和 TNF-α 的mRNA 和蛋白水平明显增加在佐剂诱导的关节炎的脊髓中。这些细胞因子主要表达在激活的星形胶质细胞和少突胶质细胞中。

综合以上结果，我们认为，某些细胞因子和它们的受体如 IL—12，TNF，CCR5 在自身免疫性疾病中起着重要的作用，如格林巴利综合症和风湿性关节炎。阻断这些细胞因子可能对这些自身免疫性疾病有治疗作用。



博士论文



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## CONTENTS

## 1. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, which are referred to in the text by their Roman numerals.

I **Lei Bao,** J. Urban Lindgren, Peter van der Meide, Shun wei Zhu, Hans-Gustaf Ljunggren, Jie Zhu. The Critical Role of IL-12p40 in Initiating, Enhancing, and Perpetuating Pathogenic Events in Murine Experimental Autoimmune Neuritis. 2002, Brain Pathol. 12: 420-429.

II **Lei Bao,** J. Urban Lindgren, Yu Zhu, Hans-Gustaf Ljunggren, Jie Zhu. Exogenous soluble tumor necrosis factor receptor type I ameliorates murine experimental autoimmune neuritis. Neurobiology of Disease. In press.

III **Lei Bao**, Yu Zhu, Jie Zhu, J. Urban Lindgren. Decreased IgG production but increased MIP-1ß expression in collagen-induced arthritis in C-C chemokine receptor 5-deficient mice (Manuscript).

IV **Lei Bao**, Yu Zhu, Adlan M Elhassan, Qinyang Wu, Baoguo Xiao, Jie Zhu, J. Urban Lindgren. Adjuvant-induced arthritis: IL-1β, IL-6 and TNF-α are up-regulated in the spinal cord. 2001, NeuroReport 12:3905-3908.

## 2. ABBREVIATIONS

| | |
|---|---|
| Ab | antibody |
| AIA | adjuvant-induced arthritis |
| APC | antigen presenting cell |
| AT-EAN | adoptive transfer EAN |
| BNB | blood-nerve barrier |
| BPM | bovine peripheral myelin |
| CCR5 | C-C chemokine receptor 5 |
| CD | cluster of differentiation |
| CFA | complete Freund's adjuvant |
| CIA | collagen-induced arthritis |
| CII | type II collagen |
| CNS | central nervous system |
| CSF | clony stimulation factor |
| EAE | experimental autoimmune encephalomyelitis |
| EAN | experimental autoimmune neuritis |
| ELISPOT | enzyme-linked immunospot |
| ELLSA | enzyme-linked immunosorbent assay |
| GBS | Guillain-Barré syndrome |
| IFA | incomplete Freund's adjuvant |
| IFN | interferon |
| IL | interleukin |
| IP-10 | interferon-γ inducible protein 10 |
| mAb | monoclonal antibody |
| MCP-1 | monocyte chemoattactant protein 1 |
| MHC | major histocompatibility complex |
| MIP | macrophage inflammatory protein |
| MNC | mononuclear cell |
| mRNA | messenger ribonucleic acid |
| MS | multiple sclerosis |
| NK | natural killer cell |
| p.i. | post immunization |
| PBS | phosphate buffered saline |
| PHA | phytohemagglutinin |
| PNS | peripheral nervous system |
| PT | pertussis toxin |
| RA | rheumatoid arthritis |
| SCID | severe combined immunodeficient |
| SD | standard deviation |
| Th | T helper |
| TNF | tumor necrosis factor |
| TNFR | tumor necrosis factor receptor |

## 3. INTRODUCTION

Autoimmune diseases constitute one of the main unsolved problems in human clinical medicine. The reason is that their etiology and pathogenesis are still not sufficient towards specify therapy. Autoimmune diseases are considered to be pathological consequences of immune reactions directed towards autologous antigens. T lymphocytes would appear to be important for this immunoregulatory effect. Cytokines seem to have a key role in activated autoreactive T cells by providing the necessary signals to turn on/off T cell specific reactions to self-antigens. A widely held belief is that, when the cytokine profile of autoreactive T cells shifts toward an inflammatory T helper (Th) 1 type, the result is pathogenicity and autoimmune diseases (Liblau et al., 1995; Tian et al., 1998). The balance of functionally distinct T-cell subsets between Th1 and Th2, separated on the basis of their cytokine expression has a direct relevance to autoimmune disease. The Th1 cells are characterized by secretion of interferon-γ (IFN-γ), and they mainly promote cell-mediated immunity able to eliminate intracellular pathogens, and the synthesis of complement-fixing antibody isotypes. Conversely, the Th2 cells selectively produce interleukin (IL)-4 and are involved in the development of humoral immunity protecting against extracellular pathogens. An efficient immune response rests on a balance between the two cell types. Any alteration of this equilibrium would affect the quality of the immune response (Fig.1). Some studies suggest that Guillain-Barré syndrome (GBS) and rheumatoid arthritis (RA) are 'Th1-associated' diseases (Miossec and van den Berg, 1997; Hughes et al., 1999; Kiefer et al., 2001). Although, the factors that initiate and sustain Th1 responses in both diseases are largely unknown, it has become increasingly clear that a series of produced cytokines play a central role in disease progression (Falcone and Sarvetnick, 1999).

### 3.1. Cytokines and cytokine network

Cytokines are soluble proteins. A variety of cells can secrete cytokines, such as macrophages, T helper cells, B cells, dendritic cells, endothelial cells, and natural killer (NK) cells, etc. (Oppenheim and Feldmann, 2000). The cytokines can be classified into five groups that are either functionally related or are expressed by common cell types. These are the interleukins (ILs), the interferons (IFNs), the clony stimulation factors (CSFs), the chemokines and the tumor necrosis factor (TNF) family. Cytokines generally function as intercellular messenger molecules that evoke particular biological activities after binding to a receptor on a responsive target cell.

The binding of a cytokine to its receptor induces numerous physiological responses including the development of cellular and humoral immune responses, induction of the inflammatory response, regulation of hematopoiesis, control of cellular proliferation and differentiation, and promotion of wound healing (Oppenheim and Feldmann, 2000).

Over 150 cytokines have been identified and cloned (Oppenheim and Feldmann 2000). Cytokines are never expressed singly by a cell or tissue. Instead, an activated cell produces a wide spectrum of cytokines. Similarly all cells express receptors for many cytokines. Unlike hormones that are expressed constitutively, most cytokines are expressed transiently after an inducing stimulus. One of the most potent signals for inducing cytokines is other cytokines, and so the concept has arisen of a cytokine network in which cytokines induce or inhibit each other (Feldmann et al., 1996b). This accounts, in part, for the complexity of cytokine expression found at some diseased tissue sites such as the rheumatoid synovium. For example, it was found that anti-TNFα antibody reduced the production of IL-1, IL-6, IL-8 in the rheumatoid synovium, whereas IL-1 receptor antagonist reduced the production of IL-6, IL-8, but not of TNFα, which led to the notion of a network or cascade in these tissues. How this complex mixture of molecules, interacting with multiple cells is currently only partly understood, but it is becoming evident that deregulation of the cytokine network contributes in a major way to the pathogenesis and pathology of autoimmune diseases (Feldmann et al., 1996b; Gold et al., 1999; Falcone and Sarvetnick, 1999)

### 3.2. The Th1/Th2 dichotomy

Mosmann et al. first introduced this concept in 1986. They found that repeated antigen-specific stimulation of murine CD (cluster of differentiation) $4^+$ Th cells in vitro resulted in the development of restricted and stereotyped patterns of cytokine secretion profiles in the generated T cell populations (Mosmann et al., 1986). Based on their distinctive cytokine secretion pattern and effector functions, $CD4^+$ T cells have been classified into two major types. Th1 cells predominantly secrete IL-2 and IFN-γ, and are primarily associated with cellular immunity and class switching to the IgG2a isotype, whereas Th2 cells produce predominantly IL-4 and IL-5, and are mainly involved in humoral immunity and class switching to IgG1 and IgE (Mosmann and Coffman, 1989; Paul and Seder, 1994).

Th1 cells develop preferentially during infections with intracellular bacteria. Type 1 cytokines, associated primarily with Th1 responses, include IFN-γ, IL-2, IL-12, IL-15 and TNF. They activate macrophages to produce reactive oxygen intermediates and nitric oxide (NO), stimulate their phagocytic functions and enhance their ability for antigen presentation by up-regulation of major histocompatibility complex (MHC) class II molecules. Moreover, Th1 cells promote the induction of complement-fixing, opsonizing antibodies and of antibodies involved in antibody-dependent cell cytotoxicity, for example, IgG1 in humans and IgG2a in mice. Consequently, Th1 cells are involved in cell mediated immunity (Mosmann et al., 1986; Abbas et al., 1996).

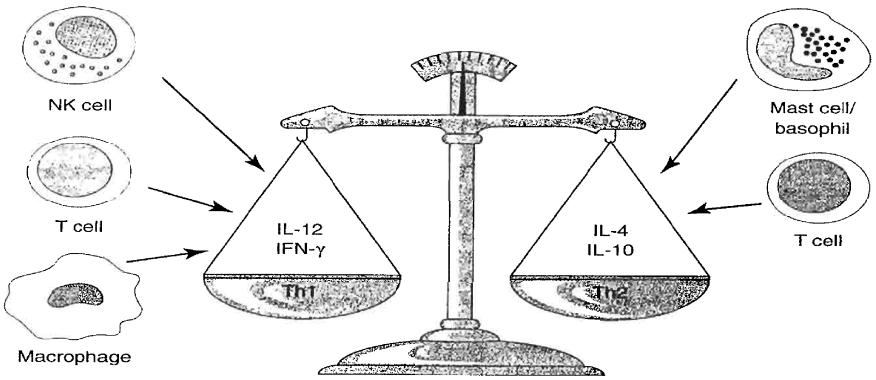


Fig.1. The Th1/Th2 cytokines balance and diseases

Th2 cells predominate after infestations with gastrointestinal nematodes and helminth. Type 2 cytokines, associated mainly with Th2 responses, include IL-5, IL-6, IL-10 and IL-13, in addition to IL-4 (Mosmann et al., 1986; Del Prete et al., 1991). They provide potent help for B cell activation and immunoglobulin class switching to IgE and subtypes of IgG that do not fix complement, for example, IgG2 in humans and IgG1 in the mouse. Th2 cells mediate allergic immune responses and have been associated with down-regulation of macrophage activation (Mosmann et al., 1986; Abbas et al., 1996). However, in contrast to the situation in mice, these

cytokines in humans are not confined to the Th2 subset but can also be produced by Th1 cells (Abbas et al., 1996).

Several factors, including the dose of antigen, the type of antigen-presenting cell (APC) and MHC class II haplotype, influence the differentiation of naive $CD4^+$ T cell into specific Th subsets. However, the best characterized factors affecting the development of Th subsets are cytokines themselves (Paul and Seder, 1994). Importantly, Th1 and Th2 cells antagonize each other (Fig.2). For instance, the generation of Th1 cells can be effectively blocked by high concentrations of IL-4, even in the presence of IL-12 (Hsieh et al., 1993). At the level of effector functions, IL-4 antagonizes much of the pro-inflammatory effect of IFN-γ and inhibits the proliferation of Th1 cells. Conversely, IFN-γ secreted by Th1 cells blocks the proliferation of Th2 cells (Falcone and Sarvetnick, 1999).

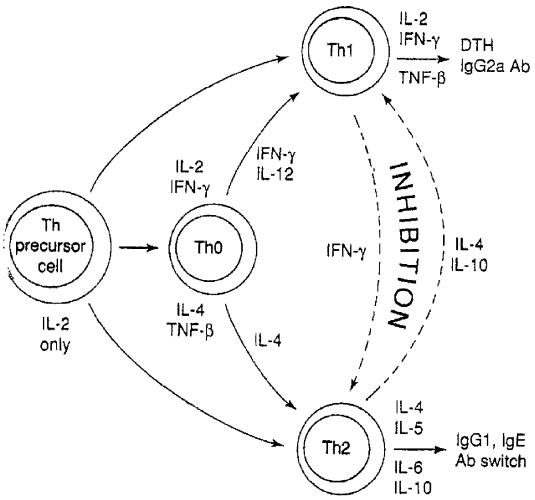


Fig.2 Diagram of the prominent regulatory interactions of cytokines between Th1 and Th2 cells

### *3.2.1. Th1 cytokines*

#### *IFN-γ*

Production of IFN-γ is the hallmark of Th1 cells. IFN-γ is produced mainly by activated T cells and NK cells and has receptors on virtually all cells of the human body. Thus, IFN-γ can exert a multitude of biological functions. The ability of IFN-γ to activate endothelial cells and

macrophages is the basis for defining IFN-γ as a proinflammatory cytokine. It increases the expression of MHC class II molecules and thereby enhances the cells' ability to present foreign antigens. IFN-γ production by T cells can be elicited by various stimuli, such as trauma or antigen-specific activation during infections or autoimmune diseases. Nevertheless, the potent pro-inflammatory activities of IFN-γ combined with its inhibitory potential for the development of Th2 cells make IFN-γ a central mediator of the signs and symptoms of chronic autoimmune inflammation (Boehm et al., 1997).

***IL-12***

IL-12 is a pleiotropic cytokine that is produced mainly by APC, such as dendritic cells, monocytes/macrophages and B cells in response to bacterial products and immune signal (Trinchieri, 1995). It enhances NK-mediated cytotoxicity and induces IFN-γ production by NK cells and T lymphocytes (Wolf et al., 1991). IL-12 plays a key role in promoting Th1 immune responses, as demonstrated both *in vitro* (Manetti et al., 1993) and *in vivo* (Sypek et al., 1993).

IL-12 plays an important role in the pathogenesis of some autoimmune diseases that are associated with aberrant Th1 activity. For example, antibodies against IL-12 have beneficial effects in experimental autoimmune diseases that are Th1-driven, such as experimental autoimmune encephalomyelitis (EAE), a model for multiple sclerosis (MS) in humans (Leonard et al., 1995) and 2,4,66-trinitrobenzene sulphonic acid-induced chronic intestinal inflammation in mice, a model for human inflammatory bowel disease (Neurath et al., 1995), murine collagen-induced arthritis (CIA) (Butler et al., 1999), Lyme arthritis (Anguita et al., 1996). Administration of IL-12 induced severe arthritis and increased the incidence of CIA (Germann et al., 1995), accelerated the onset of diabetes in non-obese diabetic mice (Trembleau et al., 1995) and increased inflammation and demyelination in chronic experimental autoimmune neuritis (EAN) in Lewis rats (Pelidou et al., 2000a). Thus, IL-12 appears to exert multiple immunoregulatory activities during the inflammatory and immune response.

***TNF and TNF receptors (TNFRs)***

TNF was initially characterized to have an antitumor activity and is a pleiotropic proinflammatory cytokine, mainly produced by activated macrophages and T cells, which mediates a wide range of biologic functions (Vassalli, 1992; Tracey and Cerami, 1993). TNF has

been found to play a pivotal role in serious acute or chronic inflammatory conditions and is thought to be involved in the pathogenesis of autoimmune and inflammatory disease (Tracey and Cerami, 1993). High levels of these molecules in body fluids and at sites of local inflammation have been associated with inflammatory processes such as RA, GBS, diabetes, and MS (Vassalli, 1992; Tracey and Cerami, 1993; Ma et al., 1998; Feldmann and maini, 2001).

The biological effects of TNF are mediated by binding to two distinct cell surface receptors. The receptor molecules are named according to their molecular weight as TNFR I (P55; about 55kDa) and TNF-R II (P75; about 75kDa) (Tartaglia and Goeddel, 1992; Smith et al., 1994). Both TNFRs are expressed on various cells types especially on the cell surface of activated CD4 and CD8 positive T cell subsets (Ware et al., 1991). After cell activation by TNF, they are cleaved by metalloproteinases and are found as soluble forms (sTNFRs) in serum and body fluids (Porteu and Nathan, 1990), which often function as TNF antagonists by competing with membrane-bound TNFR for ligand both in vitro and in vivo (Kohno et al., 1990). These receptors are thought to protect cells from TNF and to block the activity of this cytokine following its release into the circulation (Van Zee et al., 1992, Hunger et al., 1997). The results of some studies showed that sTNFRs are potent inhibitors of EAE (Baker et al., 1994; Selmaj et al., 1995a; b), autoimmune diabetes (Hunger et al., 1997), systemic lupus erythematosus (Studnicka-Benke et al., 1996), RA (Mori et al., 1996; McComb et al., 1999) and sepsis (Van Zee et al., 1992). Clinical trials with soluble TNFRs have demonstrated efficacy in human RA (Moreland et al., 1996; Moreland et al., 1997; Weinblatt et al., 1999; Feldmann and Maini, 2001). However, treatment with Lenercept, a recombinant TNF receptor P55 immunoglobulin fusion protein (sTNFR-IgG P55), failed to show benefit in MS patients, who showed a worsening of the disease (The Lenercept Multiple Sclerosis Study Group, 1999).

#### *3.2.2. Th2 cytokines*

***IL-4***

IL-4 is produced by activated T cells, mast cells, basopils, and eosinophils (Nelms et al., 1999). Its main functions on the immune system are its dominant ability to direct T cell differentiation into the Th2 subset (Seder et al., 1992; Hsieh et al., 1992) and its role in mediating immunoglobulin class switching to the IgG1 and IgE isotypes in mice (Vitetta et al., 1985; Coffman et al., 1986) and to the IgG4 and IgE isotypes in humans (Gascan et al., 1991). IL-4 is

the signature cytokine of Th2 cells and suppresses Th1 development while promoting Th2 generation. Over production of IL-4 has been associated with elevated IgE production and allergic diseases in vivo. Of importance in regulating immune responses is its ability to down-regulate the activation and inflammatory functions of monocytes and macrophages. Consequently, IL-4 has been used in vivo as a treatment of experimental autoimmune diseases. It is, to date, the most successful means for ameliorating autoimmune disorders that are caused by activated Th1 cells (Choi and Reiser, 1998; Nelms et al., 1999).

***IL-10***

IL-10 is produced by activated monocytes, NK cells, B cells and T cells (Kevin et al., 2001), and was discovered as a potent inhibitor of macrophage effector functions. In mice, IL-10 is clearly a Th2 cytokine. However, in humans IL-10 can be produced by both the Th1 and Th2 sub-sets (Del Prete et al., 1993; Beebe et al., 2002). IL-10 inhibits the production of pro-inflammatory cytokines by macrophages, such as IL-1β, IL-6, IL-8, IL-12 and TNF-α and up-regulates the production of IL-1 receptor-α and soluble p55 and p75 TNFRs (De Waal Malefyt et al., 1991a; Fiorentino et al., 1991a; D'Andrea et al., 1993). IL-10 inhibits also production of IL-2 and IFN-γ from T cells and blocks T cell proliferation (De Waal Malefyt et al., 1991b; Fiorentino et al., 1991b; De Waal Malefyt et al., 1993). Thus, IL-10 has potent anti-inflammatory functions and has, consequently, been used in the treatment of experimental autoimmune diseases with great success (Kevin et al., 1998; 2001).

### *3.2.3. Chemokine and chemokine receptors*

Chemokines (chemotactic cytokines) are low molecular weight (8-10 kDa) proteins which are classified into four families based on the position of the first two cysteine residues in the amino terminus: C, CC, CxC and Cx3C (Baggiolini, 1998; Luster, 1998; Locati and Murphy, 1999). Chemokines are multifunctional and play a major role in the perpetuation of inflammatory processes. They chemoattract a variety of inflammatory cells to sites of inflammation and injury. Chemokines also activate cells engaged in host immune responses, modulate hematopoiesis, promote foetal development, and regulate trafficking and homing of cells to appropriate tissue sites. In addition to their roles as chemoattractants, chemokines can also regulate T cell activation and differentiation as well as cytokine production (Rollins 1997; Ward et al., 1998; Zlotnik and Yoshie, 2000). Chemokines have been implicated in the regulation of normal

immune response and inflammation as well as certain physiological and pathogenic processes, including allergy, autoimmune disorders, and infectious diseases (Rollins, 1997; Ward et al., 1998; Zlotnik and Yoshie, 2000).

**Table 1. CC chemokines and their receptors**

| Chemokine receptor | Receptor expressing cells | Chemokine ligands | Major *in vivo* receptor activities |
|---|---|---|---|
| CCR1 | Activated T cells, monos, NK, immature DC, eos | MIP-1α, RANTES<br>HCC1, 2<br>MCP-2, 3, 4<br>MPIF-1 | Antifungal, antibacterial, and antiviral resistance<br>Mobilization of BM progenitors<br>Promotes mononuclear cell adhesion<br>Modulates myelopoiesis |
| CCR2 | Activated T cells, monos, basophils, immature DC, mast cells, eos | MCP-1, 2, 3, 4 | Chronic inflammation (TH1 > TH2)<br>Resistance to bacterial challenge<br>Promotes mononuclear cell adhesion<br>Histamine release, atherogenesis |
| CCR3 | Activated TH2 cells, monos, NK, basophils, eos | Eotaxin 1, 2<br>RANTES, MIP-1α<br>MCP-2, 3, 4,<br>HCC2 | Recruits eos in allergic states<br>Histamine release from basophils |
| CCR4 | Activated TH2 cells, NK | TARC, MDC | Favors TH2 responses |
| CCR5 | Activated TH1 > TH2 cells, monos, NK, immature DC | MIP-1α, MIP-1β, RANTES, MCP-2 | Coreceptor for M-tropic HIV-1<br>Enhanced antibacterial resistance<br>Favors TH2 responses |
| CCR6 | Resting memory T cells, immature DC, B cells, activated neutrophils | LARC (MIP-3α)<br>β defensins | Attracts immature DC peripherally<br>Activates resting memory T cells |
| CCR7 | Resting and activated TH1 cells, mature DC, B cells | SLC, ELC (MIP-3β) | Attracts naive T cells and mature DC to LN perifollicular areas |
| CCR8 | Activated TH2 cells, monos, B cells, immature DC | I-309, TARC, MIP-1β, HCC4 (LEC) | Promotes TH2 immune responses and chronic inflammatory reactions |
| CCR9 | Fetal thymocytes, monos, splenic DC, T cells | TECK | T cell development |

(Adapted from Howard et al., 1999)

Chemokines exert their functions through specific receptors. The final composition of leukocytes present in the inflamed sites is most likely due to both secreted chemokines and the relative expression of specific chemokine cell surface receptors on different cell types. A single chemokine can bind more than one receptor, and conversely, a given chemokine receptor can bind several chemokines (Locati and Murphy, 1999) (Table 1). The chemokine receptors may play a different role in various inflammatory reactions. For example, CC chemokine receptor 3 (CCR3) is mainly expressed by lymphocytes exhibiting the Th2, but not the Th1, phenotype, as in allergic lymphocytic infiltrates. By contrast, CC chemokine receptor 5 (CCR5), which is present on most Th1 but not on Th2-type cells, has been detected in RA synovial fluids and

tissues. CXCR3 is present on both Th1- and Th2-type cells, and this chemokine receptor is expressed in both allergic and synovial infiltrates (Qin et al., 1998).

### 3.3. Guillain-Barré syndrome (GBS)

Guillain-Barré syndrome (GBS) is an inflammatory demyelinating disease of the peripheral nervous system (PNS) in humans. The clinical features of GBS are progressive weakness and sensory dysfunction in the limbs, autonomic nerves as well as respiratory weakness. Most patients recover spontaneously and completely, but some of patients suffer subsequent relapses (McKhann, 1990). The annual incidence of GBS is between one and four cases per 100,000 throughout the world (Hughes and Rees, 1997). The pathogenesis of GBS remains poorly understood, but there is evidence that abnormal immune responses involving autoreactive T and B cells are implicated (Hartung et al., 1995; Giovannoni and Hartung 1996).

### 3.4. Experimental autoimmune neuritis (EAN)

Experimental autoimmune neuritis (EAN) is an experimental animal model of GBS and caused by immunization with heterogeneous peripheral nerve tissue, or their myelin proteins P2 or P0 or their peptides (Rostami et al., 1990; Zhu et al., 1998; Gold et al., 1999; Deretzi et al., 1999). EAN resembles many of the clinical, electrophysiological and immunological aspects of human GBS, which were first described by Waksman and Adams (Waksman and Adams, 1955). Hence, it has been widely used as a model to investigate disease mechanisms in inflammatory demyelinating disease of the PNS. Histologically acute EAN is characterized by infiltration of the nerve roots and peripheral nerves with macrophages and lymphocytes, and by primary demyelination associated with some axonal damage. Further autoantigens that have been identified in EAN models in rats and mice are P0 protein (Linington et al., 1992), myelin basic protein (MBP) (Abromson-Leeman et al., 1995), peripheral myelin protein (PMP) 22 (Gabriel et al., 1998), and myelin-associated glycoprotein (MAG)(Weerth et al., 1999).

The clinical signs of EAN in the mouse model (C57BL/6) of the study were weaker than in Lewis rats (Zhu et al., 1997a; 1998; 1999a). The C57BL/6 mice strain is reputed to be resistant to induction of EAN by several peripheral nerve myelin antigens, such as bovine peripheral myelin (BPM), and P2 protein or the P2 protein peptide 57-81. However, Zou (Zou et al., 2000b) found that the P0 peptide 180-199 is a stronger neuritogenic antigen than the P2 peptide 57-81 and that

intravenously administrated pertussis toxin (PT) had an adjuvant effect that increased the incidence of P0 peptide 180-199-induced EAN.

### *3.4.1. The role of T cells in EAN*

The central role of T cells in the pathogenesis of EAN was shown by adoptive transfer (AT-EAN) of a T cell line specific for P2 protein capable of inducing EAN in Lewis rats (Linington et al., 1984; Heininger et al., 1986) and also in Brown-Norway rats which are resistant to active EAN (Linington et al., 1986). Subsequently T cell lines specific for P2 peptides have been shown to transfer disease (Olee et al., 1990). The importance of T cells in EAN is further supported by the inability of T cell deficient animals to develop EAN upon active immunization (Brosnan et al., 1987; Zhu et al., 1999b) and by the prevention of EAN by treatment with antibodies (Abs) to the T-cell receptor (Jung et al., 1992). T cell infiltration is the first pathological sign in AT-EAN, and this infiltration is accompanied by a rapid increase in permeability of the blood-nerve barrier (BNB) (Harvey et al., 1994; Harvey et al., 1995; Hadden et al., 2001). A recent study indicated that the severity of clinical signs and histopathological manifestations of EAN in $CD4^{-/-}$ and $CD8^{-/-}$ were significantly lower than those in their wild-type counterparts, suggesting that the induction and control of murine EAN are dependent of both CD4 (+) and CD8 (+) T cells (Zhu et al., 2002). Once T cells have traversed the BNB, activated $CD4^{+}$ T cells can recognize a specific antigen in the context of MHC, and differentiate to secrete cytokines like IL-2, TNF-α, and IFN-γ. The reactivated $CD4^{+}$ T cells amplify the immune response by recruiting further T cells and macrophages via chemokines and cytokines (Hartung et al., 1996). The resulting breakdown of the BNB allows the passage of circulating autoantibodies that are thought to synergize with other cells to produce demyelination (Harvey et al., 1995; Pollard et al., 1995).

### *3.4.2. The role of B cells in EAN*

Antibodies against myelin components have been detected in the serum of EAN (Archelos et al., 1993; Zhu et al., 1994b). Although it was not possible to induce demyelination by PNS-specific antibodies, systemic administration of antibodies to galactocerebroside enhances the demyelination produced by adoptive transfer of neuritogenic T cells (Hahn et al., 1993). However, no B cell infiltrates were found in target tissue (Fujioka et al., 2000). Passive transfer of serum alone from an animal with EAN into a healthy animal does not provoke disease (Toyka and Heininger, 1987). There was no notable difference in clinical symptoms between B cell

knockout mice and wild-type mice after immunization with P0 peptide (Zhu et al., 2002). Thus, whether B cells are involved in the pathogenesis of EAN remains controversial.

### *3.4.3. The role of macrophages in EAN*

Macrophages are the predominant cell population in the infiltrates of EAN lesions (Hartung et al., 1988; Hartung and Toyka 1990a; Hartung, 1993; Jung et al., 1993). They also feature prominently in the lesions of GBS (Ho et al., 1998; Kiefer et al., 1998). Macrophages play a role as APC and as effector cells that destroy myelin. In EAN the primary MHC class II positive cells are mainly macrophages (Schmidt et al., 1990). The crucial role of macrophages in immune-mediated nerve damage were direct phagocytic attack on myelin, and the release of proinflammatory cytokines including TNF-α, IL-1 and IL-6 and other noxious molecules. If macrophages are depleted by intraperitoneal injection of sillica dust, animals are protected against the development of EAN, and the progression of disease is slowed (Craggs et al., 1984). This was confirmed by the use of dichlormethylene diphosphonate-containing liposomes that are cytotoxic for macrophages and reduce the severity of active and AT-EAN (Jung et al., 1993). Macrophages also contribute to recovery by promoting T cell apoptosis and secreting anti-inflammatory cytokines like IL-10 and TGF-β. Once inflammation has subsided they promote myelin repair and axonal regeneration (Kiefer et al., 2001).

### *3.4.4. The role of cytokines in EAN*

Abundant evidence suggests that Th1 cytokines including IFN-γ, TNF and IL-12 play pivotal roles in the pathogenesis of EAN (Hartung et al., 1992,1995; Zhu et al., 1998). The levels of IFN-γ, TNF-α, IL-12 producing cells in blood, lymph node and PNS tissue roughly parallel clinical EAN (Schmidt et al., 1992; Zhu et al., 1994a; c; 1996; 1997a). Administration of IFN-γ (Hartung et al., 1990), TNF-α (Said and Hontebeyrie-Joskowicz, 1992), IL-12 (Pelidou et al., 1999a; 2000a) markedly worsens EAN. Conversely, treatment with a monoclonal antibody (mAb) to IFN-γ (Strigard et al., 1989; Hartung et al., 1990b; Tsai et al., 1991), TNF-α (Stoll et al., 1993) ameliorates EAN. Furthermore, recombinant mouse IL-17-treated rats showed increased infiltration of inflammatory cells into the sciatic nerve, more severe demyelination, augmented proliferation of regional lymph node cells, and increased serum levels of TNF-α (Pelidou et al., 2000b). CD28-deficient mutant mice that were clearly resistant to EAN had fewer IL-12 producing cells in sciatic nerve sections and fewer IFN-gamma secreting splenic cells than

wild-type mice (Zhu et al., 2001a). The development of EAN was suppressed in IFN-γ receptor deficient mice, which is associated with fewer IL-12-producing but more IL-4-producing cells in sciatic nerve (Zhu et al., 2001b). Anti-IL-18 mAbs effectively ameliorate the clinical and pathological signs of EAN, which is associated with increased numbers of IL-4 expressing cells and decreased numbers of IFN-γ and TNF-α expressing cells in the PNS (Yu et al., 2002).

Th2 cytokines such as IL-4 and IL-10 play an important role in disease recovery. Maximal expression of IL-10 mRNA in lymph node mononuclear cells (MNC) and sciatic nerve sections was observed after clinical recovery from EAN (Zhu et al., 1997a; 1997b; Pelidou et al., 1999b). IL-10 mRNA appeared concomitantly with the proinflammatory cytokines at day 11 post immunization (p.i.), but persisted at high levels into the clinical recovery phase (Gillen et al., 1998). IL-4 peaked late in the course of EAN in lymph node (Zhu, 1994c;1997b). Treatment with IL-4 (Deretzi et al., 1999) and IL-10 (Bai et al., 1997a) amelioreates the inflammatory responses in EAN. Transfer of myelin-specific cells deviated in vitro towards IL-4 production ameliorates ongoing EAN (Ekerfelt et al., 2001). Some drugs that suppress EAN, such as solidum fusidate, Linomide and Rolipram are associated with enhancement of IL-4 and IL-10 (Di Marco et al., 1999; Zhu et al., 1999a; Zou et al., 2000a; Abbas et al., 2000; Zou et al., 2002).

Chemokines might also be involved in EAN (Fujioka et al., 1999a;b; Zou et al., 1999). Monocyte chemoattactant protein 1 (MCP-1), RANTES, interferon-γ inducible protein 10 (IP-10) and MIP-1α have been examined in EAN and it was found that RANTES and MIP-1α have similar kinetics of induction, correlating with the development of severe clinical signs (Fujioka et al., 1999a; b; Zou 1999; Kieseier et al., 2000). Administration of an anti-MIP-1α antibody suppressed the clinical signs of EAN and inhibited inflammation and demeylination in the sciatic nerves (Zou et al., 1999). The number of intraneural CCR2 positive cells followed the time course of the disease, peaking at the time of maximum of paralysis (Orlikowski et al., 2003). Moreover, increased cerebrospinal fluid levels of IP-10 and a consistent expression of several chemokine receptors have been observed in acute and chronic inflammatory neuropathies (Kieseier et al., 2002).

### 3.5. Rheumatoid arthritis (RA)

Rheumatoid arthritis (RA) is a common autoimmune disease, which is a chronic inflammatory disease that affects approximately 1% of the population in all parts of the world (Buckley, 1997). RA is characterized by chronic inflammation of synovial joints and progressive destruction of articular tissue. To date, the pathogenesis of RA is not fully understood, and treatment options are still limited to symptomatic and non-specific immunosuppressive therapies. RA is often regarded as a predominantly T cell-related disorder (Feldmann et al., 1996a; Feldmann, 2001). Much evidence favours the hypothesis that RA is an autoimmune disease. The clinical features are mostly due to inflammation and eventual damage to synovial joints of hands, feet, wrists, etc. In more severe cases, there is extra articular disease, and survival is impaired (Erhardt et al., 1989; Pincus and Callahan, 1993). The synovitis involves a massive leucocytic infiltrate chiefly consisting of macrophages, T lymphocytes, and plasma cells, and this is associated with augmented angiogenesis. The sites of major joint damage are where the synovium abuts the cartilage and bone.

### 3.6. Experimental arthritis

Arthritis induced by immunization with complete Freund's adjuvant (CFA) in rats was the first arthritic model (Pearson, 1956). Subsequently, several animal models of polyarthritis have been described which are adjuvant-induced models, cartilage antigen-induced models, spontaneously developing models and transgenic models.

#### *3.6.1. Adjuvant-induced arthritis*

The first reported principle model of experimental arthritis is adjuvant arthritis (AIA) in rats, described by Pearson (Pearson, 1956). It is induced by heat-killed mycobacteria suspended in mineral oil. The joint inflammation is characterized by accumulation of polymorphonuclear cells and MNC, pannus formation and cartilage destruction (Pelegri et al., 1995). Alternative adjuvant arthritis models are pristine-induced arthritis (Potter et al., 1981; Vingsbo et al., 1996), avridine-induced arthritis (Chang et al., 1980) and mineral oil-induced arthritis (Kleinau et al., 1991). These arthritic models are induced with adjuvants without any immunogen included. Interestingly, in spite of the fact that no immunogen is included, these models are T cell dependent (Holmdahl et al., 1992; Yoshino and Cleland, 1992; Kleinau et al., 1993; Vingsbo et al., 1995; Stasiuk et al., 1997).

### 3.6.2. Collagen-induced arthritis

Collagen-induced arthritis (CIA) was first reported by Trentham and colleagues who observed the disease in rats following a single intradermal injection of type II collagen (CII) emulsified in Freund's adjuvant (Trentham et al., 1977). Further studies demonstrated that a similar pathology could be induced in primates (Cathcart et al., 1986) and in susceptible strains of mice (Courtenay et al., 1980). CIA can be induced using native autologous or heterologous CII, which are specific to CII, since immunization with types I or III collagen fail to induce disease (Trentham et al., 1977; Courtenay et al., 1980). CIA shares many similarities with RA, such as the chronicity, large infiltrations of leukocytes in the joints, thickening of the synovial membrane with pannus formation and cartilage and bone destruction (Larsson et al., 1990).

#### 3.6.2.1. The role of T cells in experimental arthritis

The induction of CIA is associated with the dominant expression of a Th1 cytokine pattern, suggesting that the specific cellular type involved in disease is $CD4^+$ T cells (Mauri et al., 1996). The recipient mice develop arthritis after adoptive transfer of collagen-specific T cell lines (Holmdahl et al., 1985a), or $CD4^+$ T cells (Gumanovskaya et al., 1999; Plows et al., 1999). CIA can be attenuated by treatment with mAbs to CD4 and T cell receptor (TCR) (Williams et al., 1989; Goldschmidt and holmdahl, 1991; Chiocchia et al., 1991). In addition, T cells provide help to B cells for the production of arthritogenic anti-CII antibodies (Corthay et al., 1999) and T cells themselves are believed to play a role in joint inflammation through activation of other cells, e,g, synovial macrophages. However, disease induction has failed to provide a clear indication as to T cell involvement. CD4-deficient mice develop CIA with unaltered incidence and severity, whereas CD8-deficient mice showed a decreased incidence but unaltered severity (Tada et al., 1996).

#### 3.6.2.2. The role of B cells in experimental arthritis

The major role of B cells is production of arthritogenic anti-CII antibodies, which is clearly shown by the fact that antibodies reactive with CII can bind to cartilage and induce arthritis (Terato et al., 1992). A strong B cell response is activated in CIA, producing IgG directed towards CII-specific structures (Holmdahl et al., 1985b; 1990; Mo et al., 1994). Arthritis can be induced by transferring either concentrated anti-CII serum (Stuart and Dixon, 1983), purified anti-CII serum antibodies (Stuart et al., 1982) or anti-CII mAb (Terato et al., 1992) to naïve

recipient mice. B cell-deficient mice are resistant to CIA (Svensson et al., 1998), indicating a critical role for anti-CII antibodies in CIA. In contrast, some results indicated that levels of anti-CII autoantibodies in serum do not correlate with CIA development, as high levels of anti-CII antibodies can be detected in non-diseased mice (Holmdahl et al., 1986a; Reife et al., 1991).

#### *3.6.2.3. The role of a synergy of T and B cells in experimental arthritis*

Both B cells and T cells are considered to play a pathogenic role in collagen-induced arthritis (Terato et al., 1992; Hom et al., 1986; Svensson et al., 1998; Nakajima et al., 2001), but the question of which cell type acts as the initiator of the arthritic disease process remains controversial. A potent and long-lasting arthritis occurred after anti-CII antibodies had been transferred together with anti-CII $CD4^+$ T cells to T cell-depleted mice (Seki et al., 1988) or to severe combined immunodeficient (SCID) mice (Kadowaki et al., 1994), suggesting a synergy between B cells and $CD4^+$ T cells in CIA. CII-reactive T cells play a crucial role in the development of CII-induced arthritis and that anti-CII antibodies enhance the development of CII-induced arthritis. This conclusion comes from the finding that depletion of collagen-II reactive T cells and blocking of B cell activation prevents collagen II-induced arthritis in DBA/1j mice (Zhang et al., 2002).

#### *3.6.2.4. The role of macrophages in experimental arthritis*

Synovial macrophages are activated in the arthritic joints of animal models, and macrophage numbers correlate with disease severity (Holmdahl et al., 1991). The transfer of CIA into SCID mice can be blocked by treatment with anti-macrophage-specific mAbs (anti-CD11b) (Taylor et al., 1996), and a clodronate-mediated depletion of phagocytic synovial macrophages ameliorates localized inflammation in CIA (Van Lent et al., 1996).

#### *3.6.2.5. The role of cytokines in experimental arthritis*

Previous studies in mice immunized with collagen in CFA have demonstrated that Th1 cytokines are produced predominantly during the onset of disease, whereas Th2 cytokines (IL-4 and IL-10) are produced later during disease progression and remission (Mauri et al., 1996). The results are in line with the proposed concept of a pathogenic role of Th1-type immunity in both experimental murine arthritis and RA in man (Feldmann et al., 1996b; Feldmann, 2001)

Opposing effects for IFN-γ have been described on CIA, i.e. disease-promoting as well as disease-limiting. An injection of IFN-γ increased the incidence and accelerated the onset of CIA in mice (Mauritz et al., 1988), whereas another group demonstrated that the systemic administration of IFN-γ reduced the severity of the disease (Nakajima et al., 1990). On the other hand, the therapeutic efficacy of IFN-γ was demonstrated in the experimental disease and in clinical trials for the treatment of RA (Cannon et al., 1989; 1993). Furthermore, a blockade of IFN-γ using mAb exhibited paradoxical effects on CIA, with early prevention of the disease followed by late-stage disease exacerbation (Boissier et al., 1995). In another study anti-IFN-γ treatment was associated with more severe arthritic lesions (Williams et al., 1993). Interestingly, an accelerated CIA develops in DBA1 mice lacking IFN-γ receptors (Manoury-Schwartz et al., 1997; Vermeire et al., 1997), whereas mice lacking interferon regulatory factor develop a less severe arthritis (Tada et al., 1997) and reduced susceptibility to CIA in mice deficient in IFN-γ receptor (Kageyama et al., 1998).

IL-12-deficient mice have been shown to be less prone to develop severe arthritis (McIntyre et al., 1996) and anti-IL-12 antibody can suppress CIA (Butler et al., 1999). Administration of exogenous IL-12 enhanced the development of CIA in mice immunized with collagen II in IFA (Germann et al., 1995), suggesting that exogenous IL12 is capable of replacing the mycobacterium tuberculosis in CFA. The differential regulation of IL-12 in CIA depended on administration time (Joosten et al., 1997a) and dose (Tsuyoshi et al., 1999) of IL-12. Accelerated onset and increased severity of arthritis with a low dose of IL-12 (5 ng/day). In contrast, administration of a high dose of IL-12 (500 ng/day) attenuated arthritic inflammation (Tsuyoshi et al., 1999). In addition, IL-12 has a stimulatory role in early arthritic development, whereas it has a suppressive role in the established phase of CIA (Joosten et al., 1997a).

The onset of clinical symptoms and inflammation in collagen type II arthritis is TNF-α dependent, which is in line with a role of this cytokine also in human RA (Arend and Dayer, 1995). A local TNF expression is documented in the inflamed joints of experimental arthritis (Mussener et al., 1997; Marinova-mutafchieva et al., 1997) and of patients with RA (Chu et al., 1991). Studies with neutralizing anti-TNF-α Abs or soluble TNF receptors have revealed a major suppressive effect on the clinical disease activity, when treatment was started directly after onset of CIA (Williams et al., 1992; Wooley et al., 1993). Transgenic mice over expressing human

TNF spontaneously develop an erosive arthritis (Keffer et al., 1991). These results suggest that TNF is strongly implicated in the pathogenesis of RA. Although TNF-deficient mice had some reduction in the clinical parameters of CIA, however, in some individuals of $TNF^{-/-}$ severe disease still occurred. They concluded that TNF is important, but is not essential for inflammatory arthritis (Campbell et al., 2001).

IL-10 administration and intra-articular IL-10 gene transfer inhibit the progression of CIA (Walmsley et al., 1996; Lubberts et al., 2000). IL-10-deficient mice developed more severe CIA (Cuzzocrea et al., 2001; Johansson et al., 2001). Suppression of arthritis has been achieved using recombinant IL-4 (Horsfall et al., 1997; Joosten et al., 1999) and local delivery of IL-4 by adenovirus gene therapy (Lubberts et al., 1999; Tarner et al., 2002). Treatment with anti-IL-4/anti-IL-10 antibodies shortly before the onset of CIA accelerated disease expression. Moreover, IL-4/IL-10, treatment of established CIA resulted in clear suppression of the arthritis and prevented cartilage destruction (Joosten LA, 1997b). However, IL-4-deficient mice developed less acute but more chronic relapsing CIA (Svensson et al., 2002). This would implicate that IL-4 could have a role in the regulation of chronicity.

### 3.7. Immune system and nervous system

It is now clear that both the nervous system and immune system have internal homeostatic mechanisms that control and regulate the functions of these systems and their responses to various stimuli. The bidirectional communication between the nervous system and the immune system is mediated by shared neuropeptides and cytokines (Blalock, 1997; Peter and Ivan, 1998). Lymphocytes communicate with cells in the nervous system by producing neuroendocrine mediators and cytokines, and cells in the nervous system communicate with lymphocytes by producing neuroendocrine mediators and cytokines. The immune system receives a noncognitive (antigenic) stimulus, responds, and sends signals (cytokines) to the nervous system that in turn reciprocates with immunoregulatory signals (neuropeptides and cytokines) some of which are the result of behavioural responses (Blalock, 1997; Peter and Ivan, 1998). Astrocytes and microglial cells can produce some cytokines such as IL-1β, IL-3, IL-6, TNF-α, and IFN-γ (Chung and Benveniste, 1990). Peripheral stimulation can cause a changed cytokine profile in the CNS. Peripheral administration of LPS induces increased mRNA for IL-1, TNF-α, and IL-6 in the central nervous system (Ban et al., 1992; Gatti and Bartfai, 1993; Quan et al., 1998). Acute

peripheral inflammation induces moderate glial activation and IL-1 expression in spinal cord (Sweitzer et al., 1999).

## 4. AIMS OF THE STUDY

The general aims of this thesis were to improve understanding of the immunopathogenesis and immunoregulation of cytokines in experimental neuritis and arthritis by analyzing the cytokine production in the target tissues and in the peripheral lymphoid organs of experimental animals.

The specific aims were:

1) To evaluate the role of IL-12 in development and enhancement of EAN.

2) To elucidate the effect of TNF on the pathogenesis of EAN and to study the efficacy of sTNFR I in EAN.

3) To investigate the roles of CCR5 in the development of CIA and analyze effects of CCR5 deficiency on the immune system using CCR5-deficient mice.

4) To characterize the pattern of IL-1β, IL-6, TNF-α and IFN-γ in spinal cord of AIA.

## 5. MATERIALS AND METHODS

### *5.1. Induction of EAN and assessment of clinical signs*

IL-12 p40-deficient mice (IL-$12^{-/-}$ mice) (Paper I), and C57BL/6 mice (B6) (Paper I and II) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and bred at the animal-housing facilities of the Microbiology and Tumor Biology Center, Karolinska Institute. All mice were 6-8 weeks old, weighing 18-20 g and were immunized twice (designated as days 0 and 7) by subcutaneous injection into the back with 80 μg of P0 peptide 180-199 and 0.5 mg Mycobacterium tuberculosis emulsified in 25 μl saline and 25 μl FCA. All mice received 400 ng, 200 ng and 400 ng pertussis toxin by intravenous injection on days -1, 0, 3, p.i., respectively. Clinical scores were assessed immediately before immunization (day -1) and thereafter every other day until day 65 p.i.. Severity of paresis was graded as follows: 0, normal; 1, flaccid tail; 2, moderate paraparesis; 3, severe paraparesis; 4, tetraparesis; 5, death; 0.5; intermediate clinical signs.

### *5.2. Induction of arthritis and assessment of clinical signs*

CCR5-deficient ($CCR5^{-/-}$) and corresponding wild-type ($CCR5^{+/+}$) mice (Paper III) on the C57BL/6x129/Ola background were bred at the animal-housing facility of Huddinge University Hospital. All mice were 10-12 weeks of age. Arthritis was induced with chick CII, as previously described (Campbell et al., 2000). An emulsion was freshly prepared by dissolving 3 mg/ml chick CII (Sigma) overnight at 4 °C in 10 mM acetic acid and combining it with an equal volume of CFA containing 5 mg/ml of Mycobacterium tuberculosis (Strain H 37 RA; Difco, Detroit, Mich). Mice were injected twice (days 0 and 21) intradermally at several sites into the base of the tail with a total of 100 μl emulsion containing 150 μg CII and 250 μg Mycobacterium tuberculosis. The disease severity was recorded following a scoring system for each limb. Each paw was inspected and the severity of erythema and swelling was graded 0 (normal appearance), 1 (mild), 2 (moderate), or 3 (severe; maximum score of 12 for each mouse).

Adjuvant arthritis (Paper IV) was induced in female Lewis rats with body weight 160-180 g by intradermal injection of a suspension (100 μl) of heat-killed mycobacterium tuberculosis (Difco, Detroit, MI) in paraffin oil (10 mg/ml) into the base of the tail. Animals used as controls received 100 μl of paraffin oil only.

### *5.3. Immunohistochemistry*

Briefly cryostat sections were exposed to the antibodies as described in the papers. Sections were stained using the avidin-biotin technique. Omission of primary antibodies served as negative control. The tissue areas were measured by image analysis, and the numbers of stained cells were counted at × 40 magnification in the entire section area. The results were expressed as average numbers of cells per $mm^2$ of tissue section.

### *5.4. Cell culture and proliferation assay*

Spleens from each group were removed under aseptic conditions. Single cell suspensions of MNC from individual mice were prepared. MNC suspended in 200 μl portions were cultured in triplicates in round-bottomed 96-well polystyrene microtiter plates at a cell density of $2 \times 10^6$ cells/ml in a humidified atmosphere with 5% $CO_2$ at 37ºC. After 60 hours of incubation, cells were pulsed with $^3$H-methylthymidine (1 μCi/well) and cultured for an additional 12 hours. Cells were harvested onto glass fiber filters. $^3$H-thymidine incorporation was measured in a liquid β-scintillation counter, and results were expressed as counts per minute (cpm).

### *5.5. Preparation of peritoneal exudates cells (PEC)*

Sterile ice-cold PBS was injected into the peritoneal cavity of mice. After 3 minutes PEC were eluted using sterile ice-cold PBS, washed and re-suspended to 2 $x10^6$ PEC/ml in complete medium as description in Paper III. 20 μl/well of PEC were added and cultured with MNC for the T cell proliferation assay as above.

### *5.6. Enumeration of IFN-γ secreting cells by ELISPOT*

A solid-phase enzyme-linked immunospot (ELISPOT) assay was used to detect single cells that secrete IFN-γ upon antigen stimulation. Briefly, nitrocellulose-bottomed plates were coated overnight at 4°C with 100 μl (15 μg/ml) IFN-γ capture antibody. MNC were cultured as described above. After 48 h of culture, secreted and bound IFN-γ was visualized by sequential application of biotinylated detecting Ab against IFN-γ, and ABC. After peroxidase staining, the red-brown immunospots corresponding to the cells that had secreted IFN-γ, were enumerated in a dissection microscope at low magnification (x 25). Results were expressed as numbers of spots per $10^5$ spleen MNC.

### *5.7. ELISA for measurement of serum antibodies*

Serum was obtained from blood samples of test mice and determined by enzyme-linked solid-phase assay (ELISA). Antigen (P0 peptide 180-199 or CII) was coated onto ELISA plates at 10 μg/ml in a volume of 100 μl/well overnight at 4°C. Nonspecific binding was blocked with 1% normal horse serum for 2 h at room temperature (RT). After three washings of the plates, serum samples were then placed in plate wells and incubated for 2 h at RT. After another three washings, plates were incubated for 2 h with biotinylated anti-mouse IgG (Serotec), biotinylated anti-mouse IgG1 (Serotec), biotinylated anti-mouse IgG2a (PharMingen), biotinylated anti-mouse IgG2b (PharMingen), respectively, for 1 h and then with alkaline phosphatase-conjugated AB complex (Vector) for 30 min. Three additional washings followed. The reaction was visualized with p-nitrophenyl phosphate substrate (Sigma) and read at 405 nm using an ELISA reader.

### *5.8. ELISA for measurement of cytokine and chemokine production*

Spleen cell supernatants were harvested after 48 h, 72 h, 96 h and 120 h cultures, respectively. Levels of IFN-γ, TNF-α, IL-10 and MIP1-β in the culture supernatants were measured by quantitative ELISA as described in the protocol supplied by the manufacturer (PharMingen).

### *5.9. In situ hybridization*

*In situ* hybridization was performed as described previously (Zhu et al., 1994c). Briefly, synthetic oligonuleotide probes (Scandinavian Gene Synthesis AB, Köping, Sweden) were labeled with $^{35}$S deoxyadenosine-5'-α-(thio)-triphosphate with terminal deoxynucleotidyl transferase (Amersham). Cells were hybridized for 16-18 h at 42°C in a humidified chamber with $10^6$ cpm of a labeled probe per 100 μl of hybridization mixture. After emulsion autoradiography, development and fixation, the coded slides were examined by dark field microscopy at ×10 magnification for positive cells containing > 15 grains per cell in a star-like distribution over the cytoplasm. The intracellular distribution of the grains was checked by light microscopy at ×20 and /or ×40 magnification.

### *5.10. Statistical analysis*

Differences were tested by parametric one-factor analysis of variance (ANOVA) or Student's t-test and non-parametric Mann-Whitney´s u-test, respectively. The Chi-square test was used to

test the difference in incidence. All tests of significance were two-sided. The level of significance was set to $p < 0.05$.

### *5.11. Ethics*

The EAN and AIA as well as CIA models in B6 mice, transgenic mice and Lewis rats were approved by the South Stockholm Research Animal Ethics Committee, Huddinge County Court, Stockholm, Sweden.

## 6. RESULTS AND DISCUSSION

### *6.1. IL-12 p40 is vital for initiating, enhancing and perpetuating pathogenic events in EAN (Paper I)*

For the present study, EAN was established in IL-12 p40 deficient mutant (IL-12$^{-/-}$) C57BL/6 mice by immunization with P0 peptide 180-199, a purified component of peripheral nerve myelin, and CFA. In these IL-12$^{-/-}$ mice the onset of clinical disease was delayed, and the incidence and severity of EAN were significantly reduced compared to that in wild type mice. The former group's clinical manifestations were associated with less P0-peptide 180-199 induced secretion of IFN-γ by splenocytes in vitro and low production of anti-P0-pepitde 180-199 IgG2b antibodies in serum. Fewer IFN-γ and TNF-α producing cells, but more cells secreting IL-4, were found in sciatic nerve sections from IL-12$^{-/-}$ mice, consistent with impaired Th1 functions and response.

Mice lacking IL-12 p40 actively immunized with P0 peptide 180-199 developed EAN (around 50%) with a lower incidence and milder clinical signs than the wild type. In addition to the absence of IL-12, these mice were deficient in their ability to generate Th1 cells that produced IFN-γ in response to antigen stimulation, suggesting the involvement of IL-12 as an initiator of Th1 cell mediated immunity in EAN. However, this effect of IL-12 on T cells was not sufficient to produce EAN, because IL-12$^{-/-}$ mice were not completely resistant to EAN induction. Possibly IL-18 compensated for the lack of IL-12 in IL-12$^{-/-}$ mice, because IL-18 is known to work synergistically with IL-12, apparently, shares biological functions with IL-12 (Constantinescu et al., 1996; Yang et al., 1999). We previously showed that anti-IL-18 antibody treatment suppressed murine EAN (Yu et al., 2002). IL-12$^{-/-}$ mice experienced a reduced production of IFN-γ and TNF-α in the sciatic nerves, but had an elevation in IL-4 production. This outcome suggests that suppression of EAN in IL-12$^{-/-}$ mice results from an insufficiency of antigen-specific T cells available to differentiate into Th1 effector cell in the periphery, thereby altering Th1/Th2 balance in favor of Th2 selection in vivo. Furthermore, Th1 cells mediate effector functions of the immune response and help in the synthesis of IgG subclasses such as IgG2a and IgG2b that bind complement and can be especially damaging to tissues (Romagnani, 1997; O'Garra, 1998). In this current study, the significantly reduced amounts of serum anti-P0 peptide 180-199 IgG2b antibodies in IL-12$^{-/-}$ mice may have resulted from impaired Th1 cell help for B

cells and/or from direct effects of IL-12 deficiency on the B cells themselves. In either case, the reduced autoantibody response seen clearly contributed to the suppression of EAN in IL-12$^{-/-}$ mice.

### *6.2. Exogenous sTNFR I ameliorates EAN (Paper II)*

Our data from two different therapeutic regimens indicate that the administration of sTNFR I effectively ameliorated the clinical and pathological signs of EAN, i.e., decreased its severity, shortened its duration and reduced inflammatory cell infiltration into the PNS. The suppression of clinical EAN was accompanied in vitro by a marked reduction in antigen-specific T cell proliferation and IFN-γ synthesis by spleen cells from sTNFR I-treated mice, as compared to control mice treated with PBS.

Systemic administration of sTNFR I decreased the severity and the duration of clinical EAN. This ability of sTNFR I to protect from the development of EAN likely involves blocking the effects of TNF on activation and recruitment of inflammatory cells into the PNS, thereby inhibiting the downstream effects of this cytokine. It has been indicated that TNFR1 can induce T cell apoptosis and lymphocyte clearance (Bachmann et al., 1999), and TNFR1 signaling pathway plays a critical role in the control of central nervous system (CNS) demyelination and inflammation (Probert et al., 2000), which may also contribute to ameliorate EAN. Our findings consistent with previous reports by others that the administration of sTNFR I can prevent the adverse pathologic sequelae of exaggerated TNF production as observed in lethal sepsis (Van Zee et al., 1992), EAE (Baker et al., 1994; Selmaj and Raine, 1995b), autoimmune diabetes (Hunger et al., 1997) and CIA (Wooley et al., 1993; Mori et al., 1996). Recent clinical trials also showed that subcutaneous administration of sTNFR I substantially decreased the symptoms of RA (Moreland et al., 1997; Weinblatt et al., 1999). However, both sTNFR I treatment schedules, i.e., 2 days before and 14 days after P0 peptide 180-199 immunization, suppressed the effects of EAN, but neither protocol totally prevented the disease, suggesting that other pro-inflammatory cytokines, such as IL-12 and IL-18, also participate in the pathogenesis of EAN (Bao et al., 2002; Yu et al., 2002); Furthermore, sTNFR I used at dose of 3 mg/kg every other day, as in the present study, may not completely neutralize TNF activity.

### *6.3. Reduced incidence of CIA in CCR5 deficient mice (Paper III)*

$CCR5^{-/-}$ mice showed a significant reduction in the incidence of CIA after CII-immunization as compared to wild-type mice. However the severity score, once arthritis had developed, was similar in the both groups. The reduced incidence seen in $CCR5^{-/-}$ mice was associated with these animals having significantly lower IgG levels, especially IgG2a and IgG2b antibodies against CII, as well as an obviously augmented IL-10 production, as compared to wild-type mice. Higher level of MIP-1β was found in CCR5 deficient mice after CII-immunization.

Arthritis is dependent on both cellular and humoral immune responses (Ranges et al., 1985; Seki et al., 1988). Collagen-reactive antibodies, particularly of the complement-fixing IgG2a and IgG2b isotypes, have been implicated in the pathogenesis of CIA (Wooley et al., 1981; Holmdahl et al., 1986b; Durie et al., 1993). Our results show that levels of IgG2a and IgG2b against CII were obviously lower in CCR5 mutant mice when compared with wild type mice after CII-immunization. The decreased antigen-specific antibody responses of $CCR5^{-/-}$ mice may act to diminish their CIA since CCR5 deficiency may affect the primary T-dependent antibody responses. In general, RA and CIA are thought to be a typical Th1 mediated disease, whereby Th2 cytokines play a preventive role in different models of arthritis (Mauri et al., 1996; Joosten et al., 1997). CCR5 is primarily expressed on Th-1 cells (Loetscher et al., 1998). In the present study, higher levels of the Th2 cytokine, IL-10 were seen in spleen cells of $CCR5^{-/-}$ mice as compared to their wild-type counterparts. Additionally, the alteration of antibody isotypes in $CCR5^{-/-}$ mice after CII immunization shift from Th1 predominance (Th1 associated IgG2a and IgG2b antibodies) to Th2 predominance (IgG1 antibodies), which further play a protective role in $CCR5^{-/-}$ mice with CIA (Finkelman et al., 1990). In this study, one of the CCR5 ligands, MIP-1β was strongly up-regulated in serum and in supernatants of spleen cells from CCR5 deficient mice after CII immunization. This finding may partially contribute to the development of arthritis in $CCR5^{-/-}$ mice. Thus, the lack of CCR5 could be compensated for by an increased expression of MIP-1β that acts through a combination of other chemokine receptors due to the higher degree of redundancy in this chemokine subfamily.

### *6.4. IL-1β, IL-6 and TNF-α are up-regulated in the spinal cord of AIA rats (Paper IV)*

Macroglia and MHC class II immunostaining were enhanced, astrocytes expressing glial fibrillary acidic protein (GFAP) were increased in number and immunostaining intensity in the spinal cord of AIA rats. Using *in situ* hybridization and immunohistochemical methods, both mRNA and protein levels of IL-1β, IL-6 and TNF-α were significantly increased in the spinal cord of arthritic rats. Increased cytokine expression was presented in the reactive astrocytes and microglia.

We speculate that there are at least four possible mechanisms behind the enhanced cytokine expression in the spinal cord following AIA: (1) In response to peripheral inflammation, cytokines are directly produced within the CNS by activated endogenous microglia and astrocytes. (2) The upregulation of these cytokines in the CNS of arthritic rats may be intimately involved in hyperalgesic mechanisms. (3) Cytokines produced in the periphery by macrophages may be transported retrogradely via axonal or nonaxonal mechanisms (Streit, 1993). (4) Interaction between neuropeptides and cytokines: There is data showing the occurrence of bidirectional interactions between the nervous and immune systems (Blalock, 1997). The nervous system responds to and also helps to regulate the immune responses. Such interaction occurs between neuropeptides and cytokines. Neuropeptides may induce the secretion of various cytokines, including IL-1, TNF-α and IL-6 (Lotz et al., 1988; Turnbull et al., 1997). The data demonstrated that the levels of neuropeptides were increased in the spinal cord of rats with AIA (Amann et al., 1996; Calza et al., 1998; Elhassan et al., 2000). However, cytokines can also induce the secretion of neuropeptides (Heijnen et al., 1991). Neuropeptides may participate in the peripheral events of inflammation and further exacerbate inflammation, pain and hyparalgesia in AIA, at the same time, the increase of neuropeptides levels in dorsal root ganglia can be related to the structural damage of nerve fibres in arthritic rats (Calza et al., 1998). It might, therefore, be possible that the changes of cytokines in the spinal cord may also be involved in the pathogenesis of AIA via regulating the levels of neuropeptides in the spinal cord.

## 7. CONCLUSIONS

1) IL-12 has a major role in the initiation, enhancement and perpetuation of pathogenic events in EAN by promoting a Th1 cell-mediated immune response and suppressing the Th2 response. This information augments consideration of IL-12 as a therapeutic target in GBS and other T cell-mediated autoimmune diseases.

2) sTNFR I can inhibit the development of EAN, indicating beneficial effects of sTNFRs in the treatment of EAN. sTNFR I may have therapeutic potential for alleviating GBS in humans.

3) CCR5 may participate in the pathogenesis of arthritis. There was a diminished CII-specific antibody, especially IgG2a and IgG2b, response and an enhanced production of IL-10 in $CCR5^{-/-}$ mice after CII-immunization. The overproduction of MIP-1β in the serum and spleen cells of $CCR5^{-/-}$ mice may partly contribute to the development of arthritis.

4) Both mRNA and protein expression of IL-1β, IL-6 and TNF-α are up-regulated in the spinal cord of arthritic rats.

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**APPENDIX (Paper I to IV)**