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Novel Immunotherapies and Immunoregulation in a Chronic Inflammatory Disease of the Central Nervous System

Lavasani, Shahram

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From the Department of Experimental Medical Science
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Sweden

Novel Immunotherapies and Immunoregulation in a Chronic Inflammatory Disease of the Central Nervous System

Shahram Lavasani

LUND UNIVERSITY
Faculty of Medicine

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This PhD thesis will be defended on the 14th of December 2006 at 1.00 pm in the GK lecture hall, Biomedical Center, Sölvegatan 19, Lund

Faculty opponent: Professor David C. Wraith
Department of Cellular & Molecular Medicine, University of Bristol
United Kingdom

To my parents

Cover page:

Human Leukocytes
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توانا بود هر که دانا بود

"Knowledge is Power "

Ferdowsi, the greatest Persian poet (935-1020 AD)
"The Epic of Kings"

****

All human beings are different parts of the same body, who
Have inherited the same essence in creation

No part will rest in peace
If one is suffering pain

You will not deserve the name of human
If you are indifferent about other's pains

Sáadi, Persian poem (1200-1292 AD)
"Human beings"

Original papers

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. **CD1-dependent regulation of chronic central nervous system inflammation in experimental autoimmune encephalomyelitis.**
Teige A., Teige I., **Lavasani S.**, Bockermann R., Mondoc E., Holmdahl R., and Issazadeh-Navikas S.
J Immunol. 2004, 172:186-94

II. **Monoclonal antibody against T-cell receptor αβ induces self-tolerance in chronic experimental autoimmune encephalomyelitis.**
Lavasani S., Dzhambazov B., and Andersson M.
Scand J Immunol. Accepted for publication, 20 September 2006.

III. **Oral administration of unique probiotic strains successfully ameliorates experimental autoimmune encephalomyelitis.**
Lavasani S., Buske S., Fåk F., Dzhambazov B., Molin G., Alenfall J., and Weström B.
Submitted to *Nature Medicine.*

Abbreviations

Ag	antigen
APL	altered peptide ligand
APC	antigen presenting cell
BBB	blood brain barrier
CD	cluster of differentiation
CFA	complete Freund's adjuvant
CIA	collagen-induced arthritis
CNS	central nervous system
CP	cryptopatch
CSF	cerebrospinal fluid
CTL	cytotoxic T lymphocytes
DC	dendritic cell
DSM	deutsche sammlung von mikroorganismen
EAE	experimental autoimmune encephalomyelitis
Fab	Ag-binding part of Ig
FACS	fluorescence activated cell sorting
Foxp3	forkhead box p3 transcription factor
α-GalCer	α-Galactosylceramide
GALT	gut associated lymphoid tissue
HLA	human leukocyte antigen
IBD	inflammatory bowel disease
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IS	immunological synapse
IL	interleukin
i.p.	intraperitoneal
i.v.	intravenous
L.	lactobacillus
LAB	lactic acid bacteria
LN	lymph node
LP	lamina propria
LPS	lipopolysaccharide
MALT	mucosa-associated lymphoid tissues
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein
MoAb	monoclonal antibody
MS	multiple sclerosis
NF-κb	nuclear transcription factor kappa b
NK	natural killer cell
NKT	natural killer T cell
NOD	non-obese diabetic
PAMP	pathogen-associated molecular pattern
PLP	proteolipid protein
PP	peyer's patch
PT	pertussis toxin
TCR	T cell receptor
TGF-β	transforming growth factor-beta
TLR	toll-like receptor
TNF-α	tumor necrosis factor-alpha
Treg	regulatory T cell
wt	wild type

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Introduction

Our body is under constant threat of attack by viruses, bacteria and parasites. Evolution has therefore provided mammals with enormously complex and potent layers of immunological defence involving cells and molecules capable of specifically recognizing and eliminating foreign invaders, all of which act together in a dynamic network. The earliest prokaryotic microorganisms have inhabited Earth for at least 2.5 billion years, and the power of our immune system is a result of coevolution in which indigenous bacteria particularly have shaped the body's defence functions (1, 2).

In humans the critical role of the immune system, which in principle is partly open to the external environment, becomes clinically apparent when it is defective. Thus, inherited and acquired immunodeficiency states are characterised by increased susceptibility to infections.

Protection by the immune system can be divided into two related activities, recognition and response. The incredible immune recognition gives the system the capacity to distinguish foreign from self components and to identify the altered host cells. Recognition of a pathogen triggers the immune system to develop an effector response that eliminates or neutralizes the invader.

There are two systems of immunity, innate (non-specific) and adaptive (acquired or specific) immunity which function together synergistically. The adaptive immune system developed late in the phylogeny of mammals, while other species survived without it.

Despite the multitude of complexities, the immune system also requires harmonious interactions among all its components for the maintenance of homeostasis. Since every member of this community has its own "agenda", the body has also developed several means of preserving a peaceful and productive existence in order to avoid conflicts between immune responses to self (antigen-driven tolerance) and non-self (pathogen-driven immunity). An essential strategy is to rid the immune cell repertoire of self-reactive ones and maintain a wide selection of populations that take action against the foreign invaders and stressed cells. Another level of control is achieved by the active regulation of immune responses through cellular interactions and soluble mediators. Any failure in these systems can result in immune attack on the host which is termed as "autoimmunity". There are several autoimmune diseases that severely reduce quality of life and in many cases lead to the death of the individual. An example of such a disease is Multiple Sclerosis (MS) which is a disorder caused by immune

attack to the central nervous system. There is no treatment currently available that is capable of preventing the disease progression (3).

At present, all the therapies, which are used in an attempt to modify the course of the disease, have limited efficacy and in many cases substantial side-effects. For treatment of MS, there is an increasing interest for using immunotherapy, in order to modulate the patient's immune system and suppress the CNS inflammation. Successful immunotherapies require a broadening of basic research regarding the regulation of the disease and finally to administer different biological reagents to deliver or modulate a specific arm of the systemic immune responses (4).

The aim of this thesis has been to gain a better understanding of the autoimmune processes in MS and finally by using biological tools such as monoclonal antibodies, previously activated regulatory immune cells or certain lactic acid bacteria.

Innate immunity

Although the innate immune system lacks the fine specificity of adaptive immunity, it can distinguish self from nonself by three basic mechanisms; recognition of induced or altered self, detection of missing self (virally infected cells) or direct recognition of microbial nonself.

The infectious agents that enter the body will immediately be recognised and encounter the innate immune system, which consists of surface barriers, soluble factors, specialized phagocytes, dendritic cells (DCs) and Natural killer (NK) cells. The NK-cell receptors recognise structures of high molecular weight glycoproteins expressed on virus-infected cells. After activation, NK cells release their granule content, such as perforin and granzym, and kill virally-infected host cells and a variety of tumor cells without prior sensitisation (5). Components of the innate immune system use germline-encoded proteins to identify microbial substances. They recognize pathogen-associated molecular patterns (PAMPs) and this elicits rapid immune responses in professional antigen-presenting cells, such as DCs and macrophages (6). The cellular receptors of the innate immune system that recognise PAMPs as "danger signals", are called pattern recognition receptors (PRRs), many of them belonging to the so called Toll-like receptors (TLRs). They are expressed mainly by macrophages and DCs, but also by a variety of other cell types such as B cells and epithelial cells (7) leading to

the activation of nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs). Together, these functions represent a primary layer of defence against invading microorganisms, with the common goal of restricting their entry into the body by providing a physical hindrance and clearance mechanisms such as epithelial linings of skin and mucosa, chemical factors such as pH of body fluids, numerous antimicrobial peptides and proteins and phagocytic cells such as neutrophils, eosinophils, monocytes/macrophages and DCs.

Despite its evolutionary success, innate immunity has been treated with condescension by immunologists. It has been considered to be a temporary mechanism for host defence, buying time until acquired immunity takes over. It lacks the elegance of the genetic-recombination mechanisms to generate specificity. It induces no "memory", since first and second exposures to a microbial substance elicit similar responses. But increasing amounts of evidence show that innate immunity communicates with acquired immunity via the secretion of cytokines produced by macrophages and natural killer (NK) cells (8) and attachment of complement proteins to antigens (9). In addition, activation of antigen presenting cells (APCs) through TLRs results in expression of co-stimulatory molecules CD80 (B7.1) and CD86 (B7.2) (10). This costimulatory "second signal" crucial for activation of T cells and is considered to be an important link connecting innate and acquired immunity.

Adaptive immunity

Challenges to the innate system often lead to activation of the adaptive immune system, which is capable of recognizing and selectively eliminating specific foreign microorganisms and foreign antigens. Adaptive immunity displays antigen specificity, diversity, immunological memory, self-nonself recognition and involves lymphocytes (T and B cells) and APCs. In peripheral blood, the lymphocytes comprise 20–25% of the total leukocytes. Adaptive immunity depends on the functional properties of T and B cells and is directed by their antigen-specific surface receptors, T cell receptor (TCR) and B cell receptor (antibody), with a random and highly diverse repertoire (11). Further development and maturation of T and B cells occurs in the thymus and bone marrow respectively (primary lymphoid organs).

The focus of this thesis is the subsets of T cells and $CD4^+$ T cell populations in particular. Therefore activation, tolerance and immunoregulattion of T cells will be discussed in more detail.

T cells

The adaptive immune response is initiated by the interaction of a TCR with a peptide-antigen presented on major histocompatibility complex (MHC) molecules forming an immunological synapse (IS). T cell activation requires sustained TCR interaction with MHC-peptide complexes. Thus, initial adhesion between the naïve T cell and APC might require an innate signal that sets the stage for IS formation, for example, exposure to chemokines. T cells encounter chemokine gradients as they extravasate into lymph nodes and inflamed tissue. T cells will therefore be exposed to chemokines before they encounter APC. Chemokine receptor signaling results in a rapid polarization of T cells (12). The mechanism of selective TCR triggering is a hotly debated area, but it is certain that the monomeric TCR MHC-peptide interaction plays a crucial role in determining the final MHC density accumulating into the IS which results in an effective T cell activation (13). The two-signal model for T cell activation proposes that upon TCR MHC-peptide interaction, signal one is provided by the TCR/CD3 complex, while signal two is generated by engagement of T cell costimulatory receptors such as CD28 to its ligands CD80 (B7-1) and CD86 (B7-2) (14). A classic hallmark of T cell activation is early IL-2 production, upregulation of the high affinity IL-2 receptor α-chain (CD25) followed by proliferation. In addition, expression of chemokine receptors, homing receptors (e.g. down regulation of CD62L and upregulation of CD44), adhesion (e.g. VLA-4) and cytokine sensitivity (e.g. down regulation of IFN-γ receptor in effector Th1 cells) is also altered. T cells are equipped with several effector functions including cell-mediated forms of immunity characterized by cellular cytolytic activity and the production of cytokines.

The TCR is the primary trigger for the clonal expansion of antigen-specific cells from the T cell repertoire (15). Most T cells express αβ TCRs, composed of disulfide-bonded α and β chains, which typically bind themselves to the complex of the antigenic peptides presented by MHC. There are two major lineages of αβ TCR expressing T cells. MHC class II-restricted helper $CD4^+$ T cells which help other cells of the immune system such as macrophages and B

cells to activate their effector functions by providing specific cytokines and/or receptor-ligand interaction, and MHC class I-restricted cytotoxic $CD8^+$ T cells capable of killing virus infected cells and tumor cells. The cell surface proteins CD4 or CD8, expressed by T cells, act cooperatively with TCRs to induce antigen-specific cell activation. A small subset of T cells express γδ TCRs composed of disulfide-bonded γ and δ chains, which are able to recognize pathogen-derived glycoproteins without MHC assistance (16). Cell surface expression of the TCR occurs in association with, and is dependent upon, the CD3 signaling subunits (17). TCR/CD3 signaling is central to the initiation of antigen-specific T cell responses to pathogens and vaccines, as well as transplanted tissues, tumors and autoantigens. It is of major importance to increase our knowledge about T cell signaling and to evaluate these responses *in vivo*. Monoclonal antibodies (MoAbs) specific for human CD3 have been used or tested as immunomodulating agents in preventing transplant rejection and in the treatment of autoimmune diseases (18).

In our studies, we have shown that treatment with MoAb specific for anti-TCR αβ is therapeutic in experimental autoimmune encephalomyelitis (EAE), an animal model for human MS (***Paper II***).

Th1/Th2 $CD4^+$ T cells

In 1986 certain subsets of mouse $CD4^+$ T cells were identified which showed the capacity to release unique profiles of cytokines associated either with inflammatory responses or with B cell help (19-21). The T helper (Th) 1 $CD4^+$ T cell subset was shown to produce IL-2, IFN-γ, TNF-α and to mediate delayed-type hypersensitivity (DTH) responses upon transfer (22). In contrast, the Th2 subset was shown to produce IL-4 and IL-5 and provide B cell help, thus mediating a humoral immune response (23, 24). Since the original definition of the Th2 clones, several additional cytokines were associated with each subset were identified. Th2 cells are defined to produce IL-4, IL-5, IL-6, IL-10 and IL-13 (25, 26). The presence of the T helper cell dichotomy was further confirmed in species other than mice, including humans (27). The prominent aspect of these findings was that the two types of clones do not overlap but rather counteract each other (27-29) with major consequences for disease outcome. Th1 cells have been shown to be involved in the development of autoimmune diseases such as MS

(30). Interestingly, experimental data using the animal model EAE, suggested that a shift in the cytokine profile from a Th1 to a Th2 response can be used as therapy (31).
Factors influencing Th differentiation are partly determined by the antigen administration route, type of APC and, most importantly, the local cytokine environment (32). IL-12 produced by activated APCs is critical in Th1 differentiation while IL-4 is crucial in driving Th2 differentiation (33, 34).
Additional cytokines in the IL-12 family, IL-23 and IL-27, are also important for Th cell differentiation and function (35, 36). IL-23, has been shown to be involved in the pathogenesis of EAE and collagen-induced arthritis (CIA) (37, 38). Recently, distinct lineage of Th effector cell population producing IL-17 (Th-17) has been discovered (39). These cells are probably effective in the protection against extracellular bacteria, but also play a role in the amplification of autoimmune disorders by inducing a proinflammatory response (40, 41). Other studies shows that IL-27 suppresses the development of Th-17 cells (42). It has also been demonstrated that a combination of TGF-β1 and IL-6 contributes to development of Th-17 cells (43).

Tolerance

The immunologic specificity of the antigen receptors of T and B cells is the result of random shuffling of the many genes that form the DNA code for the antigen-binding site of these receptors (44, 45). Theoretically, this process could generate 10^{11} to 10^{18} different TCRs, including some that can bind to autoantigens (selfreactive T cells). Tolerance is the process that eliminates or neutralizes such autoreactive cells and a breakdown in this system can cause autoimmunity.

"The organism possesses certain contrivances by means of which the immunity reaction, so easily produced by all kinds of cells, is prevented from acting against the organism's own elements and so giving rise to autotoxins … so that we might be justified in speaking of a "horror autotoxicus" of the organism. These contrivances are naturally of the highest importance for the individual."

Paul Ehrlich

More than 100 years ago, Paul Ehrlich first defined the problem of self-reactivity "horror autotoxicus" as inherent to the adaptive immune system and postulated the existence of

mechanisms “contrivances” that could prevent harmful self-reactivity (46). Self–non-self discrimination has been considered as an important requirement of the immune system in that the immune system directs its diverse and potent effector mechanisms against foreign pathogens while ignoring the body’s own components. Only when immunity and self-tolerance are perfectly balanced is the body’s integrity safeguarded.

Central tolerance

The principal mechanism of T cell tolerance is the deletion of self-reactive T cells in the thymus. Immature T cells migrate from the bone marrow to the thymus, where they encounter peptides derived from endogenous proteins bound to MHC molecules. T cells whose receptors have very low affinity for these peptide–MHC complexes do not receive signals that would prevent spontaneous apoptosis and these cells therefore die in the thymus. T cells with high-affinity receptors for these complexes undergo apoptosis and die in a process called negative selection. The remaining T cells, which have receptors with an intermediate affinity for such complexes, mature in the thymus and migrate to the periphery, a process referred to as positive selection. The induction of central tolerance requires the presence of autoantigens in the thymus. We know that not all self-antigens occur in the thymus and this demands the existence of peripheral mechanisms that participate in T-cell tolerance (11, 47, 48). The control of self-reactive which occurs during their maturation process within the thymus is commonly known as central tolerance, while regulatory mechanisms, which dampen the responses of self-reactive T cells that have escaped to central tolerance, within peripheral lymphoid organs are known as peripheral tolerance.

Peripheral T cell tolerance

$CD4^+$ T cells are the master controllers of immune responses to protein antigens and many autoimmune diseases are thought to arise from a breakdown of immunological tolerance of these cells. Peripheral tolerance in $CD4^+$ T cells is maintained by several mechanisms, including functional anergy, deletion by apoptosis and suppression by regulatory T cell populations.

Anergy

Anergy is a state of immune unresponsiveness which is induced when a T cell's antigen receptor is stimulated but effectively freezing T cell responses, including IL-2 production, in absence of a "second signal" from the APC (49). Anergy may have widespread consequences, because certain anergic T cells produce IL-10, which suppresses the activation of T cells (50).

Deletion

The presentation of antigens in the absence of costimulation not only fails to prime T cells but can also eradicate them (51). Another mechanism of peripheral deletion results from the lack of growth factors for which all activated T cells compete (52). The death of T cells is also mediated by the pathway involving Fas (CD95) and its ligand. Engagement of the Fas receptor induces apoptosis in Fas-positive cells (53). Since T cells express both, Fas and its ligand upon activation, the interaction between the two molecules can induce apoptosis (54).

Suppression

Autoreactive clones with pathogenic potential can be kept in check by regulation mediated by dedicated lineages of regulatory T cell populations, such as regulatory T (Treg) cells and natural killer T (NKT) cells. These cells are able to control immunity by interfering with the generation of effector T cell function through cytokine production or cell-cell contact. Current evidence suggests that they are self reactive and this property plays an essential role in cellular mechanism preventing autoimmunity (55, 56). The existence of T cells with regulatory properties emphasizes the complexity of the immune system and the need for multiple levels of supervision. This control system requires a dynamic interaction with its microenvironment to achieve the ultimate goal, namely balance well-being of the body. This can be managed either naturally or through artificial intervention. Our present knowledge suggests that regulatory cells represent key factors in this balance.

Regulatory T cells

Among several classes of T cells with regulatory activity, a minor population (5-10%) of $CD4^+$ T cells that constitutively express the high affinity IL-2 receptor α chain, CD25, has been identified in mice and humans (57-59). These cells express a specific transcription factor, forkhead box p3 (Foxp3), that has been associated with their development and suppressive function (60). These cells appear to suppress a variety of reactions, including T and B cell responses, as well as DC responses (61, 62). The majority of $CD4^+CD25^+Foxp3^+$ regulatory T cells (Treg) develop in the thymus in response to self-antigens, however, cells with a similar phenotype can also arise from $CD4^+CD25^-$ T cells in the periphery (63, 64). The mechanisms by which Treg cells suppress immune responses are poorly understood, but it seems to be related to local secretion of anti-inflammatory cytokines such as TGF-β and direct cell-cell contact. This contact has been shown to be mediated by CTLA-4, expressed on Treg cells and CD80/CD86 molecules on effector T cells. The suppressive effect of Treg cells requires proper colocalization with the effector cells and seems to be triggered by signaling through the TCR (55). Because of the immunosuppressive abilities of Treg cells, they became attractive candidates for immunotherapy (Figure 1). At present, there are numerous studies in animal models demonstrating their potential in controlling autoimmune diseases (65). In one of our studies, this has been confirmed by showing that the therapeutic effect of probiotic bacteria in EAE was associated with induction of $CD4^+CD25^+Foxp3^+$ regulatory T cells.

The term Treg is used very broadly to describe distinct cellular subsets involved in immune regulation. In contrast to the naturally occurring $CD4^+CD25^+$ Treg repertoire, which develops in the thymus, distinct heterogeneous Treg subsets can also be induced in the periphery. These Tregs include subsets such as anergic $CD4^+CD25^+$ T cells, the $CD4^+CD25^-$ antigen-specific Tregs, the IL-10-induced type 1 regulatory T (Tr1) cells, the TGF β-producing Th3 cells and also a population of $CD8^+CD25^-$ Ts (suppressor) cells (66). *In vivo*, Tregs can be induced by mucosal exposure to antigen, persistent exposure to low-dose antigen, by cytokines (IL 10/TGF-β), via stimulation with dendritic cells, or co-stimulation blockade (67)

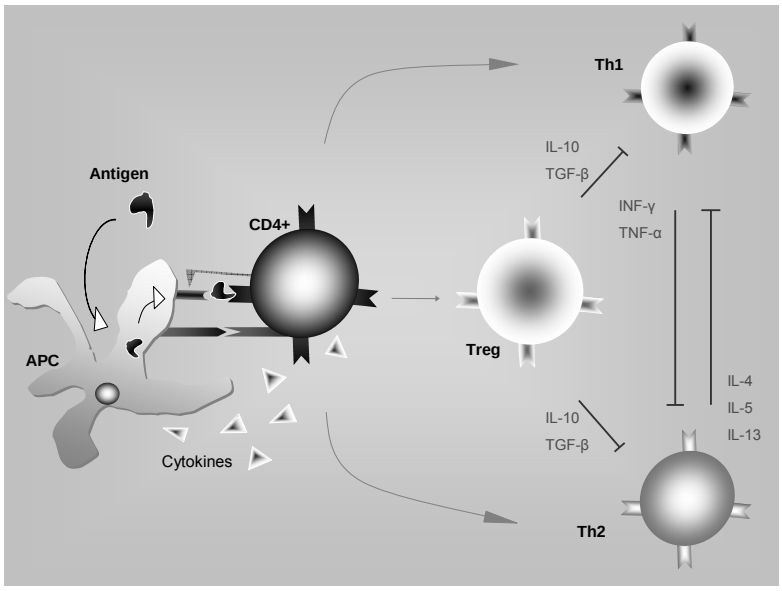

Figure 1: An overview of the development of different T cell subsets

APCs process Ags and degrade it to immunogenic peptides, which are then presented to the TCR of naïve $CD4^+$ T cells via MHC class II. If the Ag presentation is accompanied by costimulatory signals (cytokines/ chemokines/ adhesion molecules), the naïve $CD4^+$ T cells differentiate into Th1 or Th2 subsets with polarized cytokine secreting. Activated Th1 cells predominantely produce INF-γ, while the Th2 population mainly secrets IL-4, IL-5 and IL-13. The critical cytokines released, firstly promote the growth of the subset that produces them and secondly inhibit the development of the opposite subpopulation. Additionally, under certain, not yet fully understood, circumstances, immature APCs may induce Treg cells. The cytokines released by Treg cells, mainly IL-10 and TGF-β, are able to suppress both, Th1 and Th2 responses.

Natural killer T cells

In addition to Treg cells, other important self-reactive T cell sub-lineages have been identified. Prominent among these are cells that express a semi-invariant T cell TCR specific to conserved self ligands. One well characterized T-cell subpopulation is the CD1d-dependent natural killer T (NKT) cell. NKT cells are unconventional T cells that operate on the border between the innate and the adaptive immune systems, since they have characteristics of both systems. Their name is derived from the expression of NK cell-associated receptors, such as CD161 in humans and NK1.1 in mice (68, 69). Unlike conventional αβ T cells, these cells are not MHC class I or II restricted and recognize antigen presented on CD1d. CD1d is a member of the CD1 family which presents antigens to a wide variety of T cells (70). It is expressed on several cell types that function as professional antigen presenting cells, including DCs and B cells. The immunoregulatory function of NKT cells probably depends on their interaction with these cells which results to a rapid cytokine release regulating the activation of T, B and NK cells (71). The majority of these T cells are also called invariant NKT (iNKT cells) with a specific TCR α-chain rearrangement (Vα14-Jα18 in mice; Vα24-Jα18 in humans), associated with Vβ chains of limited diversity (72).

Studies on NKT cells further revealed that NK1.1 is not expressed on all CD1d restricted T cells, it downregulates *in vitro* and it can even be expressed on conventional MHC restricted T cells upon activation (73-75). Based on these data, the term NKT cell in this thesis is referred to population of CD1d restricted T cells.

Several studies have shown that NKT cells are potent regulatory T cells that have the capacity to either initiate or shut down a wide variety of immune responses (76). NKT cells are capable of producing both Th1 like (IFN-γ) and Th2 like (IL-4) cytokines rapidly upon activation, suggesting that they have important immunoregulatory functions (77). Studies from animal models indicate that NKT cells prevent autoimmunity and inflammation, either when activated naturally or when using α-Galactosylceramide (αGalCer) or related compounds (78). Stimulation of Vα14i NKT cells was beneficial in murine models of diabetes, EAE and CIA (71). Stimulating these cells was also beneficial in a chemically induced model of colitis. Moreover, the number of Vα14i NKT cells is reduced in non-obese diabetic (NOD) mice. In addition, increasing the number of NKT cells by adoptive transfer or in Vα14/Jα18 transgene mice reduced this disease.

In our study we have shown that CD1d restricted T cells have important role for immunoregulation of EAE (***Paper I***).

Mucosal immunity

Mucosal immunity is our first line of protection that reduces the need for systemic immunity. During the evolutionary modulation, the mucosal immune system has generated two non-inflammatory barriers of defence, which are, firstly "immune exclusion" performed by secretory antibodies to inhibit colonisation of microorganisms and dampen penetration of pathogenic soluble substances. The second frontier includes "immunosuppressive mechanisms" to avoid local and peripheral hypersensitivity to antigens that are normally harmless. A similar downregulatory character of the immune system normally develops against antigenic components of the commensal microbial flora (79, 80). Mucosally induced tolerance is a powerful adaptive immune function as more than a ton of food and drink may pass through the gut of an adult every year. This results in a substantial uptake of intact antigens, usually without causing any harm.

Lymphoid cells of the gastrointestinal tract are the largest lymphoid population of mammals and can be divided into loosely organized effector sites, which include the lamina propria and intraepithelial lymphocytes and more organized structures, such as mesenteric lymph nodes (LNs), Peyer's patches (PPs), isolated lymphoid follicles, and cryptopatches (CPs). These cells are located in several compartments including the organised mucosa-associated lymphoid tissues (MALT), the mucosal lamina propria (LP) and the mucosal surface epithelium (81, 82). All these structures are believed to represent inductive sites contributing to intestinal immune responses, while the lamina propria and epithelial compartment principally constitute effector sites.

MALT structures are similar to lymph nodes with B cell follicles, T-cell area and a variety of APCs such as macrophages and DCs. Exogenous stimuli are believed to come directly from the lumen mainly via M cells, perhaps assisted by DCs which may penetrate the surface epithelium with their processes (83). Therefore, induction and regulation of mucosal immunity seems to takes place primarily in the MALT.

Peyer's patches

PPs have a basic structure of a lymph node with some significant differences. The germinal centers of PPs preferentially support B cells for specific class-switching to IgA (84). The immune cells in these organs are involved in responses followed by oral tolerance by producing cytokines of Th2 phenotype mostly driven by IL-10 and induction of regulatory T cells (85, 86).

Mesenteric lymph nodes

The mesenteric lymph nodes (MLNs) of the small intestine are the largest LNs in the body and are the first LNs developing during embryogenesis. Orally exposed antigens can be presented and recognized in MLNs within a few hours followed by antigen specific T cell activation. MLNs get in contact to antigens through the afferent lymphatics draining the lamina propria and the Peyer's patches, either as free antigens or carried by DCs (87, 88). DCs are able to sample bacteria directly from the gut lumen and present them in MLNs (89).

The MLNs are therefore considered to be instrumental organs for the induction of gut immune responses and oral tolerance. Mice lacking MLNs are deficient in oral tolerance and orally exposed antigen or bacteria can be found in their spleen (90).

Oral tolerance

Mucosally induced immunosuppressive mechanisms are collectively called oral tolerance. Low doses of antigens result in the generation of antigen-specific regulatory cells following presentation by gut-associated APCs. Such presentation would preferably induce T cells that secrete downmodulatory cytokines such as TGF-β, IL-4, and IL-10. In contrast, higher doses of antigens favor clonal anergy/deletion of specific T cells in the gut and in the systemic antigen presentation (91-93).

Oral antigen induces cytokines of Th2 phenotype IL-4/IL-10 and Th3 phenotype TGF-β together with $CD4^+CD25^+$ regulatory cells (94-96). Several studies have shown that oral and nasal antigen administrations suppress autoimmune diseases including EAE, arthritis and diabetes in mice (97-99). It has further been revealed that regulatory T cells and IL-10 are fundamental key factors in protective activity followed by intranasal peptide therapy (100, 101).

In addition, oral tolerance has also been examined in human autoimmune diseases including MS, arthritis and diabetes (94). These trials demonstrated no systemic toxicity or exacerbation of disease, however, additional studies are needed to evaluate the clinical efficacy and improve the therapeutic effect of these treatment approaches.

Based on all these findings, it seems that there are two primary effector mechanisms of oral tolerance, the induction of regulatory T cells that mediate active suppression and the induction of clonal anergy or deletion.

Further studies on animals also suggest that the commensal microflora is important both for induction of oral tolerance and for reconstitution of this function after its experimental abrogation (80, 102).

Gut microflora and probiotics

"Probiotics in fermented milk have been ingested by humans for thousands of years in the belief that they have health benefits. In a Persian version of the Old Testament (Genesis 18:8) it states, Abraham owed his longevity to the consumption of sour milk. In the early 20th century, the Russian immunologist Elie Metchnikoff proposed that lactic acid bacilli may have beneficial health effects and attributed his own longevity to regular probiotic ingestion."

The human microflora is estimated to harbour about 10^{14} viable bacteria and over 500 distinct species and to have an important role in human nutrition and health, by promoting nutrient supply, preventing pathogen colonization and shaping and maintaining normal mucosal immunity (103, 104).

The influence of the resident non-pathogenic or "commensal" microflora on mucosal immune function and gut health has emerged as an area of scientific and clinical importance. In addition to the host mechanisms that control inflammation, recent evidence supports a role of the commensal microflora in maintaining immune homeostasis within the gut (105, 106).

The normal physiological response to commensal flora is immunological tolerance. A breakdown in this tolerance is thought to underlie pathological conditions, such as inflammatory bowel disease (IBD) and food allergies (107, 108). Several studies have based the so called "hygiene hypothesis", saying that a lack of early microbial stimulation (infection or exposure) results in aberrant immune responses to innocuous antigens later in life (109, 110). According to this theory, which in many ways has pushed the science of gut microbiology and immunology to the fore, reduced exposure to gut bacteria and childhood infections, alters the mechanisms and signals that determine T-cell differentiation and the susceptibility to immunological tolerance (108). For example, a bias away from Th1 towards Th2 hyper-responsiveness in the lung is thought to increase the incidence in allergic diseases, as a result of reduced exposure to Th1 respiratory pathogens. In contrast, the loss of Th2-promoting infections in the gut results in increase of Th1-dominant immunity and related gut diseases.

Mucosal DC subsets can contribute to Th1, Th2 and Treg cells. In particular, the $CD11c^+$ $CD11b^+$ $CD8\alpha^-$ DC subset preferentially polarizes antigen-specific T cells towards Th2 cytokines and IL-10, promoting T cell-dependent IgA production (111). On the other hand, the presence of $CD11c^+$ $CD11b^-$ $CD8\alpha^-$ DCs in Peyer's patches, which normally also contain bacteria, contribute to Th1-mediated responses and, in lack of tolerance, could give rise to

Th1 inflammation, characteristic for Crohn's disease (112, 113). Another mucosal DC population, $CD8^+$ plasmacytoid

DCs, are thought to be important in maintaining tolerance to harmless dietary antigens and commensal bacteria by inducing IL-10-producing Treg cells (111). In the healthy gut, elevated Th1 responses to the commensal flora are prevented by the controlling influence of Treg cells (114, 115).

Probiotics are commensal bacterial species, such as *Lactobacillus* and *Bifidobacterium*, with health promoting properties, inducing anti-inflammatory activities (102). Probiotics have shown to protect against experimental colitis and against exacerbation of inflammatory bowel disease and topical allergy in human (116-119). Since the inflammatory responses occurring in these diseases display either a Th1 or a Th2 phenotype, the immunologic effects of probiotic bacteria probably do not involve altered Th1/Th2 polarization but rather an induction of regulatory T cells.

In fact, probiotic bacteria have shown to be potent inducers of IL-10-producing DCs and inhibiting Th1 responses by promoting the appearance of Treg cells (120). In addition, the use of probiotic products, such as VSL#3 (a mixture of bifidobacteria, lactobacilli and *Streptococcus salivarius*) for the treatment of murine colitis, has shown to trigger TGF-β-bearing regulatory T cells (121).

Therefore, it is of increasing interest finding possibilities of manipulating the composition of the gut microflora by foods or food ingredients in order to increase the numbers and activities of probiotic bacteria and take advantage of their beneficial health and immunomodulatory effects.

In our study, successful therapeutic effects of selected probiotic strains have been shown on EAE. The immunosuppressive activity of probiotic on CNS inflammation has been demonstrated for the first time and this effect was attributed to activation of Treg cells (***Paper III***, Figure 2).

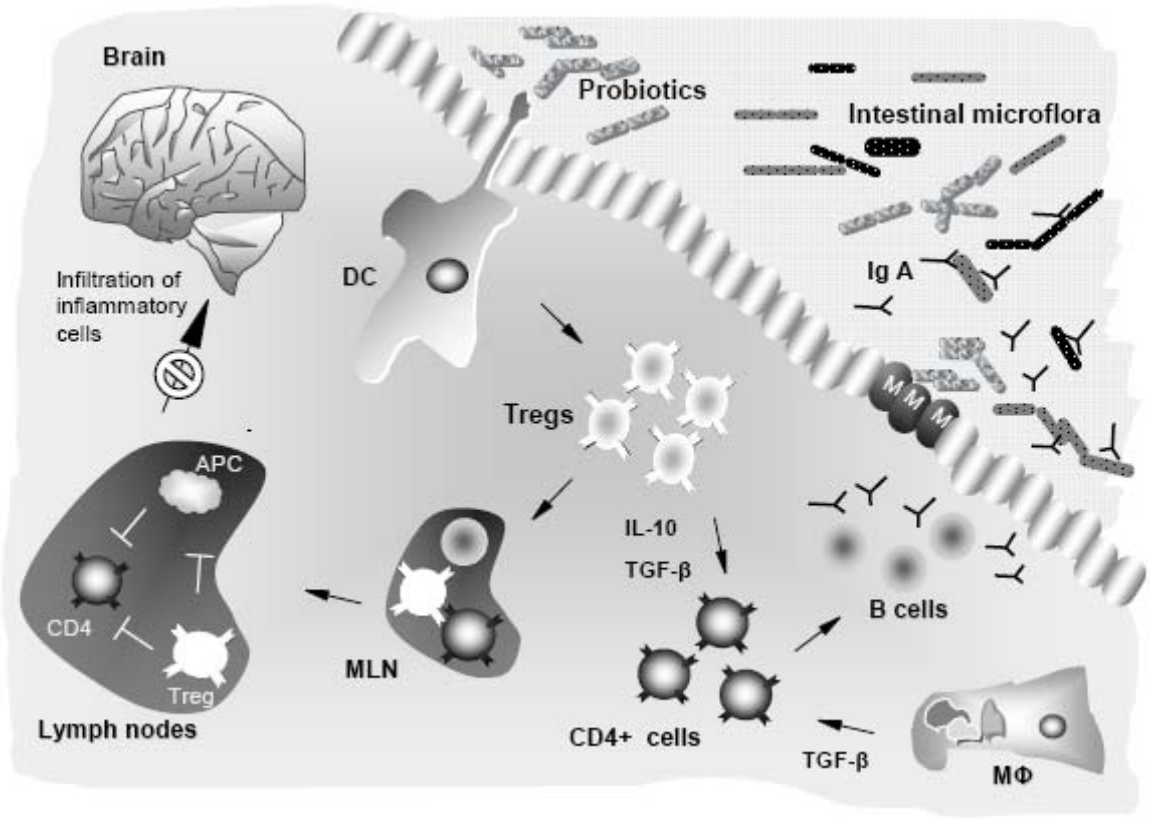

Figure 2: An overview of how probiotics exert their immunomodulating effects during inflammation

The mechanisms by which probiotic bacteria induce immunomodulating effects are yet poorly understood. It is hypothesised that probiotics prime dentritic cells (DCs) which then drive naïve T cells to differentiate into Treg cells. Once maturated, Treg cells migrate to mesenteric lymph nodes (MLNs) and, via the bloodstream, they finally end up in lymph nodes. In these sites they exert their regulatory functions by downregulating of autoreactive T cell subsets and antigen presententing cells (APCs). In addition, it has been shown that probiotic bacteria increase phagocytic activities, known as a crucial step in fighting infection. Other than that mentioned, a rice of IgA was further related to these "special" bacteria, which also remarks an initial step in punching out pathogens. In our study, we have shown that oral administration of selective *Lactobacillus* bacteria strains is therapeutic in EAE (*Paper III*). The effect was attributed to an expansion of regulatory T cells in mesenteric lymph node (MLN) and spleen followed by an increased production of IL-10, and TGF-β.

Autoimmunity *"breakdown of tolerance"*

$CD4^+$ T cells are the master controlers of immune responses to protein antigens, and various autoimmune disorders are thought to arise from a breakdown of immunological tolerance of these cells. Despite the existence of various, in fact crucial, pathways of tolerance, how can T cell tolerance, induced in the thymus and supported by several extrathymic mechanisms, be overcome and give rise to autoimmunity? Are all of these pathways essential for sustaining self-tolerance? One possible answer is that each pathway maintains tolerance to a subset of

self-antigens and so the loss of any pathway will result in a restricted set of autoimmune reactions. A possibility is that all mechanisms have to work simultaneously to preserve self-tolerance and disruption of any one mechanism alters the finely tuned balance and harmony which can lead to autoimmunity.

Another basic question is “What is self”? Some scientists describe "self" as everything encoded by the genome. Others include “everything under the skin”, including structures encoded by commensal genomes. For T cells, one definition of “self” is, the set of peptides found complexed with MHC molecules. In contrast, other argue that “self” consists only of APCs and thymic epithelium and that all other tissues are ignored or a set of bodily proteins that exist at a concentration above a certain threshold (122, 123). The definition of “non-self” does not seem to be easier than “self”. Considering the “everything outside the skin”, creates a problem with several non-self structures to which we do not raise any immune response, e.g. silicon, bone fragments, many peptides (MHC dependent) and various food particles. These definitions might discriminate “some” self from “some” non-self, but, ultimately, they all lack a fundamental explanation for autoimmunity.

Considerable evidence implicates the influence of the genetic susceptibility and/or infections that are associated with autoimmune diseases. Linkage analysis of the human genome has revealed candidate HLA complex, for susceptibility to multiple sclerosis and type-1 diabetes (124). Infection has also been suggested as causing autoimmune diseases, such as multiple sclerosis and type-1 diabetes (125). The mechanisms leading from infection to autoimmunity include the release of the appropriate autoantigens caused by tissue damage (125), the activation of a large amount of the T cell population by superantigens (126), and the induction of inflammatory cytokines and costimulatory molecules by microbial products (127, 128). Alternatively, a structural similarity between microbial and self-antigens “molecular mimicry” could also have a key role in activating autoreactive T cells (129).

Today there are more than 40 human diseases classified as either definite or probable autoimmune disorders, and they affect 5-7% of the population. Almost all autoimmune diseases appear without warning or apparent cause, and most patients suffer from fatigue. The fact that nearly 75% of autoimmune disease patients are women indicates a possible association of hormones on the disease incidence.

Multiple sclerosis

MS is one of the most common chronic and disabling disorders of the CNS, affecting 0.1% of the population. The disease generally starts in early adulthood and, despite important progress in treatment in recent years, it remains a important cause of disability in the white population (130). The etiology of MS is unknown, but many findings indicate the importance of the immune system in the disease pathogenesis, influenced by both genes and environmental factors. Family and genetic studies revealed that *HLA-DR1501* and *HLA-DQ0601* alleles are associated with a 2-4 fold greater risk of developing MS (131). However, association between relapses and viral infections and some migration studies strongly support the important role of environmental factors in development of MS (132, 133).

Since studies in EAE have presented a key role of autoreactive $CD4^+$ T cells, MS has also been recognised as a T cell-mediated disease. Any CNS tissue damage can result to activation of CNS resident immune cells, such as microglial cells, which upregulate MHC and costimulatory molecules. These cells start to release cytokines and chemokines, initiating the recruitment of monocytes, lymphocytes and DCs into the lesions. Microglial cells seem to be crucial for starting and maintaining the inflammatory milieu, while DCs are involved in presentation of antigens to infiltrating T cells (134). Simultaneously, the autoantigen from the lesions will be accessed in the periphery. It is not clear whether the antigens are passively drained or actively carried by other cells. DCs will then start an acquired immune response in the lymph nodes by processing the autoantigen and presenting peptide antigens, in complex to MHC class I and II and the presence of costimulatory molecules, to naïve T cells. Activated autoreactive T cells will then cross the blood brain barrier (BBB), guided by chemokines, and infiltrate the lesions. The distribution of the T cells is directly associated with expression of MHC molecules. MHC class II is mostly expressed on APCs, while MHC class I is expressed by all cells in the inflammatory lesions of the CNS (135, 136). Consequently, $CD4^+$ T cells are predominantly found in perivascular regions and meninges, whereas $CD8^+$ T cells are found in the center and border zones of the inflamed lesions (137, 138). Recent data confirms the fact that infiltrating T cells originate from the same precursor cell and show identical antigenic specificities (139). $CD4^+$ T cells insert their effector function by recruiting macrophages, which release proinflammatory cytokines and toxic molecules, such as nitric oxide, IL-1, IL-6, TNF-α and matrix metalloproteinases. $CD8^+$ T cells seem to directly attack MHC class I-expressing cells such as oligodendrocytes and neurons. Futhermore, the

importance of myelin-protein-specific T cells in the pathogenesis of MS is still not known since these cells exist in both, diseased and healthy controls (140).
In the lymph nodes, B cells recognize autoantigens displayed on DCs. It is still not quite clear where these cells interact with helper T cells and receive further activation signals for plasma cell differentiation. Upon activation, they infiltrate the CNS (perivascular space and meninges), release autoantigen specific antibodies which bind to self tissues and initiate the process of phagocytosis (30). Presence of activated plasma cells and antibodies in lesions and cerebrospinal fluid (CSF) during the course of the disease implies a periodical or, in the case of the CNS, a persistent recruitment (141). Unfortunately, B cells have been neglected in MS research since they appeared not to be prominent for the development of EAE (142).

Immunological changes in the lesions

Microglia and macrophages upregulate MHC class II molecules and complement receptor C3d-immunoglobulin complexes on the surface (138). Observations between 6 and 20 weeks from disease onset showed immune cell infiltration, demyelination, BBB leakage, reactive astrocytes and proliferating oligodendroglial cells in the lesions. Many cytokines, including Th1 and Th2 phenotypes, and chemokines are produced within the lesions (143). Demyelination and axonal damage were also seen during all phases of the disease but appeared more evident during the early acute phase, correlating with increased levels of cellular infiltration (144, 145).
Proliferation of oligodendrocytes and remyelination of axons are detectable in many lesions. In general, inflammation, neurodegeneration and remyelination differ between the MS patients (146). Lesions in some patients are characterized by eosinophil infiltration, in others, despite broad oligodendrocyte apoptosis and microglia activation, no inflammatory infiltrates were seen (146, 147). This indicates that in some patients and/or at certain time points, the immune system might not be the central key factor for pathogenesis of MS.

Inflammation and neurodegeneration

Axonal damage and loss seem to be important determinants of neurological disabilities. Several hypothesises have suggested a link between inflammatory responses in the CNS and

axonal damage. These include activation of $CD8^+$ T cells that directly induce apoptosis in neurons, recruitment of macrophages by activated $CD4^+$ T cells (secreting inflammatory mediators and toxic molecules) and finally presence of antibodies to neuronal surface antigens. Indirect mechanisms, such as loss of protective myelin and the release of glutamate or nitric oxide, might also result in axonal damage (148). Although all of these mechanisms could be relevant, the molecular events that cause the axonal damage in MS are still unknown.

Experimental autoimmune encephalomyelitis

Experimental animal models have provided invaluable information in our understanding of the mechanisms of various human autoimmune diseases and allow us to investigate the influence of the genetic background and environmental factors in the disease development. They also give us the ability to test and evaluate possible treatments before human trials.

EAE is an animal model for human MS used worldwide. It is an inflammatory demyelinating disease of the CNS, clinically manifested by developed tail and limb paralysis. EAE is generally induced in susceptible strains of animals by immunization with CNS antigens in adjuvants, often with additional use of pertussis toxin, or, alternatively, by the transfer of *in vitro*-cultured, CNS-specific activated T cells (149). The EAE model has been studied extensively and close clinical and histopathological similarities to MS have been found (150-152). The pathophysiological processes of EAE initiate when T cells, able to recognize myelin proteins such as myelin basic protein (MBP), proteolipid protein (PLP) or myelin oligodendrocyte glycoprotein (MOG) are activated in the periphery, migrate to the CNS and cause autoimmune inflammation leading to paralysis (153). EAE is considered a Th1, MHC-class II-restricted, $CD4^+$ T cell mediated disease of the CNS. The immunological processes in EAE are summarized in figure 3.

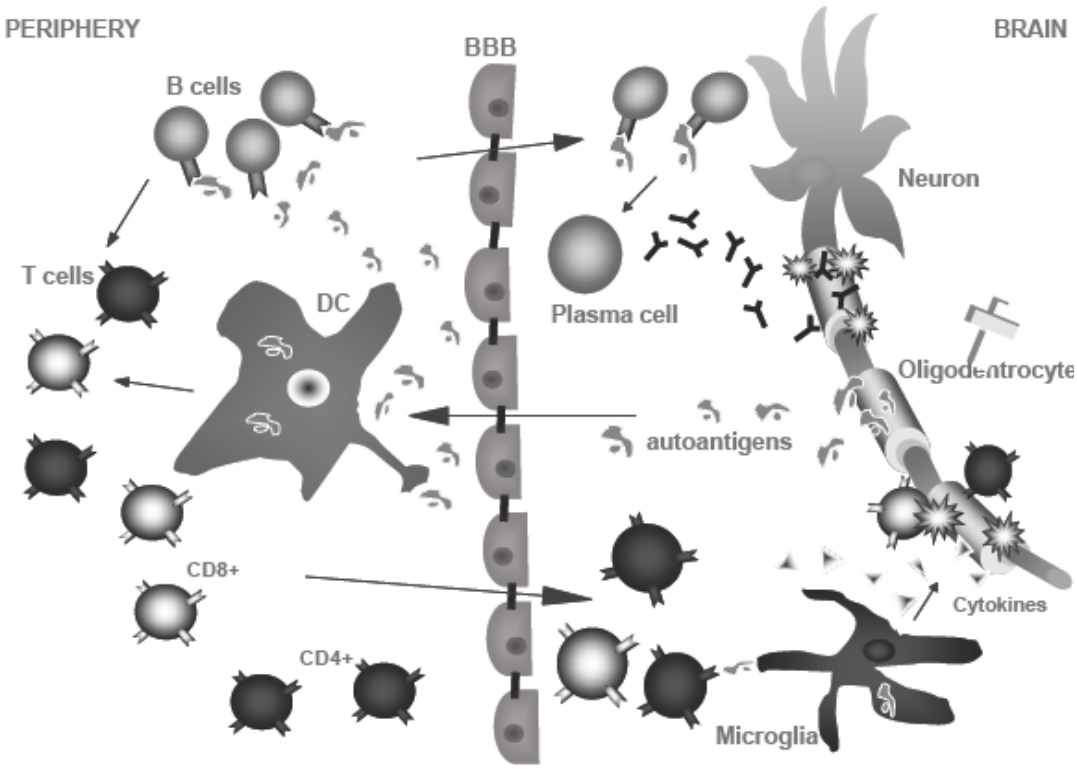

Figure 3: An overview of immunological processes in MS pathogenesis

In the periphery, autoantigens are presented by APCs such as dendritic cells to T cells. Autoreactive T cells migrate to the CNS through the BBB and are reactivated by local or infiltrating APCs, resulting in the release of proinflammatory and cytotoxic mediators, leading to tissue damage. Cooperation between $CD4^+$ T helper and B cells eventually leads to infiltration of autoreactive B cells as well. The protective myelin sheath is destroyed as a result of cytokine- and complement-mediated damage, digestion of surface myelin antigens by macrophages and direct damage by $CD4^+$ and $CD8^+$ T cells, leading to apoptosis of oligodendrocytes.

One of the critical lessons from the EAE model is the knowledge of epitope spreading. It has been shown that administration of a single myelin protein epitope into EAE mice, T cells became activated against other epitopes of the same protein which was followed by T cell activation to other myelin proteins, all capable of adoptively transferring the disease to naïve mice. The epitope spreading requires costimulation with CD28/B7, suggesting that tissue damage in the CNS is a result of a local "adjuvant" which induces high expression of the B7 molecules in association with antigen release (154). Epitope spreading has recently been shown to be initiated in the CNS by local APCs or invading DCs (155).

Recently, MOG-induced EAE has attracted increasing attention, particularly because MOG-reactive T cells are commonly found in the blood circulation of MS patients (156, 157) and it can be induced in a variety of mouse strains and leads to a chronic and relapsing demyelinating disease (158, 159). MOG-induced EAE is generally accepted as a $CD4^+$ mediated disease but can also be induced by MOG-reactive $CD8^+$ T cells. The clinical

symptoms and histological findings in $CD8^+$ T cell-induced EAE experiments indicate a major difference by showing ataxia, spinning, a loss of coordination, and neutrophil rich infiltration, instead of an ascending flaccid paralysis (160, 161).
A natural resolution is always followed by the chronic phase of the MOG-induced EAE. Mechanisms involved in this spontaneous recovery seems to be caused by IL-10 secretion and an accumulation of $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells (162-164).

As an experimental model for chronic inflammation in CNS, we immunized C57BL/6 mice with MOG_{35-55} peptide together with complete Freund's adjuvant (CFA). Pertussis toxin (PT) was also injected intra-peritoneally (i.p.) at the time of immunization, as well as two days later. It is believed that CFA and PT work as adjuvants, activating APCs priming a MOG specific Th1 response. The animals develop EAE with nearly 100% incidence which starts with an acute phase and is followed by a chronic course. The immune cell infiltration persists in the CNS tissues throughout the disease development which indicates an active inflammatory process rather than early irreversible tissue damage. Histological analysis highlights a high degree of demyelination which correlates well with MS pathogenesis.

Current disease-modifying therapies in multiple sclerosis

Over the last few years, several therapeutic agents for treatment of MS have been tested and studied, but the management of the disease still remains complex and unreliable. On the basis of the inflammatory nature of this disease, global immunosuppression was the first approach that attempted to attenuate the immune response.

Broadly immunosuppressive agents

In some early studies, treatment with ciclosporin demonstrated some minor benecicial effects in MS. In addition, mitoxantrone has also been used to treat worsening forms of MS and showed delay in disability progression of the MS patients (165).
Among currently available therapies, β-interferon (IFN-β) and glatiramer acetate (GA) have a modest effect on reducing relapses and slowing the accumulation of disability in relapsing-

remitting MS patients (166, 167). The suggested mechanisms of IFN-β are a limitation of T cell trafficking, restoration of Th1-Th2 imbalance and exhibition of anti viral properties (168). On the other hand, the suppressive mechanism of GA, synthetic polypeptide composed of the most prevalent amino acids in MBP seems to be caused by generation of myelin-reactive Th2 cells which cross the BBB and results in bystander immune suppression at lesions in the CNS (169). However, no study was able to demonstrate a significant benefit to sustained disability progression. In fact, not all patients responded to IFN-β and a substantial number of patients who initially responded experienced a reduction in treatment efficacy during the course of the disease. This has been attributed to generation of a neutralising antibody response against IFN-β (170).

There are currently no effective treatments available for MS, therefore new strategies are needed to significantly delay long-term disease progression.

New immunomodulatory drugs that ameliorate EAE have shown some promise in a few phase II trials. Statins, which exert a variety of immunomodulatory actions, are being tested in human (171). Minocycline, an immunomodulatory and possibly neuroprotective agent, is also being examined in a clinical trial (172). Hematopoietic stem-cell therapy to delete autoreactive T cells from the repertoire has also shown promising results, but this should be weighed against the high mortality associated with this treatment (173).

Selective immune intervention

Specific deletion of distinct immune populations or selective blockade or activation of immune cells and molecules have also been of interest for MS treatment. Monoclonal antibodies have been widely used targeting cell-specific surface molecules. Depletion of $CD4^+$ T cells has shown promising results in animal models but has had no impact on MS (174). On the other hand, depletion of B cells seems to be beneficial in a group of patients with high humoral activity (175). Based on the positive results in EAE, antibodies against T cell growth factor IL-2 have also been tested in a phase II trial, but this has shown no promising effect (176). In addition, anti-TNF-α therapies also successfully ameliorated EAE but were associated with increased disease activity in MS (177).

Antigen- and TCR-based therapies

Several antigen-based treatments have been developed specifically targeting the T cell responses to myelin proteins. These strategies included the tolerization of autoreactive T cells by oral administration of myelin antigens or by the administration of an altered peptide ligand (APL) based on MBP. Despite the promising results in experimental models, phase III clinical trials produced negative results and had to be stopped (178).

TCR-specific therapies have also been explored in small clinical trials. Using TCR-peptide vaccination or anti-CD4 treatment have has been shown to deplete antigen-specific T cells from the repertoire, but with no significant impact on the disease course (174, 179). A principal conceptual problem with these studies is that they targeted particular TCRs. Recent reports on a biased TCR repertoire which appears during the disease development confirm that a therapy against specific T cell clones might not be effective (180).

Modulation of immune-cell migration

Blockade of adhesion molecules, in order to prevent immune cells from passing across the BBB has also shown positive results when using antibodies against β4 integrin in EAE. This treatment had efficiently suppressed the disease progression in human trials and resulted in a product (natalizumab) but the marketing of this drug had to be stopped due to some major side effects (181).

The present study

Despite the growing number of available therapeutic approaches, it is clear that none of the existing therapies can stop the disease progression in MS. Therefore, the search for more efficient therapies has to be continued. Our improved knowledge about the mechanisms of autoimmune diseases, suggests that it is more promising to actively strengthen physiological counterregulatory mechanisms, than attempting to ‘‘delete’’ specific autoreactive cells from the immune repertoire.

Based on these facts, the general goal of this thesis was to study the immunoregulation of T cell populations in EAE in order to provide new therapeutic opportunities for the treatment of MS by using biological agents exerting an overall anti-inflammatory effect.

Our major focus was:

- The immunoregulation of CD1d restricted T cells in EAE.
- The therapeutic efficacy of monoclonal antibody against TCR αβ in EAE by targeting a broad TCR repertoire.
- The therapeutic potential of probiotic bacterial strains in EAE.

Paper I:

CD1d restricted T cells have been shown to play a protective role against autoimmune diseases. In this study we investigated the role of the CD1d antigen presentation pathway in the development of EAE, using CD1d knockout (KO) mice. We have demonstrated that mice, which were deficient in CD1d, developed a more severe and chronic EAE compared with wildtype (*wt*) mice. This was further confirmed by finding increased levels of immune cell infiltration and demyelination in the CNS of CD1d KO mice. Additional studies on the autoreactive T cells in the periphery revealed that T cells from CD1d deficient mice produced elevated levels of Th1 and Th2 cytokines. Investigation of the CNS tissues of these animals also revealed that during the course of the disease, expression of TGF-β is upregulated in *wt* mice, while it is defect in CD1d KO mice. In another attempt, using an adoptive cell transfer model to induce EAE, we did not find any difference in the disease development between the two groups, whereas priming the immune system with CFA prior to the transfer restored the partial protective activity in the *wt* mice.
We therefore conclude that CD1d restricted T cells play a regulatory role in EAE. This regulation is mediated both through inhibition of the encephalitogenic T cell responses and induction of anti-inflammatory TGF-β at the inflammatory site. The CD1d antigen presentation pathway requires activation which in our approach has been achieved by CFA immunization.

Paper II:

Monoclonal antibodies against TCRs have been extensively used to eliminate or modulate the function of T cell-mediated autoimmune diseases. Despite some promising prophylactic treatments in animals, little success was achieved when TCRs were applied therapeutically or in human trials. In this study, we have evaluated the therapeutic efficacy of monoclonal antibody (H57-597 MoAb) against TCR αβ in EAE. Mice were treated with three i.p. injections of 100 μg antibody for three consecutive days. In an attempt to treat EAE prophylactically, administration of anti-TCR αβ immediately after the immunization protected the mice from EAE and inhibited the inflammatory cell infiltration into the CNS. In another experiment, we further examined the tolerogenic capacity of this MoAb by treating highly diseased animals. We showed that highly diseased animals treated with anti-TCR αβ completely recovered from EAE, shortly after the treatment. Further investigation showed that the therapeutic effect of MoAb treatment was attributed to a transient depletion of T cells and an expansion/activation of NKT cells in the periphery. T cells were restored 17 days after treatment but the tolerance against autoreactive T cells remained. We have demonstrated that this tolerogenic effect was transferrable by splenocytes from MoAb treated mice to EAE mice. For the first time, we have demonstrated the therapeutic effect of anti-TCR αβ MoAb in EAE and showed that this antibody via TCR signaling activated a population of immunoregulatory T cells. This represents a promising approach in the treatment of MS.

Paper III:

Probiotic bacteria, including Lactobacilli, have been shown to exert beneficial health effects in infectious and inflammatory diseases by modulating the immune system. In the case of chronic autoimmune diseases, such as MS, a major goal of treatment is to suppress inflammation. The aim of our study was to investigate whether oral administration of selected *Lactobacillus* strains could affect the systemic immune response by suppressing the T cell-mediated chronic inflammation in the CNS. To this end, the immunomodulatory potential of a range of *Lactobacillus* was evaluated in EAE. Daily oral administration of *Lactobacillus paracasei* DSM 13434, *Lactobacillus* plantarum DSM 15312 and *Lactobacillus* plantarum DSM 15313, starting 12 days prior to immunization, prevented EAE development in mice.

Further analysis of *L. paracasei* DSM 13434 treated mice revealed reduced amounts of autoreactive T-cells both in CNS and periphery in concert with increased production of IL-4, IL-10 and TGF-β and increased numbers of regulatory $CD4^+CD25^+Foxp3^+$ T cells in the MLNs and the spleen. Furthermore, TGF-β producing regulatory $CD4^+$ T cells in MLNs were shown to be essential for the protective effect of the probiotics since tolerance could be transferred by MLN cells but was abolished after CD4+ T cell depletion. Finally, a therapeutic treatment with a mixture of *L. paracasei* DSM 13434, *L. plantarum* DSM 15312 and *L. plantarum* DSM 15313 successfully suppressed established chronic EAE. These studies indicate the therapeutic potential of selected probiotic bacteria on T cell-mediated chronic inflammation in CNS which can be applicable to the treatment of human autoimmune disorders (Figure 2).

Concluding remarks

Multiple sclerosis is one of the most common inflammatory and neurological diseases of young adults in Europe and North America. It is likely that MS has multiple causal factors that differ between individuals. Both genetics and the environment are supposed to play roles. In particular, the immune system seems to be a key factor in the disease progression. Current drugs shut down the patients' immune system, which limits their ability to fight against infections. New compounds and immunosuppressive drugs have shown only a limited impact on the disease course, but biological agents are capturing the spotlight now. Researchers are harnessing the power of the body's own immune system to help fight diseases.

Bringing immunology to medicine offers exciting and real scientific challenges such as immunotherapy. Restoring tolerance via immunotherapies not only continues to fascinate immunologists, but, beyond its experimental aspects, remains an attractive goal for clinicians who treat MS patients and other autoimmune diseases. These approaches should allow us to arrest completely the disease process with minimal side effects. As evidenced by our scientific achievements, our research has contributed to a better understanding of a few immunoregulatory pathways which could be targets for immune interventions. In addition, we have introduced unique therapeutic approaches for chronic inflammation using a monoclonal antibody or probiotic bacteria. The latter indicates the powerful therapeutic potential of these healthy micro-organisms which can be used for the management of autimmune diseases.

Populärvetenskaplig sammanfattning

”Summary in Swedish”

Kroppen har en mycket imponerande arsenal för att avvärja sjukdomar, alltifrån huden och magsäckens syror till slem i andnings-, urogenital- och mag-tarmsystemet. Men det mest invecklade och, ända fram till våra dagar, mest gåtfulla försvarsvapnet är vårt immunsystem. Detta består av ett nätverk av olika celler så kallade vita blodkroppar och substanser (t.ex. antikroppar) som tillsammans skyddar kroppen från främmande ämnen som tenderar att störa kroppens inre balans eller skada vävnader. Immunsystemets reaktion på skada eller angrepp kallas för inflammation som kännetecknas av en ansamling av immunceller och molekyler. Immunförsvarets mekanismer är anmärkningsvärt komplicerade men kan delas in i den medfödda och den inducerbara adaptiva immuniteten. Den medfödda immuniteten finns färdigt redan från födelsen och har en generell skyddseffekt mot mikroorganismer. Den adaptiva immuniteten utgör ett specifikt immunnsvar mot olika främmande ämnen. Systemet består av T-lymfocyter och B-lymfocyter som kan skilja mellan vad som är främmande och vad som tillhör kroppen med hjälp av speciella receptorer. Dessa receptorer har stor diversitet som kodas slumpmässigt av varierande gener. För att kunna känna igen och skilja åt främmande från kroppsegna ämnen måste lymfocytcellerna utbildas i en tuff skola i benmärgen (B-celler) och i brässen (T-celler).

Det är anmärkningsvärt att de flesta lymfocyter inte klarar av detta och underkänns och dör. Men ibland smiter vissa icke godkända celler undan och genom att uppfatta kroppsegna celler som främmande angriper de friska vävnader och orsakar ogynnsamma effekter vilka leder till autoimmuna sjukdomar.

Multipel skleros (MS), är en sjukdom som angriper centrala nervsystemet (CNS), d.v.s. hjärna och ryggmärg, och tros vara en autoimmun sjukdom. Den som har MS får återkommande inflammatoriska reaktioner som angriper och förstör isoleringsskiktet (myelin) runt nervtrådarna. Detta orsakar störning i de elektriska nervimpulserna utmed nervtrådarna vilken slutligen leder fram till neurologiska funktionsnedsättningar med vanligtvis förlamning och synskada. Insjuknandet sker huvudsakligen i 20-40 årsåldern. Kvinnor drabbas oftare än män. Cirka 80% insjuknar med skovvis förlöpande sjukdom, s.k. relapsing-remitting MS. Prevalensen i Skandinavien är cirka 100/100 000, medan den i Nordamerika är cirka 200/100 000. Idag finns det ingen behandling som kan bota MS. Under de senaste åren har det emellertid utvecklats så kallade sjukdomsmodifierande läkemedel som bromsar

inflammationsprocesserna och leder till mindre handikapputveckling. Men dessa måste individanpassas och är oftast inte är tillräckligt effektiva.
Förutom T-lymfocyter som startar igång inflammatoriska processer, finns det andra T-celler som har till uppgift att reglera och dämpa immunsvaret för att hålla det under kontroll. Dessa kallas för regulatoriska T-celler och tros fungera sämre hos MS-patienter. Därför kan en aktivering av dessa regulatoriska celler vara ett effektivt sätt för att behandla MS.
Målet för denna avhandling har varit att studera hur de inflammatoriska processerna i MS regleras och vidare utveckla nya biologiska behandlingsmetoder för att lindra sjukdomen.
För detta ändamål har vi använt oss av en djurmodell för MS, experimentell autoimmun encefalit (EAE), som induceras i möss genom att aktivera myelin-reaktiva lymfocyter.

I det första arbetet har vi studerat betydelsen för CD1 reglering för utvecklingen av EAE/MS. CD1 är ett gåtfullt protein som presenterar lipid- och glykolipid-partiklar för T-celler, i synnerhet till vissa regulatoriska populationer. Vi har visat att CD1-aktiverade celler utför kontrollmekanismer som är viktiga för att begränsa inflammatoriska T-celler i CNS.

I jakten efter effektiva behandlingsmetoder för MS, har vi behandlat EAE/MS-sjuka möss med en antikropp riktade mot T-cell receptorn. Behandlingen kunde skydda djuren från EAE/MS och även behandla och fullständigt återställa djur med svår grad av sjukdom. Denna sensationella behandling har visat sig dämpa alla de sjukdomsalstrande T-cellerna och aktivera en viss population av regulatoriska T celler.

I vårt sista försök har vi behandlat EAE/MS-sjuka möss med utvalda probiotiska bakterier. Probiotiska bakterier har förmågan att kolonisera mag-tarmkanalen och därigenom medföra positiva effekter till värdens hälsa. De senaste årens forskning om probiotiska bakterier föreslår en förstärkande effekt på immunförsvaret vilket kan reducera oönskade inflammatoriska processer i kroppen. Vi har selekterat vissa unika probiotiska stammar som tidigare har isolerats från människans tarmslemhinnor, här i Lund. Behandlingen gick ut på att låta mössen få de bakterierna i dicksvattnet. Möss som hade fått bakterier i förebyggande syfte utvecklade en mildare sjukdom. Slutligen visade vi att möss med svår grad av sjukdom framgångsrikt kan behandlas med en blandning av olika probiotiska bakterier. Därmed visade vi för första gången att vissa probiotiska bakterier har en stark potential att förebygga och till och med bota MS.

Min förhoppning är att dessa studier ska kunna läggas till grund för design av framtidens immunosuppressiva läkemedel för att behandla autoimmuna sjukdomar och i synnerhet MS.

Acknowledgements

In this thesis, I am only presenting a fraction of my scientific work and achievements during the past few years! All these experiences helped me to grow and develop as a teacher and scientist. There are several wonderful people who helped me with their support and guidance to stay on track and reach where I am. They supported me with my ups and downs, while I tried to find a cure for other people's ups and downs.

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