Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 608

CNS-Targeted Cell Therapy for Multiple Sclerosis

MOA FRANSSON
Dissertation presented at Uppsala University to be publicly examined in Rudbecksalen, Rudbecklaboratoriet, Dag Hammarskjöldsväg 20, Uppsala, Friday, December 3, 2010 at 09:15 for the degree of Doctor of Philosophy. The examination will be conducted in English.

Abstract

Multiple sclerosis (MS) is an autoimmune disorder of the central nervous system (CNS). In the current thesis, we have performed an immunological investigation of patients with MS and developed an immunosuppressive cell therapy that could be beneficial for these patients.

MS has been considered to be driven by T helper type 1 (Th1) lymphocytes but new data indicate the involvement of Th17 responses. T cells from patients with MS that were evaluated for immunological status secreted both interferon-γ and interleukin-17 upon stimulation. However, T cells from patients with MS in remission, in contrast to relapse, had poor proliferative capacity suggesting that they are controlled and kept in anergy.

T regulatory cells (Tregs) are important to maintain self-tolerance and the role of CD4+CD25+Foxp3+ Tregs in autoimmunity has been extensively investigated. We analyzed Tregs from patients with MS in relapse and remission by multicolor flow cytometry for the expression of CD3, CD4, IL2R (CD25), FoxP3 and the IL7R (CD127). Patients in relapse exhibited higher levels of FoxP3-positive Tregs lacking CD25 compared to healthy controls, indicating that Tregs might attempt to restrain immune activity during relapse.

In the murine experimental autoimmune encephalomyelitis (EAE) model of MS, therapy with suppressive cells such as Tregs or mesenchymal stromal cells (MSCs) has proven beneficial. However, systemic administration of such cells may immunologically compromise the recipient and promote infections due to general immunosuppression. We hypothesized that suppressive cells can be equipped with a CNS-targeting receptor and be delivered intra-nasally to avoid systemic exposure. CD4+ T cells were modified with a lentiviral vector system to express a myelin oligodendrocyte (MOG)-targeting receptor in trans with the FoxP3 gene that drives Treg differentiation. Genetically engineered Tregs demonstrated suppressive capacity in vitro and localized to the brain and suppressed ongoing encephalomyelitis in vivo. Cured mice were rechallenged with an EAE-inducing inoculum but remained healthy.

MSCs are a heterogeneous population of stromal cells residing in most connective tissues and have the capacity to suppress effector cells of the immune system. MSCs were engineered to express MOG-targeting receptors using lentiviral vectors. Genetically engineered MSCs retained their suppressive capacity in vitro and successfully targeted the brain upon intranasal delivery. Engineered MSCs cured mice from disease symptoms and these mice were resistant to further EAE challenge. Encephalitic T cells isolated from cured mice displayed an anergic profile while peripheral T cells were still responsive to stimuli.

In conclusion, MS patients have peripheral CNS-reactive T cells of both Th1 and Th17 type that, while in remission, are kept in anergy. Also, MS patients in relapse exhibit increased levels of CD25 negative Tregs indicating an attempt to restrain immune activity. Finally, immunosuppressive cells can be genetically engineered to target CNS and efficiently suppress encephalomyelitis in an active EAE model upon intranasal delivery.

Keywords: CAR, Targeting, Suppressive cells, Foxp3, Tregs

Moa Fransson, Department of Oncology, Radiology and Clinical Immunology, Clinical Immunology, Rudbecklaboratoriet, Uppsala University, SE-75185 Uppsala, Sweden.

© Moa Fransson 2010

ISSN 1651-6206
urn:nbn:se:uu:diva-132364 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-132364)
To Henrik, Hugo and our baby
List of Papers

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


III Fransson, M.*, Piras, E.*, Wang, H., Burman, J., Harris, R., Brittebo, E and Loskog, A Engineered T regulatory cells target CNS and suppress active experimental autoimmune encephalomyelitis (EAE) upon intra nasal delivery. *Manuscript*

IV Fransson, M.*, Piras, E.*, Wang, H., Burman, J., Duprez, I., Harris, R., LeBlanc, K., Brittebo, E and Loskog, A. Human mesenchymal stroma cells expressing a CNS-targeting receptor can be administrated intranasally and cure EAE. *Manuscript*

Reprints were made with permission from the respective publishers.

1 Copyright © 2009 John Wiley and sons
2 Copyright © 2010 Informa healthcare communications
* The authors contributed equally
Is it possible to treat autoimmune immunity with immunotherapy? If the origin of autoimmunity lies in the immune system why not set everything right by triggering the signaling pathways already existing? The idea is simple – create a situation where the immune system acquire help to do what it is supposed to do and everything goes back to normal. There are, however, a few obstacles to overcome before it is a reality and with every try there is a risk. A risk to cause more damage than remedy. Adoptive cellular immunotherapy as treatment for autoimmunity has been evaluated in experimental models during the past decade and many successful trials have been conducted. However, there are still some important questions that need thorough investigation: where do the therapeutic cells end up in the patient, how long are they lasting and is there a possibility that they will turn into malignant cells? Because of the potential biohazard of therapeutic cells one interesting option is to genetically engineer therapeutic cells with inducible suicide genes so the treatment can be aborted at will. In this thesis we have constructed immunosuppressive cells that can target the brain by genetic engineering and evaluated them in a model of Multiple Sclerosis (MS). These cells completely blocked autoimmunity and may be a candidate drug for human trials. Today there is no cure for autoimmune diseases such as MS but autologous hematopoietic stem cell transplantation has shown that a selection of patients clearly benefits and may even be cured. Patients with life-threatening paralysis that received stem cell transplantation regained their ability to walk and the lack of immediate degeneration after treatment demonstrated the possibility for resetting the immune system. In conclusion, there is great possibility to remodel the immune system without causing damage to the patient; we just have to find the right treatment combined with the best safety systems. Search and you will find!
Results and discussion .................................................................38
  Paper I .........................................................................................38
    Background and significance .....................................................38
    Results and discussion ..............................................................38
  Paper II .........................................................................................39
    Background and significance .....................................................39
    Results and discussion ..............................................................40
  Paper III .........................................................................................41
    Background and significance .....................................................41
    Result and discussion ...............................................................41
  Paper IV .........................................................................................42
    Background and significance .....................................................42
    Result and discussion ...............................................................42

Conclusions ..................................................................................44

Future perspectives ........................................................................45

Acknowledgements .......................................................................46

References ....................................................................................50
Abbreviations

BBB Blood brain barrier
CAR Chimeric antigen receptor
CD Cluster of differentiation
CD25 IL2 receptor alpha chain
CD127 IL7 receptor alpha chain
CAF Carcinoma-associated fibroblasts
CMV Cytomegalovirus
CNS Central nervous system
CSF Cerebrospinal fluid
CTL Cytotoxic T lymphocytes
CTLA-4 T lymphocyte-associated antigen-4
DC Dendritic cell
iDC Immature dendritic cell
pDC Plasmacytoid dendritic cell
EAE Experimental autoimmune encephalomyelitis
EBV Epstein-Barr virus
ES Embryonic stem cell
FoxP3 Forkhead box P3
GA Glatiramer acetate
G-CSF Granulocyte-colony stimulating factor
GFP Green fluorescent protein
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICAM</td>
<td>Intracellular adhesion molecule</td>
</tr>
<tr>
<td>IDO</td>
<td>Indoleamine 2,3-dioxygenase</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>i.n.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>i.n.</td>
<td>Intranasal</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IVIG</td>
<td>Intravenous immunoglobulin</td>
</tr>
<tr>
<td>LAG-3</td>
<td>Lymphocyte activation gene-3</td>
</tr>
<tr>
<td>LFA</td>
<td>Lymphocyte function-associated antigen</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MBP</td>
<td>Myelin basic protein</td>
</tr>
<tr>
<td>MIP</td>
<td>Macrophage inflammatory protein</td>
</tr>
<tr>
<td>MOG</td>
<td>Myelin oloidendrocyte glycoprotein</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stroma cell</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer cell</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>PLP</td>
<td>Proteolipid protein</td>
</tr>
<tr>
<td>RRMS</td>
<td>Relapsing remitting multiple sclerosis</td>
</tr>
<tr>
<td>ScFv</td>
<td>Single chain variable fragment</td>
</tr>
<tr>
<td>TcR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory cell</td>
</tr>
</tbody>
</table>
Introduction

The immune system

Overview

The immune system is a complex network of cells and molecules that provide protection against invading pathogens such as bacteria, viruses and parasites. The first line of defense is provided by the *innate immunity*. Physical and chemical barriers such as epithelia, skin and anti microbial substances hinder many pathogens from entering. However, if entered, phagocytes, NK-cells and proteins of the complement system become activated in response to recognition of certain molecular structures shared by different groups of microbes and viral agents such as unmethylated DNA found in bacteria and double stranded RNA found in replicating viruses. Cells and molecules of the innate immune system are preformed and ready to operate rapidly. If re-challenged by the same pathogen they do not remember the pathogen but respond in a similar manner. *Adaptive immunity*, involving T- and B-lymphocytes and their products, mobilizes first after a few days but is highly specific and more effective in eliminating pathogens. In contrast to innate immunity, adaptive immunity has the ability to respond more rapidly and more efficiently when re-encountered with a pathogen because of their capacity to remember the pathogen by a process called memory. Lymphocytes can be divided in to two major sub populations (B cells and T cells) depending on surface protein expression and mode of action. B cells are a part of the humoral response that upon activation release antibodies and function as antigen-presenting cells, and T cells, that are a part of the cellular response that releases cytokines to direct immune responses and/or function as effector cells that release cytotoxic substances and express death receptor ligands. Innate and adaptive immunity work in concert by communicating via cell-to-cell interactions and by releasing signaling molecules such as cytokines and chemokines.
T cell activation

T cells originate from the bone marrow, migrate to the thymus for proper education and after a selection process they are released into the circulation. T cells are phenotypically described by their surface expression of CD3 and CD4 or CD8, they conduct adaptive immunity by receptor interaction and cytokine production. Professional antigen presenting cells (APC) are responsible for presenting antigens to T cells via MHC and T cell receptor (TcR) interaction. APCs are very efficient at internalizing antigen, either by phagocytosis or by receptor-mediated endocytosis. They clear the tissues from both dying cells and infectious agents. The internalized material is degraded and protein peptides are loaded onto MHC molecules to display pathogens or dead self cells to the T cell. If the APC is in a mature status and the T cell recognize the loaded peptide, the T cell will become activated against the presented pathogen or self peptide. On the other hand, if the APCs are immature they will induce tolerance [1, 2]. DCs mature upon so called danger signals produced by the microenvironment during pathological conditions. Danger signals can come from cytokines, bacteria, viruses, hypoxia, mechanical damage, heat shock etc. Viral and bacterial ligands have the capacity to activate APCs through ligation of Toll like receptors (TLRs), which recognize microbial motifs such as lipopolysaccaride (LPS). Ligation of TLRs on DC induce maturation and upregulation of costimulatory molecules and the matured DC will then have the capacity to activate T cells [3].

Mature DCs upregulate the chemokine receptor CCR7 which interacts with the chemokines CCL19 and CCL21. This will initiate migration of DCs into secondary lymphoid tissue where they meet and activate naive T cells[4]. DCs present antigen peptides via MHC class I to CD3^+CD8^+ T cells and via MHC class II to CD3^+CD4^+ T cells. Simultaneously, costimulatory molecules interact with responder molecules and form complexes such as CD80/86-CD28 and CD40-CD40L. To further strengthen the interaction, adhesion molecules interact i.e.(LFA3-CD2 and ICAM1-LFA1)[5]. Hence, under normal conditions immature DCs are presenting autoantigens to tolerize T cells and in a danger milieu the same DCs will instead induce activated T cells. In MS patients, myelin debris is present in the blood and is constantly phagocytized and presented by APCs to surrounding T cells. MOG-reactive T cells will become activated and eventually
migrate into the CNS and cause damage. What causes myelin breakdown and the danger signaling in the initial stage is yet unknown.

Microglia cells are considered to be potent APCs in the CNS and are consistently phagocytosing myelin components and presenting them on both MHCI and MHCII [6]. When microglia are activated by the CNS microenvironment they secrete pro inflammatory cytokines (IL1, IL6 and TNFα) and undergo oxidative bursts. This will result in production of reactive oxygen such as superoxide (O$_2^-$) that will serve as a danger signal. However, super oxide and nitric oxide (NO) will rapidly react and form peroxynitrite [7]. Peroxinitrite inhibits T cell proliferation by CD3ζ down modulation [8]. At the same time adult oligodendrocytes are sensitive to peroxynitrite-mediating killing[6].

![Diagram](image)

Figure 1: Danger signals stimulate APCs, such as dendritic cells (DC), to mature, upregulate MHC- and co stimulatory molecules and to present antigens to T cells. T cells receiving both TcR and costimulatory signals (CD28-CD80/86) become activated. If, however, APC are resting (immature) or presenting an antigen that the T cell does not recognize, the T cells remains anergic to stimulation.

T helper cells

CD3$^+$CD4$^+$ T cells are generally called T helper cells and can be divided into distinct lineages on basis of their cytokine production profile. Cells of the Th1 lineage produce IFNγ and are potent activators of CTLs, NK cells and macrophages. Th1-responses are important to combat intracellular bacteria, viruses and deformed atypical cells such as cancer cells. Cells of the Th2 lineage are characterized by their production of IL4, IL5 and IL13, which are potent activators of B cells
and their antibody production [9, 10]. Th2 response is important to control extracellular pathogens such as bacteria and free viruses yet to infect cells. The innate immune responses, including APCs, are important to define the type of T helper response needed. In some autoimmune diseases CTLs, as a result of Th1 activation, are a major source of cells disturbing and damaging normal tissue. In MS CTLs are targeting nerve cells in the CNS and will upon migration through the BBB destroy CNS tissue [11].

Recently, a new helper cell population distinct from the Th1 and Th2 types was identified. These cells, called Th17, are IL23-dependent and produce the cytokine IL17[12]. IL17 has major pro-inflammatory effects on epithelial cells and is important for the recruitment of neutrophils [13, 14] which are phagocytes involved in inflammation and infection. Expression of IL17 was first associated with many inflammatory or autoimmune conditions in humans such as rheumatoid arthritis, asthma, systemic lupus erythematosus and allograft rejection [15]. The murine experimental autoimmune encephalomyelitis (EAE) model is the most commonly model used for MS (for details; see materials and methods). In EAE, responses of both Th1(IFNγ)- and Th17(IL17)-type have been studied with a general consensus that Th17 seem to be the driving force behind development of EAE [16-18]. It has been demonstrated that Th17 cells exhibit poor proliferative capacity, low cytotoxicity and reduced susceptibility to the suppressive activity of CD4+FoxP3+ T regulatory (Treg) cells compared to Th1 and Th2 cells. Th17 cells are able to help B cells to induce the production of IgM, IgG and IgA. [19]. Another study showed that Th17 cells express the B cell chemoattractant CXCL13 [20]. Thus, the Th17/B cell interaction could lead to the production of Igs by B cells and be part of the accumulation of B cells producing anti-myelin antibodies in MS patients.
Figure 2: Naïve CD4+ T cells develop into Th1, Th2 or Th17 cells depending on exogenous stimuli. Th1 T cells become activated upon IL12 stimulation and will result in CTL activation. Th2 cells are activated by IL4 and will result in B cells activation and antibody secretion. Th17 become activated after stimuli from IL23 and will secret the pro-inflammatory cytokines IL17 and IL22 upon activation. In the absence of IL6 (but still in the presence of TGFβ or IL10) naïve CD4+ T cells can turn into Tregs.

T effector cells

CD3+CD8+ CTLs are mayor players in viral and tumor clearance but also in lesion formation in MS. CTLs are promoted by Th cells to become cytotoxic and kill their target by releasing perforin and granzymes or by death receptors. In MS, CTLs are activated as a response of Th1 activation and target myelin coated cells. CD8+ T cells are found proximal to damaged and demyelinated axons in the CNS and present at the edge of the lesion as well as is perivascular regions[21].

T regulatory cells

Treg cells play an active part in establishing and maintaining immunological tolerance and restraining various immune responses post inflammation/infection [22]. Tregs are commonly recognized by their surface expression of the IL2 receptor (CD25) and the intracellular transcription factor Forkhead boxP3 (FoxP3). Recently, Tregs have become of great interest in many fields of basic and
clinical immunology [23]. It has been demonstrated that the removal of Tregs leads to spontaneous development autoimmune diseases in normal mice and it is interesting to note that in several autoimmune diseases there is a selective decrease in the number of Tregs [24]. Tregs can be classified according to their surface phenotype or their cytokine secretion profile. There are two main subsets of Tregs: natural Tregs (nTregs) and inducible Tregs (iTregs). Natural Tregs are generated in the thymus and has been reported to be essential of maintaining tolerance to tissue-specific self-antigens [25] whereas the iTregs are induced in the periphery from non-regulatory T cells and they may or may not express FoxP3 [26]. However, both CD25 and FoxP3 are induced upon general T cell activation [27]. On the other hand, CD127 which is down regulated by the transcription factor FoxP3 [28, 29] and, hence, barely expressed by Tregs is highly expressed by activated T cells. Thereby, low CD127 can be combined with high Foxp3 to define a more accurate Treg population. Treg cells, can be found among both CD4 and CD8 T cells but CD4+Tregs have been more extensively investigated [30]. In this thesis, we have focused mainly on CD4+Tregs.

T regulatory cell suppression

Tregs can suppress target cells via inhibitory cytokines such as IL10, IL35 and TGFβ. IL35 is a newly discovered inhibitory cytokine that is thought to be required for maximal suppressive activity of the Treg cell [31]. IL10 can inhibit the synthesis of cytokines by Th1 and Th2 cells as well as inhibit DCs from maturing and producing pro-inflammatory cytokines and presenting antigens. TGFβ does not seem to be crucial for Treg function but it is involved in the suppressive activity and has been shown to inhibit IL17 production [32]. It has been suggested that Tregs also practice suppressive abilities via interaction of CTLA-4 and CD80/86. Via these interactions, Tregs can mediate down-regulation of co-stimulatory molecules on DCs leaving them, for the moment, unable to activate T cells [33]. Tregs might also be able to promote DCs to express IDO, an important molecule known to induce production of pro-apoptotic metabolites resulting in suppression of T effector cells[34]. Recent studies have suggested that lymphocyte activation gene-3 (LAG-3) may block DC maturation. LAG-3 is a CD4 homolog that binds MHCII with high affinity. Binding of LAG-3 to MHCII on immature DCs will mediate an
inhibitory signal suppressing DC maturation[35]. The expression of the ectoenzymes CD39 and CD73 has been shown to induce perivascular adenosine, which suppresses T effector cell function through activation of adenosine receptor 2A[36]. Binding of adenosine to A2A R appears not only to inhibit T cell function but also to enhance the generation of induced Tregs by inhibiting IL6 and promoting TGFβ[37]. Finally, Tregs are highly dependent on IL2 but can not produce IL2 them selves. Tregs deprive the environment of IL2 to proliferate and as a consequence T effector cells become prone for apoptosis in a milieu lacking IL2 [38].

![Diagram](image)

Figure 3: Treg suppression of DCs, T cells, NK cells and T helper cells. 6a) Treg suppression via cytokine release of IL10, TGFβ and IL35. 6b) Tregs targeting DCs via CTLA4 - CD80/86 mediate induction of IDO which is a immunosuppressive molecule made by DCs. Also, inhibition of DC maturation via LAG3 – MHC interaction. c) Metabolic disruption including IL2 deprivation and CD39 and/or CD73 generated adenosine receptor (A2A R)-mediated immunosuppression.

T regulatory cell therapy

Transfer of Tregs into animals with autoimmunity provides protection but to this date there are no records of clinical trials using adoptive transfer of Tregs in humans with autoimmune diseases. Tregs have, however, been used in the clinic to induce transplantation tolerance (listed in table 1) [39]. One problem with Tregs is sorting and expanding the population since Foxp3, the most reliable marker is intracellular. It is a major challenge to produce antigen-specific Tregs for cell therapy. Tregs can be generated by gene transfer of FoxP3 [40]. Chai et al have achieved Tregs by retroviral transfer of murine FoxP3 into CD4+CD25+ T cells and used them in a animal model for
transplantation with promising results[41]. Genetically engineered and cultured Tregs have been evaluated for treatment efficacy in a wide variety of adoptive transfer models of autoimmune disease with promising results [41-44]. One problem with adoptive transfer of Tregs is systemic immunosuppression. Tregs in the wrong environment can be harmful. Immune cells can be hampered and not be able to combat infections. Mekala et al redirected murine CD4⁺CD25⁺ Tregs by using a chimeric antigen-MHC/ζ receptor targeting myelin basic protein (MBP) in the EAE model. In their study MBP-specific Tregs were able to suppress EAE-inflammation in comparison to conventional Tregs[45]. Hombach et al have redirected human CD4⁺CD25⁺Tregs by using retroviral transfer of a recombinant anti CEA-immunoreceptor to target inflamed intestine[46]. In our study we have produced Tregs with a CNS-targeting receptor making the suppressive effect local in the brain and leaving the rest of the body unhampered.

Mesenchymal stroma cells

Stem cells are characterized by their ability to renew themselves through mitotic cell division and differentiate into a diverse range of specialized cell types[47]. In a developing embryo, stem cells can differentiate into all of the specialized embryonic tissues. In adults, stem cells act as a repair system for the body, replenishing specialized cells and maintaining the normal turnover of regenerative organs such as blood, skin and intestinal tissue. Stem cells can be isolated from bone marrow or umbilical cord and are routinely used in the clinic for medical therapies [48]. In comparison to embryonic stem cells, adult stem cells are lineage restricted and generally referred to by their tissue origin e.g. mesenchymal stroma cells, adipose-derived stem cells and endothelial stem cells [49, 50].

Mesenchymal stroma cells (MSCs) are a heterogeneous population of stromal cells residing in most connective tissues including bone marrow, adipose tissue, umbilical cord, blood and perivascular tissues [51]. MSCs can differentiate into cells of the mesenchymal lineage, such as bone, cartilage and fat [52, 53]. Within the bone marrow they are tightly intermingled with and support haematopoiesis and the survival of hematopoietic stem cells. MSCs are highly suppressive cells and have been demonstrated to suppress both innate and adaptive
**Immunity.** MSCs impair in vitro maturation of monocytes and myeloid derived dendritic cells through the down regulation of MHC class II, CD11c, CD83 and co-stimulatory molecules resulting in impaired antigen presentation as well as impaired IL12 production [54]. Recently, MSCs have been demonstrated to significantly inhibit proliferation of resting NK cells, NK-mediated cytotoxicity and cytokine production. MSCs also inhibit proliferation of T cells through the induction of cell cycle arrest [55, 56]. Further, MSCs have been reported to promote the generation of Treg cells [57, 58], inhibit B cell proliferation, differentiation and their constitutive expression of chemokine receptors.

![Diagram of interactions between MSCs and immune cells](image)

**Figure 4:** Schematic figure of the interactions between MSCs and immune cells. After activation, MSCs secrete soluble mediators such as nitric oxide (NO), prostaglandin (PGE2), indoleamine 2,3-dioxygenase (IDO), IL6 and human leukocyte antigen (HLA)-G. Production of these mediators regulates the proliferation and function of a variety of immune cells as well as the induction of Tregs, either directly or indirectly through the generation of immature DCs.

**Mesenchymal stroma cells as therapy**

The trafficking and homing properties of MSCs are of particular interest for clinical applications aiming at using non-invasive systemic cell administration to treat inflammation. MSCs have been shown to express a variety of chemokine receptors and can home to site of inflammation by migrating towards inflammatory chemokines and cytokines. Homing of cultured MSCs, however, is inefficient compared with lymphocytes. This inefficiency has been attributed to a lack of cell adhesion and chemokine receptors but also to the size of MSCs that promote passive cell entrapment and reduce trafficking
To overcome this limitation several laboratories have developed non-genetically engineering methods to enhance stem cell delivery such as enzymatic modification of cell surface glycoproteins into E-selectin ligands or by biotinylating cell surface proteins and coat cells with streptavidin-linked ligands [60, 61]. Others have coated MSCs with antibodies to enhance delivery [62]. Risk factors involved with using MSCs in human trials have been described [63-65]. In contrast to mouse and human embryonic stem (ES) cells, bone marrow (BM)-derived undifferentiated MSCs do not pose a risk of producing teratomas that contain derivatives of all three germ layers. However, they can be a source of carcinoma-associated fibroblasts (CAF), which may contribute to the promotion of tumor growth and metastasis[63, 66]. New strategies including introduction of suicide genes or rendering the cells unable to divide mitotically by irradiation are warranted and currently under construction of several research groups including us. Both murine and human MSCs have been examined for treatment efficacy in the animal model EAE with different results. When administrated i.v. post immunization, MSCs were only able to suppress inflammation to some extent but did not cure animals completely [67, 68]. When administrated i.p. six days post immunization, MSCs were able to suppress EAE inflammation but only a few cells were found in the brain[69]. In our experiments we saw that MSCs carrying a CNS-targeting receptor could cure mice with EAE when administrated both i.p. and i.n. as late as fourteen days post immunization. Today there are several ongoing and completed trials with MSCs for MS patients and other conditions (Table I). All this taken together, MSCs have opened a new field for possible MS therapies.
<table>
<thead>
<tr>
<th>Study</th>
<th>Condition</th>
<th>Treatment</th>
<th>No of patients</th>
<th>Dose</th>
<th>Injection</th>
<th>ClinicalTrials.gov identifier</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase I/II</td>
<td>MS</td>
<td>Autologous MSC</td>
<td>10</td>
<td>2x10^6/kg</td>
<td>i.v.</td>
<td>NCT00395200</td>
<td>Currently recruiting</td>
</tr>
<tr>
<td>Phase I/II</td>
<td>MS</td>
<td>Autologous MSC</td>
<td>24</td>
<td>2x10^6/kg</td>
<td>i.v.</td>
<td>NCT00813969</td>
<td>Not yet recruiting</td>
</tr>
<tr>
<td>Phase I/II</td>
<td>MS</td>
<td>Autologous MSC</td>
<td>30</td>
<td>4x10^6/kg</td>
<td>i.v.</td>
<td>NCT01056471</td>
<td>Currently recruiting</td>
</tr>
<tr>
<td>Phase I/II</td>
<td>SLE</td>
<td>Allogeneic MSC</td>
<td>13</td>
<td>1x10^6/kg</td>
<td>i.v.</td>
<td>NCT00698191</td>
<td>Completed</td>
</tr>
<tr>
<td>Phase I/II</td>
<td>Sjögrens disease</td>
<td>Allogeneic MSC</td>
<td>20</td>
<td>10^6/kg</td>
<td>i.v.</td>
<td>NCT00953485</td>
<td>Currently recruiting</td>
</tr>
<tr>
<td>Phase II</td>
<td>Chron's disease</td>
<td>adult human MSC</td>
<td>10</td>
<td>2x10^6/kg/8x10^6/kg</td>
<td>i.v.</td>
<td>NCT00294112</td>
<td>Completed</td>
</tr>
<tr>
<td>Phase I/II</td>
<td>Heart Failure</td>
<td>Autologous MSC</td>
<td>60</td>
<td>20-30x10^6</td>
<td>Intra myocardial</td>
<td>NCT00644410</td>
<td>Not yet recruiting</td>
</tr>
<tr>
<td>Phase II</td>
<td>Chron's disease</td>
<td>Allogeneic MSC</td>
<td>20</td>
<td>2x10^6/kg weekly for 4 weeks</td>
<td>i.v.</td>
<td>NCT01090817</td>
<td>Currently recruiting</td>
</tr>
<tr>
<td>Phase I</td>
<td>Type 1 Diabetes</td>
<td>Autologous polyclonal Tregs</td>
<td>-</td>
<td>-</td>
<td>i.v.</td>
<td>NCT01210664</td>
<td>Not yet recruiting</td>
</tr>
<tr>
<td>Phase II</td>
<td>Allogeneic BM</td>
<td>Autologous Tregs with SCT</td>
<td>300</td>
<td>-</td>
<td>i.v.</td>
<td>NCT00303719</td>
<td>Currently recruiting</td>
</tr>
</tbody>
</table>

MS, multiple sclerosis; SLE, systemic lupus erythematosus; MSC, Mesenchymal stromal cell; i.v., intravenous injection; BM, bone marrow; SCT, stem cell transplantation
Multiple sclerosis

In the late 19th century a systematic investigation of human neurology was completed and neurological diseases were from this time point recognized as a separate group of diseases. The birth of neurology took place in Paris where Jean-Martin Charcot was treating women with undefined neurological conditions [70]. In 1886, Charcot was the first to describe a neurological condition that caused multiple patches of scar tissue in both brain and spinal cord. This led to the term “Sclérose en plaques disséminées” i.e. scattered sclerotic plaques, later known as multiple sclerosis (MS). The observation in 1948 by Elvin Kabat of increased oligoclonal immunoglobulin in the cerebrospinal fluid (CSF) of patients with MS provided evidence of an inflammatory nature of the disease [71].

MS is nowadays considered to be an inflammatory demyelinating disease of the central nervous system (CNS)[72]. The precise pathogenic mechanisms are not clearly understood but in the early stage of MS, neural damage is considered to arise due to T cell-mediated destruction of myelin expressing cells[73]. 80% of patients will initially present with a relatively benign relapsing-remitting disease course (RRMS). Half of them will ultimately convert to a second progressive form (SPMS) that gradually become more severe [74]. This suggests that initially most patients have the capacity to limit their inflammatory episodes by using drugs that down modulate the immune system [75]. A smaller percentage of patients will from onset slowly deteriorate in disease progression without any recovering phases of remission. This type, primary progression (PPMS), is the most severe of the three types and has the lowest life expectancy[76, 77] (Figure 5). Finding and understanding the mechanisms responsible for controlling these inflammatory episodes is crucial in the development of new therapies for MS. In this thesis we have focused on patients with RRMS.
Figure 5: Different courses of disease activity in MS. Patients with relapsing-remitting (RR)MS experience periods of remission in between relapses. Phases of relapse can be months or years apart. With time phases of relapses occur more frequently and it is common that patients convert to a secondary progressive form (SPMS) that gradually become more severe. Primary progressive (PP)MS is constantly progressive and is most severe of the three types.

MS etiology

MS has a peak incidence between the ages of 20 and 40 years with a female predominance [74]. There are many theories how to explain the pathogenicity of the disease but the etiology of the condition is still uncertain. Although viral infection has been postulated, no specific viral agent has been consistently detected or directly implicated to the disease[70]. Immunological mechanisms are central to disease pathogenesis and an active immunological response is present in areas of demyelination but the cause of the immune response remains unclear[78]. Association with certain HLA antigens such as HLA-DR15 (associated with transforming growth factor (TGF)-β family members, cytotoxic T lymphocyte-associated antigen (CTLA)-4 and the tumor necrosis factor (TNF)) has also been demonstrated[78, 79]. It is possible that the disease is a result of a genetic susceptibility predisposing to mounting inappropriate immune response against CNS structures post viral infections or other events.
MS pathogenicity

A general consensus is that the initiation of MS is the formation of inflammatory lesions in the CNS caused by activated autoreactive T cells and macrophages[80]. Proinflammatory cytokines and chemokines activate resident immune cells in the CNS, such as microglia and astrocytes. They recruit other immune cells including monocytes, T cells, B cells and mast cells from the peripheral blood as the blood brain barrier (BBB) allows passage during inflammation. Larger lesions may now be seen by magnetic resonance imaging (MRI) scans of the brain and spinal cord (Figure 6)[81]. CNS damage (i.e., the myelin sheet, oligodendrocytes and axons) occurs at this inflammatory stage. Oligodendrocyte precursors still present in the adult CNS mature and surviving oligodendrocytes begin to reproduce myelin, although the original thickness is never recovered. Hence, the nerve conduction velocity in those areas are permanently damaged [82].

Figure 6: MRI scan of an MS patient presenting with white lesions compatible with MS (Courtesy of Anders Fransson MD, Radiology Department, Gävle hospital)

MS symptoms and current treatment options

Patients with RRMS experience periodic episodes where new functional deficits arise spontaneously over days or a few weeks and then resolve over the next coming weeks or months. During one of these episodes or relapse, patients experience loss of function in different areas resulting in different symptoms as difficulty walking, incontinence and/or reduced vision. Between relapses, patients can be symptom free depending on progression of the disease. Today, there is no cure for patients with RRMS. Patients are offered different treatment options to reduce disease severity. Treatment with type I
interferon is the most common option and has been shown to decrease inflammatory activity and reduce relapse rate by approximately 30% [83-85]. There are some other options as well. Glatiramer acetate (GA) is a mixture of four random amino acids resembling myelin basic protein (MBP). Its mode of action is unclear but patients seem to experience a shift in the T cell population from a Th1- toward a Th2 response[86, 87]. Treatment with intravenous immunoglobulins (IVIG) result in unspecific reactions such as attenuation of antibody/complement complexes and they might act as receptors for activated complement components and prevent their attachment to myelin proteins [88]. Natalizumab (Tysabri) interact with α4-integrins (adhesion molecule acquired for passage of the BBB) on lymphocytes and thereby hinder them from entering the brain. By not allowing T cells to migrate into the brain, a high concentration of activated T cells is gathered in the periphery and as a result, when patients stop treatment, they fall back into a relapse[89]. An option for patients with severe MS is autologous hematopoietic stem cell transplantation (HSCT). Patients with life-threatening paralysis that received stem cell transplantation regained their ability to walk [90].

Blood brain barrier

The blood brain barrier is a cellular structure in the CNS that restricts passage of various cells, chemical substances and microscopic object, such as bacteria between the bloodstream and the neural tissue, while allowing the passage of substances essential for metabolic function, such as oxygen and glucose. The selectivity of this barrier is a result of the tight junctions between endothelial cells in the CNS vessels that restrict passage (for review see [91]). Infections or inflammatory damage in the CNS or direct damage to the blood brain barrier will result in a breakdown of the barrier. The intracellular content of necrotic cells leak out into the extracellular environment which is a signal of danger and the blood brain barrier will allow passage of lymphocytes to deal with a possible infection agent. Unfortunately, in an autoimmune disease, autoreactive T cells passing the blood brain barrier contribute to the damage of CNS instead of being protective[92]. It should be noted that, even though it has been debated, it is unlikely that MS is a result of a breakdown in the blood brain barrier.
Cells of the central nervous system

The fundamental cellular units of the CNS consist of neurons and their axonal and dendritic processes embedded in a glial network providing additional structure and function. Macroglia (oligodendrocytes and astrocytes) and microglia contribute to the cellular architecture of the CNS. Oligodendrocytes synthesize and maintain the myelin sheath that extensively coats nerve fibers in white matter and strengthen saltatory conduction of the nerve impulse [93, 94]. These are terminally differentiated cells with a limited response to injury. Astrocytes provide architecture for neurons and define anatomical boundaries. They act as a source of growth factors and cytokines and assume many physiological roles including those necessary for conduction of nerve impulses and participate in the response to injury. Microglia are bone marrow derived cells of the macrophage lineage providing the nervous system with a degree of immunological competence. Both astrocytes and microglia are highly reactive cells that play important roles in health and disease, contributing both to tissue injury and to repair (for review see [95]). Early loss of oligodendrocytes has been associated with MS lesions that rapidly expand in the absence of IgG positive plasma cells in the lesion and with relatively few lymphocytes present in the perivascular spaces and the parenchyma [21]. This might indicate that break down of oligodendrocytes is a part of the early stages of MS, even before T- or B-cell mediated inflammation.

Autoimmunity

A key event in the development of autoimmunity is the activation of autoreactive lymphocytes which leads to proliferation and differentiation into effector cells causing tissue injury [96]. Why self-tolerance fails is a fundamental issue in autoimmunity. Alternations in the tissue microenvironment such as infection, inflammation, ischemic injury or trauma may lead to the exposure of self antigens on mature DCs leading to activation of autoreactive T cells. Why some individuals manage to restrain such T cells while others do not may be due to the strength of the infection and/or inflammation but also to genetic differences (Figure 3)[97].
Much attention has focused on the role of T cells in autoimmunity mainly because T helper cells are key regulators of both cytotoxic T cells (CTLs) and antibody-producing B cells [98]. They can also boost the effector function of natural killer (NK) cells and macrophages. T cells cause tissue injury either by triggering delayed type hypersensitivity (DTH) reactions or by direct killing of target cells. DTH reactions are elicited by CD4+ T cells or Th1 type which secrete cytokines that activate other immune cells such as macrophages and induce inflammation. T cells that cause tissue injury in MS might be autoreactive and/or specific for foreign protein antigens that are present or bound to cells or tissue in the CNS [99]. Myelin-specific T cells can be isolated from patients with MS and it has been shown that these T cells can cross react with peptides derived from viral proteins[100]. Cross-reactive T cells can become activated by non self antigens during the course of an infection [101]. Molecular mimicry of viral or bacterial proteins could initiate autoimmune reactions. Thus, many studies provide evidence for this theory and a number of viral epitopes that trigger autoreactive T cell clones have been identified in cytomegalovirus (CMV) [102], Epstein-Barr virus (EBV) [103], Herpes simplex [104] and Corona virus [105]. Also, what could be described as a contributing factor, autoreactive T cells isolated from patients with autoimmune an disease have a lower threshold of activation than in healthy individuals[106].

B cells originate and develop in the bone marrow. After reaching IgM positive immature status they migrate to the spleen and lymph node where some differentiate into mature B cells. The function of B cells in MS has been well studied. Although antibodies specific for myelin proteins may contribute to loss of myelin it is not certain if the antibodies initiate the immune attack or if they develop as a consequence of CNS inflammation. B cells are not only antibody producers, they may also participate in MS biology as APCs. However, a trial of a therapeutic antibody specific for CD20 on B cells, rituximab, has been shown to reduce disease activity[107]. These findings indicate involvement of B cells, either as antibody producers or as antigen presenters to T cells. Antibodies specific for myelin basic protein (MBP) were identified in MS CSF 25 years ago[108, 109] although several other studies have reported their absence [110]. Antibodies directed to proteolipid protein (PLP) are also found in MS CSF but never simultaneously with anti-MBP [111].
In MS, both B cells and T cells have been extensively studied and found to be part of the immune response responsible for the disease. B cells secreting antibodies to myelin antigens may contribute to loss of myelin but the function of B cells in the acute lesion is not yet known. It has been suggested that the B cell mechanism in MS is to regulate T cells via antigen presentation. However, it seems to be a consistent finding that T cells are to a higher extent involved in the pathogenesis of MS and therefore, in this thesis, we have focused on the role of T cells and their sub populations during health and disease.

T regulatory cells in MS

The importance of Tregs in MS and EAE is becoming increasingly significant. It has been shown that Tregs play a critical role in the protection and recovery of EAE. Depletion of Tregs inhibits naturally recovery from EAE whereas transfer of these cells to recipient mice reduces disease severity [112]. Human Tregs need to be activated through their TCR in order to be functionally suppressive. However, when activated they suppress surrounding immune cells in a non-specific manner [113]. Vandenbank and co-workers showed decreased levels of FoxP3 in Tregs from MS patients and found that this decrease correlated with Treg loss of function [114]. Additionally, Venken and co-workers reported that relapsing remitting (but not secondary progressing) MS patients express lower levels of FoxP3 than healthy controls [115]. It is not clear from these studies whether lower FoxP3 expression in MS patients is due to a reduced frequency of FoxP3+ cells in the CD4+CD25+ population or due to a decreased FoxP3 expression at a cellular level. A consistent finding seems to be that Tregs are not functional in MS patients [116-118]. However, Michel and co workers found that patients with RRMS have normal Treg function when excluding IL7R+ (CD127) cells[119]. Hence, some of the previous studies testing the suppressive function of CD25high cells may have been contaminated by effector T cells which also have high CD25 expression. Even if the Treg role is not completely clear in the pathogenesis of MS, Tregs are clearly involved in the different stages of remission and relapse.
Summary of the thesis in Swedish

Populärvetenskaplig sammanfattning på svenska

Multipel skleros (MS) är en autoimmun sjukdom där kroppens eget immunförsvar attackerar och bryter ner kroppsegen vävnad i hjärnan. Immunförsvaret försvarar oss vanligen mot exempelvis bakterier och virus men i vissa fall riktas attacken mot kroppsegen vävnad och autoimmunitet uppstår. När frisk vävnad i det centrala nervsystemet (hjärna och ryggmärg) bryts ner påverkas nervsignalerna. Den vävnad som bryts ner är det myelinrika isolerande höljet som finns runt nervcellerna. Utan isolering går nervsignalerna inte lika fort och de kommer inte fram som de ska vilket leder till brister i funktion i muskler såväl som syn och urinblåsa.

Det har diskuterats vilka typer av immunceller som är ansvariga för den riktade attacken mot myelinet och varför attacken uppstår. Mycket är fortfarande oklart i frågan om uppkomst men de typer av celler som är inblandade börjar bli allt mer klarlagt. I det här avhandlingsarbetet har vi tittat på så kallade T celler som reagerar med myelinproteiner och vad de utsöndrar när de blir aktiverade. Reagerar T celler med att utsöndra IFNγ kallas de Th1 celler med utsöndrar de IL17 kallas de Th17 celler. Det har diskuterats om uppkomsten till MS är driven av aktiverade Th1 eller Th17 celler. Vi såg att patienter med MS har T celler som när de blir aktiverade av myelinprotein utsöndrar IFNγ samtidigt som det fanns T celler från samma individ som på samma sätt utsöndrade IL17. Med andra ord så var det inte bara en typ av T celler var involverade utan båda.

Man har även ägnat stor uppmärksamhet åt en tredje typ av T celler som kallas T regulatoriska celler (Tregs) och deras roll hos patienter med MS. Tregs är traditionellt beskrivna som hämmade celler som reglerar inflammation. De blir speciellt intressanta i det här avseendet eftersom MS uppkommer av en autoimmun inflammation som inte slås av tillräckligt snabbt. Att det skulle vara något fel på Tregs hos
MS patienter är en trolig anledning till varför de aggressiva T cellerna inte kan hållas i schack. Olika forskare har hittat olika svar. Många menar att MS patienter inte har funktionella Tregs medan andra hävdar att de har det. Vi har tittat på olika undergrupper av Tregs och hur de förändras från när patienten mår bra och när denne hamnar i ett sjukdomsskov (när den autoimmuna inflammationen pågår). Vi upptäckte att en viss typ av Tregs ökade när patienten hade ett sjukdomsskov medan andra typer minskade. Resultaten pekar på att det kan vara mer komplicerat än vad man tidigare trott att titta på och bedöma dessa cellers roll i MS. Oavsett vilka T celler som är skyldiga till attacken mot myelinet eller hur väl Tregs fungerar hos MS patienter kan man konkludera att dessa patienter skulle vara gagnade av en terapi som tillförde fler hämmande celler. I den sista delen av detta avhandlingsarbetet har vi utvecklat en cellterapi med immunhämmande celler. För att de immunhämmande cellerna ska hitta till hjärnan utrustade vi dem med en målsökande receptor. Tanken är att behandla ett område utan att det ska påverka resten av kroppen. I en djurmodell kunde de målsökande hämmande cellerna hitta myelinrika områden i hjärnan och lokalt hämma den autoimmuna inflammationen utan att påverka resten av kroppen. Syftet med våra studier var att utvärdera om cellterapierna kunde bota en MS-liknande sjukdom hos möss. Målsökande celler var så som MSCs effektiva både på att hämma olika typer av aggressiva celler och att få behandlingseffekten att hålla i sig. MSCs utan målsökande receptorer har redan nått kliniken och testats idag i kliniska prövningar med MS patienter. Med vår målsökande receptor hoppas vi på att göra behandlingen mer lokal eftersom spridning av hämmande celler i hela kroppen kan göra patienter känsliga för infektioner då hela immunförsvaret blir täta. Förhoppningen är att denna nya terapi ska kunna användas i kliniken för att lindra den autoimmuna inflammation som uppstår hos patienter med MS.
Present investigation

General aim
The aim of this thesis was to perform an immunological investigation of patients with RRMS to gain a greater understanding of the disease and to develop an immunosuppressive cell therapy that could be beneficial for these patients.

Specific aims of the study

I  To investigate T cell subsets (Th1) and (Th17) as well as their release of cytokines in MS patients in remission.

II  To investigate different T regulatory cell subsets in the different phases of relapse and remission.

III To develop CNS-targeted suppressive cells by genetically inserting a CNS-targeting receptor into T regulatory cells to investigate their immunosuppressive function and efficacy in the animal model for MS called EAE.

IV To develop CNS-targeted mesenchymal stromal cells and to investigate their immunosuppressive function in and their capacity to cure EAE.
Materials & Methods

Vectors and viruses

The CARαMOG vector was constructed as follows: a single chain variable fragment (scFv) was cloned from the 8.18C5 hybridoma producing anti-rat myelin oligodendrocyte glycoprotein (MOG) antibodies (kind gift from Dr Robert Harris, Karolinska Institute, Sweden). The MOG scFv was inserted into a conventional chimeric antigen receptor (CAR) receptor [120] which gives stable cell surface expression of the targeting device. The final CAR construct was inserted into the lentiviral vector pRRL-CMV. Lentiviruses (Lenti-CARαMOG, Lenti-CARαMOG/FoxP3, Lenti-Mock, Lenti-GFP) were produced by cotransfecting 293FT cells with helping plasmids pLP1, pLP2 and pLP/VSVG from Invitrogen (Paisely, UK).

Figure 8: Four different vectors used in paper III & IV. (A) CMV-CAR was used in paper IV to produce CAR expressing MSCs. (B) CMV-CAR-Foxp3 used in paper III to differentiate CD4+ cells into Tregs while simultaneously expressing the CARαMOG receptor. (C) The CMV-GFP was used to produce GFP expressing cells in paper III & IV to control transduction efficiency and for tracing GFP positive cells after i.n. administration. (D) The Mock vector (empty virus vector) was used in the control groups to exclude a possible treatment effect of the viral backbone alone.

Virus supernatant was harvested on day 2 and 3 and concentrated by ultracentrifugation. 50ul viral supernatants were added to 5x10^4 MSCs or 5x10^5 CD4+ T-cells in 100ul RPMI-1640 medium supplemented with 1% sodium pyruvate, 1% nonessential amino acids, 10% fetal bovine serum, 1% penicillin/streptomycin (all from Invitrogen,
Paisley Scotland) and 8 μg/ml Polybrene (Sigma-Aldrich Inc, Saint Louis, MO, USA). Cells were incubated for 4h at 37°C, 5%CO2 followed by addition of 300ul of media (as above) the following day, media was replaced. Cells were cultured for 7 days before analyzing transgene expression.

Animal model for MS

Our understanding of MS as an inflammatory disease has advanced due to the animal model EAE which is similar to human MS [121]. EAE is induced in experimental animals by initial injection of an isolated autoantigen from the spinal cord together with complete Freund’s adjuvant and influenza virus boosts. The vaccination initiates an inflammatory reaction in the brain that causes a progressive paralysis affecting first the tail and hind limbs before processing to forelimb paralysis. When evaluating recovery animals are given score values depending on their state of paralysis (listed in table II). The occurring inflammation in the brain and spinal cord resembles the autoimmune reaction in MS and the pathology is similar to human disease [122]. However, there are differences between EAE and MS. First EAE, in contrast to MS, is not a spontaneous disease but induced by active sensitization with brain tissue antigens. Furthermore, strong adjuvants are required to induce disease and it seems unlikely that similarly immunological boosts occur under physiological conditions. Despite these limitations, most of our current knowledge regarding principal mechanisms of brain inflammation has been gathered from studies on EAE [123].

Table II: EAE score (0-5) with corresponding clinical manifestations

<table>
<thead>
<tr>
<th>Score</th>
<th>Clinical manifestations</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Healthy mice</td>
</tr>
<tr>
<td>1</td>
<td>Flaccid tail</td>
</tr>
<tr>
<td>2</td>
<td>Hind limb weakness</td>
</tr>
<tr>
<td>3</td>
<td>Partial hind limb paralysis</td>
</tr>
<tr>
<td>4</td>
<td>Hind limb paralysis</td>
</tr>
<tr>
<td>5</td>
<td>Forelimb paralysis</td>
</tr>
</tbody>
</table>
Intra nasal administration

In paper III and IV injections were made using intra nasal (i.n.) administration of the therapeutic cells. Intranasal cell administration facilitate bypass of the BBB by permitting migration from the nasal mucosa through the cribriform plate along the olfactory neural pathway into the brain and cerebrospinal fluid (CSF) [124]. CAR or Mock-transduced cells diluted in 10uL PBS were administrated by i.n. instillation using a plastic catheter connected to a pipette during anesthesia.

Figure 9: Therapeutic cells were administrated i.n. using a plastic catheter connected to a pipette during anaesthesia.
Results and discussion

Paper I
Background and significance

Recently, a novel T helper cell subset (Th17) has been identified and pointed out as a contributing factor to the inflammatory process in murine MS. In this paper we investigated the presence of CNS-reactive T cell responses with emphasis on the Th1 and Th17 cell profiles in patients. Patients with RRMS display oscillating phases of relapse and remission with a constant deteriorating prognosis. During relapse, an acute inflammatory process is ongoing. What initiates phases of relapse has been debated; is it because of a general reduced number and/or a reduced suppressive ability of the Treg cells or is it due to loss of some other tolerogenic mechanism? We investigated the presence of Treg cells in MS patients and their ability to suppress allo-reactive T cells in vitro. In summary, we combined functional assays with analysis of cytokines and cell surface molecules to identify the role of circulating T cells of both effector and regulatory subtypes involved in RRMS.

Results and discussion

Th1 cells traditionally activate CTLs that are capable of directly killing their target cells. Upon ligation of the TcR/MHC complex IFNγ is released. A Th17 cell, on the other hand, secretes IL17. Peripheral cytokine analysis of the Th1 and Th2/Treg cytokines IL12 and IL10, respectively, and Th17-related cytokines IL17 and IL23 demonstrated similar levels in MS patients as in healthy controls except for the immunosuppressive cytokine IL10 that was significantly lower in MS patients. The low IL10 levels may contribute to MS patients’ inability to maintain tolerance toward self antigens. The effector cytokine levels may be too low for detecting in
plasma. Therefore, cytokine production by peripheral T cells in MS was investigated by intracellular flow cytometry. T cells incubated with a CNS-specific peptide mix (MOG 1-125) reacted with both IFNγ and IL17 production. Hence, neither response can be excluded in MS.

Conflicting data has been published on the Treg cell level in patients with RRMS due to comparison of different subtypes of Treg cells. In this paper we compared levels of $\text{CD}^4^+\text{CD}25^+\text{CD}127^-\text{Foxp}3^+$ in MS patients and healthy controls. In this patient cohort, there was no significant difference of Treg levels compared to controls. We also investigated the Treg cells suppressive capacity by co-culture with polyclonally stimulated T cells and observed a similar suppressive capacity as Tregs from healthy controls. In conclusion, our findings demonstrate simultaneous presence of the Th1 and Th17 effector cells in patients with RRMS and functionally active Treg cells that may keep T cells in proliferative anergy during remission.

Paper II
Background and significance

Dysregulation of inflammatory responses is considered to be a key element in the autoreactive immune responses seen in patients with MS. Tregs are important to maintain self tolerance. High expression of the IL2 receptor (CD25) has been the best marker for Tregs and is still used for in vitro sorting of Tregs. However, CD25 expression is increased in activated T effectors which complicates the identification in patients with T cell-mediated autoimmunity or in infectious diseases. The identification of the transcription factor FoxP3 as a specific marker for Tregs enabled to distinguish Tregs from other T cells and Tregs were subsequently described as $\text{CD}^4^+\text{CD}25^+\text{Foxp}3^+$ T cells. Recently, it was shown that CD127 expression is negatively correlated to FoxP3 expression and since effector T cells express high levels of the IL7R (CD127) which is low on Tregs it is still possible to distinguish these two populations using multicolour flow cytometry. In this study we compared Tregs of different sub populations in a patient cohort to determine which population that should be analyzed in MS. Further, Treg levels were correlated to disease duration and age to enlighten the importance of a well characterized patient cohort.
when discussing the levels of Tregs in MS patients. Finally, we investigated the presence of activated effector cells and how they correlate to Tregs and disease duration.

Results and discussion

In this pilot study, blood from patients in remission and patients during clinical relapse was investigated by flow cytometry for the expression of CD3, CD4, IL2R (CD25), FoxP3 and the IL7R (CD127). T cells in patients with RRMS were evaluated for their suppressive or effector phenotype. Patients in clinical relapse exhibited higher levels of FoxP3 positive Treg cells lacking both CD127 and CD25 compared to patients in remission (p<0.01) indicating that Tregs might attempt to restrain the immune activity in RRMS patients during, or shortly after, relapse. Interestingly, the proportion of Tregs was negatively correlated with disease duration while CD25+CD4+ and CD25+CD8+ effector T cell populations were elevated and positively correlated to overall disease duration. In conclusion, while MS patients in remission had normal levels of Tregs, relapsing patients show an increased proportion of systemic CD25 FoxP3+ Tregs. With time, as could be expected, the proportion of Treg cells decreased while effector T cells expanded.

Erratum Paper II:
Figure 4A “Treg suppression (% proliferation) instead of Treg suppression (%)”

Materials and Methods, Tregs separation and suppression: “Both populations were irradiated at 25Gy and mixed with stimulating 15ug/ml OKT3 antibodies” should be: 1,5ug/ml OKT3 antibodies.

Statistical evaluation: “p-values <0.05(*), <0.001(**) and <0.0001(***)) were considered significant.” Should be: <0.01(**) and <0.001(***)
Paper III
Background and significance

Transfer of antigen-specific Tregs derived from TCR transgenic mice have shown to be more effective than polyclonal Tregs in controlling murine models of both autoimmune gastritis and multiple sclerosis. However, antigen specific Tregs are difficult to achieve in an adequate number for adoptive transfer and culture of Tregs might alter the suppressive phenotype. Further, Tregs can differentiate into so called Th17 effector cells which would increase disease severity. It has been shown that the transcription factor Foxp3 is fundamental for the differentiation and maintenance of murine Tregs. Stable FoxP3 expression may render Tregs insensitive of further differentiation signals and secure the regulatory phenotype. By transferring an organ-specific receptor and the gene for Foxp3, Tregs can be targeted to the site of autoimmunity without systemic Treg exposure. In the present study, a MOG-reactive CAR was expressed in trans with Foxp3 to generate CNS-specific Tregs. These engineered Tregs were analyzed for their capacity to suppress T cell proliferation in vitro and the autoimmune like disorder in the EAE model.

Result and discussion

Genetically engineered Tregs were able to suppress polyclonally activated T cells in vitro. To investigate if our target-specific Tregs were efficient against autoimmune-like inflammation the cells were infused into animals with EAE. At peak of EAE inflammation, 1x10^5 CAR-FoxP3 or Mock transduced CD4^+ T cells or PBS alone were administrated i.n. Eleven days post treatment all mice in the Treg-CAR group (n=10) were cured (score value = 0). At en point (16 days post treatment) mice in the CD4-mock group had a mean score value of 0,1 and were not completely cured. Histological examination of the brain revealed axonal damage of PBS-treated mice, in the CAR- and mock-treated groups no or very little damage to the axons could be detected. Inflammatory cytokines in the brain (IL12 and IFNγ) revealed a hampered response in both CAR- and Mock-treated mice compared to PBS-treated mice. At end point (16 days post treatment), cured mice were re-challenged with EAE using CFA and pertussis toxin. Mice treated with CNS-specific Tregs gained a mean score
value of 0.5 while control mice exhibited a mean score value of 1.5 that at end point (16 days post immunization) increased to a mean score of 3 indicating that CNS-specific Tregs were able to keep CNS T cells in anergy even after end of inflammation. In conclusion, we have developed CNS-specific Tregs that when administrated i.n. in mice with active EAE can suppress the local CNS environment leaving immune cells in the periphery unhampered to combat infections.

Paper IV
Background and significance

There is a considerable interest in the use of cell-based therapies for a variety of chronic diseases such as MS. Due to their therapeutic plasticity, adult stem cells such as MSCs have been proposed as a possible treatment for MS. Infused therapeutic MSCs can migrate to sites of inflammation and protect damaged tissues, including the CNS. MSCs can be utilized in xenotransplantation models meaning that human MSCs can be used in murine disease models such as EAE where they have been demonstrated to have excellent suppressive effect. Intra peritoneal (i.p.) or intravenous (i.v.) infusion of MSCs have shown that only a low number of MSCs were able to infiltrate inflamed areas. Homing of in vitro-cultured MSCs have been shown inefficient in comparison to T cells due to lack of adhesion molecules, chemokine receptors and their large size. By engineering MSCs with a CNS-targeting receptor we aimed to increase the homing of therapeutic MSCs to the CNS. The cells were then tested in the EAE model for treatment efficacy.

Result and discussion

Genetically engineered MSCs were able to express the CARaMOG receptor and transfer of the CAR receptor did not alter MSCs suppressive abilities. In a thymidine-based assay, CAR transduced MSCs were able to suppress polyclonaly stimulated T cells (p<0.01) in the same manner as untransduced cells (p<0.01). Since genetically engineered MSCs were able to suppress activated T cells in vitro we sought to investigate whether they could cure mice with an active
EAE inflammation. At peak of EAE inflammation, $1 \times 10^4$ CAR or Mock transduced MSCs or PBS alone were administrated i.n. Thirteen days post treatment all mice in the MSC-CAR group (n=10) were cured (score value = 0). At end point (16 days post treatment) mice in the MSC-mock group had a mean score value of 0.5 and were not completely cured. At end point mice were re-challenged with EAE. Mice in the CAR-treated group retained a score value of zero until eight days post new EAE challenge when they presented a mean score value of one. Mock-treated mice immediately displayed a mean score value of 0.5 that at end point (eighteen days post EAE) had increased to a mean score value of 3. T cells from the brain of CAR- and Mock-treated mice were stimulated with MOG-peptides and analyzed for effector cytokine release. T cells from Mock-treated mice were more effective in releasing IL17 than T cells from CAR-treated mice indicating that brain T cells from CAR treated mice had a more restricted response even if they were not completely in anergy. Inflammatory cytokines in the brain (IL12 and IFN$\gamma$) revealed a hampered response in both CAR- and Mock-treated mice compared to PBS-treated mice. Also, CAR-treated mice showed little or no axonal damage in the cerebellum and brain stem when analyzing histology of the brain compared to both Mock- and PBS-treated mice. In conclusion, we have developed CNS-specific MSCs that when administrated i.p. in naïve mice can transmaigrate into the CNS and when infused i.n. in mice with active EAE, suppresses the local CNS environment leaving immune cells in the periphery unhampered to combat infections.
Conclusions

I RRMS patients have peripheral CNS-reactive T cells of both Th1 and Th17 type that, while in remission, are kept in anergy.

II RRMS patients in relapse exhibit increased levels of CD25 negative Tregs indicating an attempt to restrain immune activity. As the disease progresses, the proportion of Tregs decline while effector T cells are increasing.

III CNS-specific Tregs suppressed activated T cells in vitro and cured mice with EAE when administrated both i.n. and i.p.

IV CNS-targeted MSCs were able to home to myelin structures in the CNS, suppress activated T cells and cure mice with active EAE when administrated i.n. and i.p.
Future perspectives

In this thesis we have performed an immunological profiling of patients with RRMS and developed immunotherapy with treatment efficacy in the EAE model. We are currently enrolling patients in a longitudinal study where patients will be monitored during different phases of relapse-remission-relapse. This study is a collaboration between the Division of Clinical Immunology, the Department of Neurology and the Division of Radiology, both at the Uppsala university hospital. Patients enrolled in the study will be monitored and examined for a various of disease markers over time.

To further prove treatment efficacy of treated EAE mice in the performed immunotherapy trials, we are adding histological markers for myelin regeneration (GFAB) and inflammatory cytokines (IL17) to the protocol. Further, the survival of the therapeutic cells is being monitored. Naïve mice have been administrated i.n. with GFP-transduced MSCs/Tregs and sacrificed at different time points (24h, 20d, 40d, and 80d). This will provide information of receptor/target specificity and for how long cells with the targeting receptor are able to stay on target. We also hope to gain information of the percentage of cells administrated that end up in the brain. In future studies, we are going to investigate how the cells migrate from the nose into the brain by using luciferase carrying vectors. By adding luciferase to the CMV-CAR-vector we will be able to document migration of the transduced cells. Naïve mice will be administered with luciferase-positive cells i.n. and monitored at different time points in a luminescence camera.

To improve our targeting receptor we will add an inducible suicide gene to the vector. Every cell transduced with the targeting receptor will thereby carry a suicide gene that can be activated at will and force the cell into apoptosis allowing the treatment to be aborted if/when needed. Such safety systems will enhance security of cell therapy in humans.
Acknowledgements


Många personer har på ett eller annat sätt bidragit med inspiration, stöd och uppmuntran under arbetet med denna avhandling och ni förtjänar alla att nämnas med värme och tacksamhet.

Till följande personer vill jag rikta ett särskilt stort tack:

Min handledare Angelica Loskog som är ett outtömligt energiknippe! Du har fått mig att utvecklas inte bara på lab utan också som person och arbetsledare. Tack för att du har utmanat mig att göra mitt bästa och samtidigt varit förstående för att man som forskande mamma inte alltid kan eller hinner vara på lab.

Min bihandledare Thomas Tötterman som ställde upp som handledare innan Angelica kunde ta över på heltid och som fanns tillgänglig för diskussioner under Angelicas mammaledighet.


Elena Piras för ett roligt samarbete som jag hoppas kan fortgå efter våra respektive disputationer. Du har varit ett stöd och en rolig samarbetspartner. Utan dig hade vi inte lyft projektet till där det är idag.

Pella, du har varit en ovärderlig person för mig under mina år på klinimm. Under de första åren, var du en stor hjälp på lab för en ny
och förvirrad student och under de senaste även en god vän som alltid har tid att dricka kaffe och prata. Tack för all tid du har lagt ner på mig!


Magnus, för att du alltid har ställt upp med att diskutera virusvektorer och primerdesign. Tack för alla plasmider och virussystem som vi har fått använda i projektet!

Binfeng Lu, University of Pittsburgh, who offered great help in optimizing our protocols in the EAE studies.


Mina studenter som har varit involverade i projektet på olika sätt: Hao – you have been a great resource to this project! I really enjoyed working with you, good luck in Singapore with your medical studies. Kicki, vilken härlig person du är, det är tur att du är kvar i korridoren så att man kan få en daglig dos av dig! Vroni – you helped me a great deal in this project when I was pregnant, I hope everything works out for you in the future. Summer, du var en ambitiös student som fick en
svår uppgift att lösa. Angelos – it was fun having you here, I hope you will succeed with the EAE-model in Create.


Maj-Britt, Maj-Britt, Anna och Viola på rutin som har låtit mig köra FACSCanton och hjälpt till med inställningar vid paniksituationer.

Jan Grawe, för ovärderlig hjälp med FACS-inställningar

Roger Festin, för hjälp med en situation med kladdiga monocyter.

Våra samarbetspartner på neurologen: Joachim Burman och Jan Fagius som har försett oss med patentprover och värdefull information genom åren. Särskilt tack till Joachim som både har visat mig kliniken och diskuterat neurologi. Ett stort tack även till alla patienter som varit involverade i studien!

Anna Lobell, tack för all hjälp när vi först skulle sätta upp EAE-modellen och för utbyte av såväl reagenser som tankar kring MS. Jag ser fram emot ett framtida samarbete!

Till min familj vill jag rikta ett särskilt stort tack – det är ni som har format mig till den person som jag är idag! Först vill jag tacka min farmor som har alltid har ställt upp som sömmerska, bullbakerska och som alltid har haft saftigt skvaller att komma med. Du visar att man aldrig behöver bli gammal i sinnet! Morfar som alltid har haft poesi, rim och musik nära till hands. Du har alltid satt en extra guldkant på middagar med hemskrivna visor. Mormor som betydde mycket för mig under min uppväxt och som jag saknar så att det gör ont. Familjen Collander med Magnus och Carina i spetsen som har gjort underbara personer av mina kusiner Ida och Sanna. Alla i familjen Sköld, Mats och Malin och mina kusiner Adam och Alicia som har växt upp till härliga och mysiga vuxna, det ska bli spännande att se vad ni hittar på i framtiden! Familjen Appelsved, Ingela, Per-Arne, Alexander och Philip som man har fått hälsa på både i Portugal och på landet i Sverige. Familjen Fransson, Stefan och Marie, Simon och Matilda, ni är en jordnära familj som har nära till skratt. Det är synd att vi inte umgås ofta, är det inte dags att flytta uppåt?
Familjen Björke, Ulla, Hans och Erik. Tack för allt roligt vi har när vi träffas och för allt roligt vi kommer att ha! Tack för att ni är så bra farmor, farfar och farbror till Hugo!

Till min allra närmaste familj, snacka om att jag hade tur när jag föddes in i den här familjen! Livet är roligt när jag får vara med er och jag hoppas att jag kan göra detsamma för mina barn som ni gjort för mig. Mamma som alltid stått upp för mig och pappa som hjälppte mig komma igenom gymnasiet när allt var jobbigt. Sara, du har tagit hand om mig, varit min vän, vet nästan alla steg jag har tagit i livet och är en av de bästa personer som jag vet. Tillsammans med Micke har du dessutom gett mig de bästa syskonbarnen som man kan tänka sig – Alva och Theo, moster tänker skämma bort er i hela era liv!

Sköna Uppsalavänner som gör att man aldrig vill flytta härifrån: Anna, särskilt tack för all hjälp med allt kring disputationen och att du tillsammans med Calle alltid är redo att hoppa in som extra föräldrar till Hugo! Henrik, Jonas och Elina med lilla Ruth ni är supersköna personer, varje middag med er blir liksom kul! Från Örebro, Tomas, Malin med söta Wilhelm, det är få personer som man har så otvunget roligt med som man har med er!

Till mina allra bästa: Henrik, Hugo och bäbisen i magen. Jag älskar mitt liv tillsammans med er, ni är de bästa personer jag vet. Så länge vi fyra får vara tillsammans kommer livet att vara lätt. All kärlek ♥
References


77. Lublin, F.D. and S.C. Reingold, *Defining the clinical course of multiple sclerosis: results of an international survey.* National Multiple Sclerosis Society (USA) Advisory Committee on


Acta Universitatis Upsaliensis
Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine 608

Editor: The Dean of the Faculty of Medicine

A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)