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**Regulation of immunity in Multiple Sclerosis:
CD4+ T cells and the influence of natalizumab**

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*“That which can be asserted without evidence,
can be dismissed without evidence”*

– Christopher Hitchens

ABSTRACT

Multiple sclerosis (MS) is an autoimmune disease targeting the central nervous system (CNS) and the most common neurological cause of disability in young adults. In most cases, the disease course is characterised by the cycling of relapses and remissions, so called relapsing-remitting MS (RR-MS). Although extensively studied, the underlying mechanisms are not fully elucidated, yet CD4⁺ T cells have been shown to be of importance in disease pathology. A range of treatments are available; the most effective to date being natalizumab, a monoclonal antibody directed against the adhesion molecule VLA-4 on the lymphocyte surface, thereby preventing entry into the CNS.

The aim of this thesis was to assess the nature of lymphocyte populations in MS. This was achieved by studying CD4⁺ T helper cells (T_H) and regulatory T cells (T_{REG}) in peripheral blood. In addition, the influence of natalizumab was also investigated, both regarding the effect of the drug on the composition of the peripheral lymphocyte compartment as well as its effects on CD4⁺ T cells *in vitro*.

We showed an imbalance in the mRNA expression of CD4⁺ T helper cell lineage specific transcription factors in peripheral blood. While T_H1 and T_H17 associated *TBX21* and *RORC* expression was comparable in MS and healthy individuals, the T_H2 and T_{REG} associated *GATA3* and *FOXP3* expression was decreased in RR-MS. Given the reciprocally inhibitory nature of T_H subsets, this might imply not only diminished function of T_H2 and T_{REG} cells but also a permissive state of harmful T_H1 and T_H17 cells. The size of the peripheral T_{REG} population was unaltered in RR-MS. When analysed in detail, activated and resting T_{REG} were distinguished, showing clear differences in FOXP3 and CD39 expression. Furthermore, when investigating these subpopulations functionally, the ability of activated T_{REG} to suppress proliferation of responder T cells was found to be decreased in RR-MS patients compared to controls. To further investigate this defect, the global gene expression of T_{REG} was compared between patients and controls. Gene set enrichment analysis revealed an enrichment (over-expression) of chemokine receptor signalling genes in RR-MS T_{REG}, possibly suggesting a role for chemokines in T_{REG} function.

A sizable effect of natalizumab treatment was seen in the composition of peripheral lymphocyte populations after one year of treatment. While the number of lymphocytes increased over all, the largest increase was seen in the NK and B cell compartments.

ABSTRACT

Furthermore, T cells from patients with MS displayed decreased responsiveness towards antigens and mitogens *in vitro*. Natalizumab treatment was able to normalise the responsiveness in blood, an effect not solely dependent on the increased number of cells.

The importance of CD4⁺ T cells in human disease, including MS, was shown by a systems biology approach; using GWAS data, genes associated with CD4⁺ T cell differentiation were enriched for many, not only immune-related, diseases. Furthermore, global CD4⁺ T cell gene expression (by microarray) could discriminate between patients and controls. Lastly, using *in vitro* treated CD4⁺ T cells, we could show that natalizumab perturbed gene expression differently in patients responding to the drug compared to those not responding.

In conclusion, our results demonstrate an imbalance of peripheral CD4⁺ T cells in MS, along with a functional deficiency in the case of T_{REG}. Taken together, these aberrations might result in differentiation and activation of harmful T_H1 and T_H17 cells, resulting in CNS tissue damage. The importance of CD4⁺ T cells was further demonstrated by the finding that genes associated with CD4⁺ T cell differentiation constitute a pleiotropic module common to a number of diseases. Investigation of natalizumab revealed drastic changes in the peripheral lymphocyte compartment caused by treatment. It also appears as treatment might influence the responsiveness of peripheral T cells to antigens. In addition, by using CD4⁺ T cell transcriptomics after *in vitro* drug exposure, prediction of treatment outcome may be possible.

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ORIGINAL PUBLICATIONS

I. Transcriptional characteristics of CD4⁺ T cells in multiple sclerosis: relative lack of suppressive populations in blood.

Måns Edström, Johan Mellergård, Jenny Mjösberg, Maria C. Jenmalm, Magnus Vrethem, Rayomand Press, Charlotte Dahle, Jan Ernerudh
Mult. Scler. 2011 Jan; 17(1): 57-66

II. Regulatory T cells in multiple sclerosis – Indications of impaired function of suppressive capacity and a role for chemokines

Måns Edström, Charlotte Dahle, Magnus Vrethem, Mika Gustafsson, Mikael Benson, Maria C. Jenmalm, Jan Ernerudh
Manuscript

III. An increase in B cell and cytotoxic NK cell proportions and increased T cell responsiveness in blood of natalizumab-treated multiple sclerosis patients

Johan Mellergård, Måns Edström, Maria C. Jenmalm, Charlotte Dahle, Magnus Vrethem, Jan Ernerudh
PloS ONE. 2013 Dec; 8(12): e8168. doi:10.1371/journal.pone.0081685

IV. Integrated genomic and prospective clinical studies show the importance of modular pleiotropy for disease susceptibility, diagnosis and treatment

Mika Gustafsson, Måns Edström, Danuta Gawel, Colm E Nestor, Hui Wang, Huan Zhang, Fredrik Barrenäs, James Tojo, Ingrid Kockum, Tomas Olsson, Jordi Serra-Musach, Núria Bonifaci, Miguel Angel Pujana, Jan Ernerudh, Mikael Benson
Genome Med. 2014 Feb; 6(2): 17

SAMMANFATTNING PÅ SVENSKA

Multipel skleros (MS) är en sjukdom där kroppens immunförsvar angriper den egna vävnaden i centrala nervsystemet (CNS), ett exempel på en s.k. autoimmun sjukdom. Den vanligaste varianten av MS karakteriseras kliniskt av skov av symptom som följs av remissioner, s.k. skovvis förlöpande MS. Immunförsvaret, som annars är utformat för att oskadliggöra yttre hot såsom bakterier och virus, identifierar av misstag kroppsegna ämnen som främmande.

Eftersom det kroppsegna ämnet inte kan oskadliggöras då det fortsätter produceras tenderar många autoimmuna sjukdomar att vara kroniska, så även MS. Centralt i immunreaktionen återfinns vita blodkroppar som kallas $CD4^+$ T-celler. Huvudfunktionen hos dessa celler är att instruera och kontrollera övriga celler i immunförsvaret. Vid MS finns flera indicier som tyder på att $CD4^+$ T-celler är viktiga för att sjukdomen ska uppstå och progrediera. Det finns ett flertal subtyper av $CD4^+$ T-celler varav de viktigaste är T-hjälparcell (T_H) 1, T_H2 , T_H17 och regulatoriska T celler (T_{REG}). Vid behandling av MS är den mest effektiva behandlingen idag en antikropp, natalizumab, som blockerar trafik av immunceller in i CNS.

Syftet med avhandlingsarbetet var att studera olika aspekter av immunförsvaret vid skovvis förlöpande MS. I de två första arbetena undersöks $CD4^+$ T-celler i blodet med fokus på T_{REG} i arbete II. Arbete III handlar framför allt om effekten av natalizumab och vilka effekter det har på immunförsvaret. I arbete IV undersöktes signifikansen av $CD4^+$ T-celler, inte bara vid MS utan i en rad sjukdomar. Vidare studerades även effekten av natalizumab experimentellt i cellkultur.

Vi kunde visa att det finns en obalans i blodet mellan olika subtyper av $CD4^+$ T-hjälparceller vid MS, jämfört med friska kontroller. Av de fyra huvudpopulationerna av $CD4^+$ T-celler sågs en minskning av uttryck av gener som styr utveckling av T_H2 -celler och T_{REG} . Detta är intressant eftersom en minskad funktion av dessa celler kan innebära minskad kontroll av skadliga T_H1 - och T_H17 -celler. Vid närmare studie av T_{REG} såg vi att andelen celler i blodet inte skiljde sig mellan patienter och kontroller. En av huvudfunktionerna hos T_{REG} är deras förmåga att hämma konventionella $CD4^+$ T-celler. Hos patienter med MS hade dock aktiverade T_{REG} en nedsatt förmåga att hämma konventionella T-celler. För att förstå bakgrunden genomfördes "microarray"-analys, med mätning av genuttryck av samtliga gener. Vi fann att gener som reglerar celltrafik (kemokiner) var kollektivt uppreglerade i T_{REG} hos

patienter jämfört med T_{REG} hos friska. Detta fynd, tidigare inte känt, skulle möjligen kunna vara en orsak till varför aktiverade T_{REG} fungerar sämre vid MS.

När vi undersökte vilka effekter natalizumab hade på lymfocyter sågs en tydlig ökning av dessa celler i blodet efter ett års behandling. Den största ökningen syntes för naturliga mördarceller och B-celler, även om T-celler också ökade drastiskt. Vi undersökte även effekten av läkemedlet genom att studera aktiveringsförmågan hos T-celler *in vitro*. Det visade sig att innan behandling hade T-celler hos patienter en lägre förmåga att aktiveras än T-celler hos kontroller, något som normaliserades efter ett års behandling, delvis på grund av det ackumulerade antalet T-celler.

I sista arbetet återkommer vi till CD4⁺ T-celler. Vid analys av stora mängder publika data över association av genmutationer till olika sjukdomar kunde vi visa att gener förknippade med CD4⁺ T-celler var associerade till ett flertal sjukdomar, inte enbart begränsat till sjukdomar med en känd koppling till immunförsvaret. Vidare, genuttrycksdata från CD4⁺ T-celler kunde användas för att skilja patienter och kontroller vid en rad sjukdomar, inklusive MS. Detta talar för att det finns gemensamma sjukdomsmekanismer och att CD4⁺ T-celler verkar vara den gemensamma nämnaren. Vi kunde även visa att natalizumab påverkade globalt genuttryck *in vitro* hos MS-patienter olika beroende på huruvida svarade eller inte svarade på behandlingen. I förlängningen kan denna forskningslinje göra det möjligt att skraddarsy behandling vid MS.

ABBREVIATIONS

AIRE	autoimmune regulator
AP-1	activator protein 1
APC	antigen-presenting cells <i>or</i> allophycocyanin
ATP	adenosine triphosphate
aT _{REG}	activated T _{REG}
BBB	blood-brain barrier
Bcl-6	B cell lymphoma 6
B _{REG}	regulatory B cells
cAMP	cyclic AMP
cDNA	complementary DNA
CCL	C-C motif ligand
CCR	C-C chemokine receptor
CFSE	carboxyfluorescein succinimidyl ester
ChIP	chromatin immunoprecipitation
CIS	clinically isolated syndrome
CMV	cytomegalovirus
CNS	central nervous system
CNS1-3	conserved non-coding sequence 1-3
CREB	cAMP-responsive element binding
cRNA	complementary RNA
CSF	cerebrospinal fluid
CTLA-4	cytotoxic T lymphocyte antigen 4
cT _{REG}	classically defined T _{REG}
CXCL	C-X-C motif ligand
CX3CL	C-X3-C motif ligand
DC	dendritic cell
DE	differentially expressed
DP	CD4 ⁺ CD8 ⁺ double-positive
EAE	experimental autoimmune encephalomyelitis
EBI3	Epstein-Barr virus induced gene 3
EBV	Epstein-Barr virus
EDSS	expanded disability status scale
EDTA	ethylenediaminetetraacetic acid
ES	enrichment score
FACS	fluorescence-activated cell sorting
FCS	fetal calf serum
FDR	false discovery rate
FITC	fluorescein isothiocyanate
FSC	forward-scatter

ABBREVIATIONS

GA	glatiramer acetate
GATA3	GATA binding protein 3
GC	glucocorticoids
GO	gene ontology
GSEA	gene-set enrichment analysis
GWAS	genome-wide association study
HDAC	histone deacetylase
HLA	human leukocyte antigen
HR	high-responders
ICOS	inducible T cell co-stimulator
IDO	indolamine-2,3-dioxygenase
IFN- β/γ	interferon β/γ
Ig	immunoglobulin
IL	interleukin
IMDM	Iscove's modified Dulbecco's medium
IPA	Ingenuity Pathways Analysis
IPEX	immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
iT _{REG}	induced T _{REG}
IvIg	intravenous immunoglobulin
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LR	low-responders
MBP	myelin basic protein
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein
MRI	magnetic resonance imaging
mRNA	messenger RNA
MS	multiple sclerosis
MSIS-29	multiple sclerosis impact scale
MSSS	multiple sclerosis severity score
NES	normalised enrichment score
NFAT	nuclear factor of activated T cells
NF κ B	nuclear factor κ B
nT _{REG}	natural T _{REG}
PBMC	peripheral blood mononuclear cells
PCA	principal component analysis
PD1/PD2	programmed death 1 and 2
PE	phycoerythrin
PerCP	peridinin chlorophyll
PHA	phytohaemagglutinin
PLP	proteolipid protein
PPD	purified protein derivate
PPI	protein-protein interaction
PP-MS	primary-progressive multiple sclerosis

PWM	pokeweed mitogen
ROR γ / α	retinoic acid-related orphan receptor γ / α
RPMI	Roswell Park Memorial Institute
RR-MS	relapsing-remitting multiple sclerosis
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
rT _{REG}	resting T _{REG}
RUNX1-CBF β	runt-related transcription factor 1-core binding factor β
SAR	seasonal allergic rhinitis
SDMT	single-digit modality test
SMAD	mothers against decapentaplegic, drosophila homolog
SNP	single nucleotide polymorphism
SP	CD4 ⁺ or CD8 ⁺ single-positive
SP-MS	secondary-progressive multiple sclerosis
SSC	side-scatter
STAT	signal transducer and activator of transcription
T-bet	T-box expressed in T cells
T _{CM}	central memory T cells
TCR	T cell receptor
T _{EM}	effector memory T cell
T _{FH}	follicular T helper cell
TGF- β	transforming growth factor β
T _H 1/2/17	T helper 1, 2 and 17 cell
TLR	Toll-like receptor
T _M	memory T cell
TNF	tumor necrosis factor
T _{REG}	regulatory T cells
T _{RESP}	responder T cells

1. INTRODUCTION

1.1 Multiple Sclerosis

1.1.1 Clinical diagnosis

Multiple sclerosis (MS) is a neurological disorder, exclusively affecting the central nervous system, where the anatomical localisation of injury determines the clinical manifestations. MS was first described by Charcot in the 19th century (Charcot 1868). In clinical practice, three major subgroups may be identified. The most common form of disease, called relapsing-remitting MS (RR-MS) is characterised by episodes of clinical relapses, followed by spontaneous remission of symptoms. The cycling of relapses and remissions can persist for decades, but eventually there usually is a transition into secondary-progressive MS (SP-MS), where a steady progression of neurological deficit ensues (Lublin et al. 1996). In SP-MS, there may still be additional relapse-remission cycles, although the deterioration continues irrespectively. Alternatively, the progressive component may be present at disease onset, accounting for the third major phenotype; primary-progressive MS (PP-MS) (Lublin et al. 1996, Thompson et al. 2000). Around 80-85% of patients experience a relapsing-remitting course of disease at onset, while the remaining 15-20% present with PP-MS (Compston et al. 2008) (Fig 1).

The diagnosis of MS has traditionally been made based on the symptoms present at neurological examination. Today, in addition to clinical examination, so called paraclinical disease manifestations have become important for early and accurate diagnosis, including magnetic resonance imaging (MRI) and analysis of cerebrospinal fluid (CSF) (McDonald et al. 2001, Polman et al. 2011).

The fundamentals of diagnosis are observations of dissemination in time and space, *i.e.* presence of evolution of symptoms (relapses) in time and signs and symptoms indicative of different anatomical locations. Both time and space dissemination of neuroinflammation may be determined by MRI (Barkhof et al. 1997, Tintore et al. 2000). CSF analysis is important for establishing the inflammatory component of the disease, a hallmark of MS. In MS, there is commonly a mild mononuclear pleocytosis in CSF, as well as the presence of oligoclonal bands not mirrored in serum, signifying intrathecal antibody production. Additional parameters of interest include albumin index, indicating blood-brain barrier leakage

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(Freedman et al. 2005). At the time of clinical presentation of RR-MS, *i.e.* the first overt relapse, dissemination in space and time might be difficult to establish. Before the definite diagnosis is made, the disease is classified as clinically isolated syndrome (CIS).

Assessment of disability in MS is based on careful clinical examination. Commonly, the expanded disability status scale (EDSS) is used (Kurtzke 1983). The scale ranges from 0 to 10, where 0 corresponds to no signs of damage to the CNS and 10 to death caused by MS. EDSS 0 through 4.0 is determined by focal signs of disability in any of the following areas: pyramidal tract function, cerebellar function, brain stem function, sensory function, bowel and urinary functions, visual functions higher cerebral function and other functions (including paroxysmal manifestations). Scoring over 4.0 is mainly based on walking ability. The multiple sclerosis severity score (MSSS) combines EDSS and disease duration to give an approximate estimate of disease progression and prognosis and may be useful when evaluating treatment options (Roxburgh et al. 2005). MS impact scale 29 (MSIS-29) is a validated questionnaire used by patients' for self-assessment of impact of disease on physical and psychological aspects of daily life (Hobart et al. 2001). In addition, there are numerous instruments for measuring cognitive impairments following CNS involvement. Single-digit modality test (SDMT), due to its simplicity, is suitable for rapid assessment of cognitive impairment in outpatient care (Smith 1991).

Figure 1. The natural progression of MS with a relapsing-remitting clinical course (RR-MS).

1.1.2 Prevalence, incidence and prognosis

MS is the most common disease causing neurological disability in young adults, with a worldwide estimated prevalence and incidence of 30/100 000 and 2.5/100 000/year, respectively (WHO 2008). In Scandinavia, the incidence and prevalence is among the highest in the world. Using the National Swedish MS register, the recorded prevalence was 189/100 000 inhabitants, with a female to male distribution of 2.4-2.6 to 1 (Ahlgren et al. 2011, Bostrom et al. 2013). The incidence of MS in Sweden is 4.0-6.4/100 000/year (Andersen 2012) and the adjusted mortality rate, *i.e.* death caused by MS adjusted for risk of death of other reasons, nation-wide is 2.0/100 000/year (Bostrom et al. 2012).

1.1.3 Genetic risk factors

Observations of MS prevalence have revealed a 15-35% concordance among monozygotic twins (Mumford et al. 1994, Willer et al. 2003, Baranzini 2011, Westerlind et al. 2014). There is also an, albeit much lower, increased risk to develop MS among dizygotic twins (Dyment et al. 2004, Westerlind et al. 2014). These observations suggest a complex disease etiology with interactions between genetics, epigenetics and environmental factors. Early investigations of genetic associations identified certain haplotypes of the human leukocyte antigen (HLA) locus on chromosome 6, in particular the HLA-DRB1*15 haplotype, to be strongly associated with disease (Dyment et al. 2004, Lincoln et al. 2005) and it is estimated that the HLA locus accounts for 17-62% of the genetic burden in MS (Haines et al. 1998). The HLA-DRB1 locus encodes part of the major histocompatibility complex (MHC) class II molecules present on antigen-presenting cells (APC), which are of great importance in the adaptive immune response. In addition to HLA-DRB1*15, other haplotypes conferring disease risk include HLA-DRB1*17, whereas the HLA-DRB1*14 appears to be protective and negatively associated with disease risk (Ramagopalan et al. 2007). HLA-DRB1*01 also seems to be protective, but only when transmitted together with HLA-DRB1*15 (Dyment et al. 2005). As expected, homozygosity of HLA-DRB1*15 is associated with the highest risk increase in disease development. The HLA-DRB1*17 haplotype is recessive, with high risk of disease only in homozygotes, but no risk increase in heterozygotes (Modin et al. 2004, Dyment et al. 2005, Barcellos et al. 2006, IMSSG 2007, Ramagopalan et al. 2007). Haplotypes of the HLA class I locus, encoding MHC class I have also been implicated in the susceptibility to MS. In

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particular, the HLA class I A haplotype HLA-A*02 has been found to be protective, and there is also an additional synergy between lack of HLA-A*02 and carriage of HLA-DRB1*15, greatly increasing the risk of developing MS (Brynedal et al. 2007, Bergamaschi et al. 2010). Other loci outside the HLA locus displaying high association with disease include the *IL2RA* (encoding the α -chain of the interleukin 2 (IL-2) receptor) and the *IL7R* (encoding the α -chain of the IL-7 receptor). Although not as strongly associated with MS as the HLA locus, the presence of polymorphisms in *IL2RA* and *IL7R* has an OR of approximately 1.1-1.3 (IMSGC 2007, Lundmark et al. 2007). Recently, several other genes have been implicated; GWAS studies on ~25 000 patients and controls revealed weaker genetic relationships not detectable in previous, smaller materials (IMSGC 2011, IMSGC 2013). These studies have created a more comprehensible picture of the genetics behind MS, as well as confirming previous findings. Interestingly, the *HLA-DR*, *IL2RA*, and *IL7R* genes, as well as a sizable portion of newly identified genes, are intimately associated with adaptive immune responses (IMSGC 2011), highlighting the importance of CD4⁺ T cell immunity in MS.

1.1.4 Environmental risk factors

The relatively low concordance of MS in monozygotic twin studies has directed attention towards exogenous factors as etiologically important in MS development. The uneven prevalence and incidence ratios found in different geographic locations point towards a genetic risk-association. However, studies investigating the migration of people have contradicted this notion, particularly if migrating at a low age. Migration from a low-risk to a high-risk area confers a higher risk of disease, and if migrating from a high-risk to a low-risk area, the opposite is true (Dean et al. 1997, McLeod et al. 2011, Ahlgren et al. 2012), implying that environmental factors in high-risk geographic areas are of relevance. Geographic latitude, correlated with sunlight exposure, has been suggested as a predictor of MS incidence (Staples et al. 2010). UV radiation might have immunomodulatory effects in itself, but is also one of the major determinants of vitamin D levels. MS patients have been reported to have subnormal levels of the main form of circulating vitamin D, 25(OH)D (Hiremath et al. 2009, Salzer et al. 2012). Interestingly, the biologically active form of vitamin D, 1,25(OH)₂D have multiple effects on immune cells and can deviate T cells from a deleterious to an immunosuppressive phenotype. In addition, T cells express the enzyme 1 α -hydroxylase, which cleaves the circulating 25(OH)D to 1,25(OH)₂D (Correale et al. 2009,

Bartosik-Psujek et al. 2010). Mechanistically, there is thus evidence of a connection between the immune response in MS and epidemiological data, although the exact mechanisms to a large extent are still not yet shown.

Similarly, there is a clear relationship between tobacco smoking and MS where a higher smoking rate correlates to MS incidence (Hernan et al. 2001, Hedstrom et al. 2009, Salzer et al. 2013). As in the case of vitamin D, there have been speculations as to why this is the case, and an increase in cerebral blood flow following nicotine intake was shown decades ago (Hans et al. 1993), with the corollary that this might increase cerebral immune trafficking. Also, direct effect of nicotine on T cells has been shown in rodents (Kalra et al. 2000). Interestingly, oral snuff entails protection to MS, as shown in a Swedish population (Hedstrom et al. 2009), indirectly indicating that the risk-bearing elements associated with smoking is not attributable to nicotine. In addition to the direct effects of smoking on MS incidence, there is also a suspected interaction with certain genotypes. Individuals bearing the risk-alleles HLA-DRB1*15 who also smokes have a greatly increased risk of developing MS. The co-occurrence of protective MHC class I haplotype HLA-A*02 decreases the influence of HLA-DRB1*15 (Hedstrom et al. 2011).

The role of infectious agents in MS development has been investigated in depth, and the clearest association has been shown for Epstein-Barr virus (EBV). Although the increased risk is modest, the relationship has been thoroughly investigated (Thacker et al. 2006, Handel et al. 2010).

1.2 Adaptive immune responses

1.2.1 CD4⁺ T cells

1.2.1.1 Thymic maturation of CD4⁺ T cells and central tolerance

T cells originate in the bone-marrow. After migration to the thymus they undergo a selection process, after which they enter the circulation as naïve thymus-derived cells, or T cells. The selection in the thymus is constituted of two interdependent processes known as positive and negative selection, respectively. Initially, the immature T cells are double-negative for the lineage-markers CD4 and CD8, and express a crude form of the T cell receptor (TCR). During the thymic maturation process the T cells first become double-positive (DP) for CD4 and

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CD8, and later, before release, most of the T cells are single-positive (SP); CD4⁺ or CD8⁺ T cells, respectively. Before positive selection, the cells, now called thymocytes, are expressing both chains of the TCR (α and β), and have become DP. During positive selection, DP cells interact with cortical thymic stromal cells expressing both MHC class variants, presenting self-antigens to the T cell. Only cells that show an appropriate affinity towards either MHC class I or II are selected for survival. Cells binding with inadequately low affinity die by neglect, and high affinity clones undergo deletion. At this stage, the fate of the T cell as CD8⁺ cytotoxic or CD4⁺ helper T cells is determined. Cells whose TCR interact with MHC class I are chosen to become CD8⁺ T cells and stop expressing CD4, and the reverse is true for cells with appropriate affinity for MHC class II. Negative selection occurs in the thymic medulla, where a large number of endogenous antigens are expressed under autoimmune regulator (*AIRE*). Clones with high affinity for MHC-bound endogenous peptides are deleted, one of the more important processes in regulation of autoimmunity. Thus, the thymic selection process aims to ensure that naïve thymic emigrant T cells have TCRs with an adequately high affinity and reactivity, but only directed against foreign antigens (Macian 2005, Klein et al. 2014).

1.2.1.2 Differentiation of naïve CD4⁺ T cells

In the activation and differentiation of a naïve CD4⁺ T cells, three distinct signals are required. The first signals (1 and 2) involve interaction with an APC, and is cell-to-cell contact dependent. The third signal (3), ultimately determining differentiation fate, consists of cytokines acting in a paracrine fashion.

Tissue-resident dendritic cells (DC) migrate to lymph nodes after Toll-like receptor (TLR) activation and phagocytosis of proteins in peripheral sites. In the lymph node, foreign peptides (antigens) are presented on MHC molecules, MHC class II in the case of CD4⁺ T cells. Only naïve CD4⁺ T cells with TCR specificity for the presented antigen can engage their TCR to the MHC class II-antigen complex. This ligation between the T cell antigen-specific TCR, the antigen and the MHC class II on APC constitutes signal 1, and initiates a cellular program in the naïve T cell. Necessary for this process, however, is the presence of signal 2. DC phagocytosis and TLR activation leads to surface expression of co-stimulatory molecules, of which CD80 and CD86 are the best characterised. Binding of co-stimulatory molecules with

CD28 on the T cell mediates signal 2. Other co-stimulatory molecules include inducible T cell co-stimulator (ICOS), cytotoxic T lymphocyte antigen 4 (CTLA-4) and programmed death 1 and 2 (PD1/2). The function of ICOS and DC-expressed ICOS ligand are partly overlapping with those of CD28 and CD80/86, while engagement of CD28 and PD1/2 with CTLA-4 and PD1/2 ligand, respectively, on the APC work in an inhibitory manner (Chen et al. 2013). CTLA-4 is covered more in depth under the discussion on regulatory T cells.

In the presence of signal 1 but absence of signal 2 at the time of antigen presentation, T cell anergy is induced (Chen et al. 2013). TCR engagement results in the Ca^{2+} signalling-dependent activation of nuclear factor of activated T cells (NFAT), the major transcription factor responsible for early naïve T cell activation (Shaw et al. 1988, Macian 2005). Signal 2, co-stimulation, enhances NFAT activation, but also induces other transcription factors, including activator protein 1 (AP-1), regulating the transcriptional profile of NFAT (Podojil et al. 2009). NFAT-induced IL-2 acts in an autocrine manner through ligation on IL-2R on the cells surface, resulting in clonal expansion (Shaw et al. 1988). Activation of the naïve cell through signals 1 and 2 also results in surface expression of CD40L on the T cells, necessary for interaction with B cells. In addition, CD40L on T cells interact with CD40 on DC, creating a positive feedback loop of reciprocal activation.

1.2.1.3 CD4^+ T helper phenotypes

During antigen presentation, the presence of signal 3 directs the differentiation path the cell will take. Signal 3 is a collective term representing the presence of cytokines, acting on the developing T cell and directing its lineage commitment. The CD4^+ T cells are subdivided into four distinct lineages; T helper 1 ($\text{T}_\text{H}1$), $\text{T}_\text{H}2$, $\text{T}_\text{H}17$ and regulatory T cells (T_REG) (summarised in Fig. 2). More recently, follicular T helper cells (T_FH) have also been acknowledged as a discrete phenotype after identification of the lineage-specific transcription factor Bcl-6 (Yu et al. 2009). Naturally, the concept of discrete subsets is an oversimplification. When studying CD4^+ T cells, observed phenotypes many times display characteristics of one or more of the above mentioned phenotypes, perhaps representing a transition between phenotypes or an overlapping phenotype with characteristics of two or more T_H phenotypes. Previously, other CD4^+ T cell subpopulations, including $\text{T}_\text{H}9$, $\text{T}_\text{H}22$, and regulatory subsets $\text{T}_\text{H}3$ and $\text{T}_\text{R}1$, have

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been suggested, but these have faded in the face of time due to failure to identify lineage-specific transcription factors (Jonuleit et al. 2003, Veldhoen et al. 2008, Eyerich et al. 2009).

As the names suggest, T_H1 and T_H2 were the first CD4⁺ helper T cells identified (Mosmann et al. 1986, Killar et al. 1987). T_H1 cells are characterised by the secretion of interferon γ (IFN- γ) under the lineage-specific transcription factor T-box expressed in T cells (T-bet), encoded by *TBX21* (Szabo et al. 2000). T_H1-directed immune responses are primarily targeting intracellular pathogens, where *M. tuberculosis* often is used as model organism. Development of the T_H1 phenotype is under control of IL-12, secreted from macrophages and DC at the time of presentation (Hsieh et al. 1993). In addition, an additive effect of IL-18 has been shown (Stoll et al. 1998). IL-12, binding the IL-12R on the naïve T cells leads to expression of T-bet, which in turn induces IFN- γ expression. T-bet also directs transcription of IL-12R β 2 which increases the T cells responsiveness to further IL-12 stimulation (Mullen et al. 2001). Studies on *Tbx21*^{-/-} mice underline the importance of T-bet in T_H1 development; the generation of T_H1 cells is severely limited with attenuated IFN- γ response to infection with *Leishmania major* (Szabo et al. 2002). In addition, absence of T_H1 immunity in mice results in exaggerated T_H2 responses with increased T_H2 cytokine production and development of spontaneous airway hypersensitivity (Finotto et al. 2002, Szabo et al. 2002). IFN- γ acts on APC in several ways, one being stimulation of further IL-12 expression (Frasca et al. 2008). In this way, a positive feedback loop is established between the T_H1 cell and the APC, potentiating the commitment to the T_H1 phenotype. Signal transducer and activator of transcription (STAT) is a class of transcription factors important for T_H cell polarisation and the T_H subpopulations are typically associated with separate classes of STATs. T_H1 cells are expressing STAT4 which is induced by IL-12, similar to T-bet (Kaplan et al. 1996, Thierfelder et al. 1996). T_H1-associated immune responses are primarily associated with the activation of macrophages. IFN- γ from Th1 cells activates the macrophage, increases the phagocytic potential as well as increases phagolysosome fusion, thereby greatly potentiating the pathogen-clearing ability (Cross et al. 1995, Ismail et al. 2002). Another important effector mechanism of T_H1 responses is the generation of immunoglobulin (Ig) G₁ and IgG₃ antibodies from plasma cells, acting as complement activating and opsonising agents, further increasing the clearance of pathogens through Fc γ R-mediated phagocytosis (Holdsworth et al. 1999).

T_H2 cells play an important role in the defence against parasites, and are also central in IgE-mediated allergy. The differentiation of T_H2 is dependent on IL-4 as signal 3, leading to

expression of GATA binding protein 3 (GATA3) (Le Gros et al. 1990, Swain et al. 1990). Similarly to the T_H1 case, a positive feedback loop is established; under GATA3, IL-4 is expressed and secreted, which further enhances T_H2 commitment (Zhang et al. 1997, Zheng et al. 1997). In addition to IL-4, GATA3 also controls expression of IL-5 and IL-13. The latter, in conjunction with IL-4, is important in B cell development into IgE-secreting plasma cells (Ishizaka et al. 1990). IL-5 acts on developing cells in the bone-marrow, resulting in differentiation of eosinophils (Nakajima et al. 2007). Plasma cells secreting IgE and eosinophils are at the heart of the T_H2-associated immune response. In accordance with its crucial role, GATA3^{-/-} knockout mice have an impaired T_H2 response (Ouyang et al. 2000). Transcriptional control of IL-4, -5 and -13 expression differs, where GATA3 binds the promoters of the *Il5* and *Il13* genes, but only enhancer regions of the *Il4* gene. The relevance of this was highlighted in a GATA3 conditional knockout model, where GATA3 silencing in developed T_H2 impaired IL-5 and IL-13 production while IL-4 production only was decreased (Siegel et al. 1995, Agarwal et al. 2000, Kishikawa et al. 2001). Similar to the role of STAT4 in T_H1 cells, STAT6 is associated with T_H2 cells (Kaplan et al. 1996, Takeda et al. 1996). STAT6, downstream of IL-4 signalling, induces GATA3 expression as well as regulating the transcription of IL-4 and IL-13 (Kurata et al. 1999, Lee et al. 2004). In addition, the transcription factors interferon regulatory factor 4 (IRF4) and c-Maf affects T_H2 development (Kim et al. 1999, Lohoff et al. 2002, Rengarajan et al. 2002). During naïve CD4⁺ T cell differentiation into T_H1 or T_H2, the strength of TCR ligation also influences the outcome; a high affinity binding favours T_H1 development whereas weak binding is characteristic for T_H2 differentiation (Constant et al. 1997).

T_H17 cells were discovered much later than T_H1 and T_H2 cells. The designation of this cell type stems from the secretory profile with the signature cytokines being IL-17 family members (Aggarwal et al. 2003). The lineage specific transcription factor was later found to be retinoic acid-related orphan receptor γ t (ROR γ t) in mice (Ivanov et al. 2006) while the human orthologue is called RORC. The cytokines profile leading to differentiation of T_H17 cells is not as clear as for T_H1 and T_H2. In mice, it has been convincingly shown that the combination of IL-6 and transforming growth factor β (TGF- β) is sufficient for T_H17 induction *in vitro* and *in vivo* (Bettelli et al. 2006, Mangan et al. 2006, Veldhoen et al. 2006). However, studies on IL-6^{-/-} mice denied the necessity of IL-6 in T_H17 development, and an alternate differentiation pathway was discovered where IL-6 was substituted by IL-21 (Korn et al. 2007, Nurieva et al. 2007). IL-21, which is also produced by T_H17 cells, thereby

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establishes an autocrine loop to further enhance T_H17 development (Zhou et al. 2007). In addition to IL-6, TGF- β and IL-21, an auxiliary role for IL-23 was shown. IL-23R expression, not constitutively expressed on naïve $CD4^+$ T cell, is induced by TGF- β and IL-6/IL-21 and is required for maintenance of T_H17 -associated immunity *in vitro* and *in vivo* (Veldhoen et al. 2006, McGeachy et al. 2009). In humans, it was initially thought that the combination of IL-1 β and IL-6/IL-23 was sufficient for T_H17 differentiation, although TGF- β was later discovered as paramount (Manel et al. 2008, Yang et al. 2008). Binding of IL-6, IL-21 and IL-23 all lead to the activation of STAT3 which induces transcription of IL-17, IL-21 and ROR γ t (Chen et al. 2006, Wei et al. 2007, Yang et al. 2007). ROR γ t transcription also requires TGF- β -activated mothers against decapentaplegic, drosophila homolog (SMAD) 2 (Martinez et al. 2010). T_H17 -associated immune responses are primarily directed against fungi and certain extracellular bacteria, thereby filling a niche not covered by T_H1 - and T_H2 -associated immunity. IL-17 has several functions, but recruitment and activation of neutrophils are especially important in T_H17 immunity; the neutrophil chemoattractant C-X-C motif ligand (CXCL) 8 is produced by T_H17 cells (Pelletier et al. 2010).

During maturation of naïve B cells in secondary lymphoid tissues germinal centres, the recently discovered T_{FH} cells play a crucial role (King et al. 2008). The lineage specific transcription factor responsible for T_{FH} function and phenotype appears to be B cell lymphoma 6 (Bcl-6). In addition, Bcl-6 acts as a transcriptional repressor of cytokines associated with other types of $CD4^+$ T_H immune responses (Nurieva et al. 2009, Yu et al. 2009).

Figure 2. Schematic representation of $CD4^+$ T helper cell differentiation.

1.2.1.4 Memory T cells

The concept of immunological memory is of key importance in immunology. In adaptive immunity, longevity of cells able to recall responses towards previously encountered antigens provides a rapid route of clearing foreign pathogens. The cell-surface markers CD45RA/RO have long been known to be dichotomous in the division of naïve and memory T cells. Memory T cells (T_M) are CD45RO⁺CD45RA⁻ whereas naïve T cells are CD45RO⁻CD45RA⁺ (Michie et al. 1992). After the development of an effector T cell clone and clearing of the inflammatory stimulus, there is a phase of contraction, where the number of cells is rapidly declining. Approximately ~70% of CD4⁺ effector T cells die due to apoptosis during this phase; the remainder of the cells enter the memory T cell pool (Wojciechowski et al. 2006). The CD4⁺ T_M population may be subdivided into effector memory T cells (T_{EM}) and central memory T cells (T_{CM}) based on migratory and functional characteristics. T_{CM} appear to be localised in the T cell zone of secondary lymphoid organs, as they express C-C chemokine receptor (CCR) 7, a homing receptor for C-C motif ligand (CCL) 19 and CCL21 which are expressed by stromal cells in lymph nodes, while T_{EM} cells lack CCR7 expression, thus being more prone to circulation and tissue residence (Sallusto et al. 1999). In addition, T_{CM} express CD62L, another marker facilitating migration to secondary lymphoid organs (Tedder et al. 1990). Due to the complexity of CD4⁺ T cell development and differentiation into many distinct phenotypes, knowledge on the specifics of CD4⁺ T_{CM} cells is limited, but they are known to produce IL-2 upon stimulation and they exhibit a low cell turn-over (MacLeod et al. 2008). Expression of low levels of the transcription factor Bcl-6 in CCR7⁺ cells has been shown in mice after infection with *L. monocytogenes* (Pepper et al. 2011), but without concurrent PDx1 expression, thereby distinguishing T_{CM} cells from T_{FH} cells (King et al. 2008). In contrast, T_{EM} show closer resemblance to effector T cells, in that they display aggressive activation and proliferation upon stimulation with recall antigen. Furthermore, the T_{EM} cells retain the phenotype they acquired upon induction. Both *in vitro* and *in vivo* T_{EM} differentiated under $T_H1/T_H2/T_H17$ inducing conditions show a propensity to produce cytokines associated with their respective induction milieu within hours of recall antigen reactivation (Harrington et al. 2008, Lohning et al. 2008, Zielinski et al. 2012). Both T_{EM} and T_{CM} cells express the IL-7R constitutively, and IL-7 signalling have been implicated in the long-term survival of T_M cells (Seder et al. 2003). Although T_M cells can survive and proliferate under IL-7 and TCR stimulation (Seddon et al. 2003), an additive, albeit smaller, effect has been shown for IL-15 (Purton et al. 2007).

1.2.2 Regulatory T cells

In prevention of autoimmune disease, in addition of thymic central tolerance, peripheral tolerance is of key importance. One of the main constituents of peripheral tolerance is the suppression of immune responses mediated by T_{REG}. In a seminal paper by Sakaguchi and colleagues, CD4⁺CD25⁺ T cells were found to be instrumental in prevention of autoimmune disease (Sakaguchi et al. 1995). These cells were characterised by high expression of CD25 (the α -chain of the IL2R). CD4⁺CD25⁺ cells were able to prevent development of autoimmune disease in thymectomised mice after transfer of either thymocytes and splenocytes or CD4⁺CD25⁺ cells from non-thymectomised mice. Unlike in mice, a human T cell expressing CD25 may be either a T_{REG} or an activated effector T cell. T_{REG} in human were later identified as being CD4⁺CD25^{high} (Baecher-Allan et al. 2001, Baecher-Allan et al. 2006), a discovery hindered by the role of CD25 in human as a marker of activation of conventional CD4⁺ T cells (Malek 2008).

The origin of human T_{REG} is still a subject of debate. Initially, it was thought that all T_{REG} stemmed from thymic precursors, a derivation from the initial studies in mice (Sakaguchi et al. 1995). These CD4⁺CD25^{high} cells, called ‘natural’ T_{REG} (nT_{REG}), differed from the previously known T_R1 and T_H3 cells. Later it was shown that the T_{REG} phenotype could be derived *in vitro* and *in vivo* in mice, giving rise to the term ‘induced’ or ‘adaptive’ T_{REG} (iT_{REG}). The possible differences between the functionality of nT_{REG} and iT_{REG} are still a subject for debate in the field. It has been shown that peripheral iT_{REG} can be generated through the TCR activation in the presence of TGF- β and although this differentiation remains stable in mice (DiPaolo et al. 2007), iT_{REG} induced by TGF- β in human only display transient suppressive characteristics and limited lineage stability (Tran et al. 2007). Expression of the transcription factor Helios was proposed as a marker to differentiate between human nT_{REG} and iT_{REG} (Thornton et al. 2010) but this was later rejected since Helios⁺ and Helios⁻ cells were identified both in the thymically and peripherally derived T_{REG} compartments (Gottschalk et al. 2012, Himmel et al. 2013). The issue of peripheral generation of functionally competent and long-lived human T_{REG} is thus so far unsolved.

Both the previously described T_H3 and T_R1 cells could be classified as adaptive T_{REG}. T_H3 cells are characterised by secretion of TGF- β (Weiner 2001) while T_R1 cells secrete both IL-10 and TGF- β (Roncarolo et al. 2006). TGF- β has profound immunomodulatory effects,

including controlling the activation and survival of T cells, B cells, NK cells, macrophages and granulocytes (Li et al. 2006).

1.2.2.1 FOXP3 and transcriptional control

The lineage-specific transcription governing T_{REG} differentiation and maintenance of the phenotype is called forkhead box P3 (FOXP3). Numerous studies in mice have demonstrated the importance of FOXP3 in T_{REG} function. Retroviral transduction of FOXP3 to CD4⁺CD25⁻ cells induces differentiation into a T_{REG}-like phenotype, with the ability to suppress conventional CD4⁺CD25⁻ cells, as well as being hypoproliferative (Fontenot et al. 2003, Hori et al. 2003). Deletion of FOXP3⁺ cells results in aggressive autoimmune disease (Lahl et al. 2007). In humans, the importance of FOXP3 in immune regulation is highlighted by studies on familial cases of FOXP3 deletion. In case of loss-of-function mutations, an autoimmune syndrome called immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX), resembling the pathology of FOXP3^{-/-} mice, emerges. Any FOXP3 mutation resulting in IPEX presents as an aggressive multi-organ autoimmune disease in males, present at birth, leading to death before two years of age if untreated (Bennett et al. 2001, Wildin et al. 2001, van der Vliet et al. 2007).

FOXP3 is acting both as a transcriptional repressor and activator (Lopes et al. 2006, Zheng et al. 2007). Several of the phenotypical characteristics of T_{REG} cells have been found to be under direct transcriptional control of FOXP3, including expression of CTLA-4, The glucocorticoid-induced TNFR-related protein (GITR), CD25 and CD73 (Zheng et al. 2007). In addition, FOXP3 act as a transcriptional repressor for numerous markers of conventional T cells, including IL-2, IFN- γ , tumor necrosis factor (TNF) and IL-4 (Chen et al. 2006, Gavin et al. 2007). In a recent analysis, more than 350 proteins interacting with FOXP3 have been identified; directly, indirectly or as part of multi-protein complexes (Rudra et al. 2012). Although the FOXP3 interactome is far from being fully understood, a few important observations have been made. The FOXP3 protein consists of four regions; a repressor domain, a zinc finger domain, a leucine zipper domain and the forkhead DNA-binding domain. Histone deacetylase (HDAC) 7 and HDAC9, together with the histone acetyltransferase KAT5 was shown to be bound to the repressor domain of FOXP3 (Li et al. 2007, Li et al. 2007). Later, Eos, a member of the Ikaros transcription factor family, was

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found to bind the same domain (Pan et al. 2009). These data suggest that a chromatin remodelling complex assembled of Eos, HDAC7 or HDAC9 and KAT5 mediates FOXP3 gene silencing. In addition, sequences of the repressor domain have been shown to interact with, and inhibiting the activity of, ROR γ t, thereby promoting commitment of naïve CD4⁺ T cells towards the T_{REG} lineage in place of T_H17 differentiation (Du et al. 2008, Zhou et al. 2008). In IPEX, mutations in the leucine zipper region was identified as preventing homodimerisation of FOXP3, thereby inhibiting its function (Lopes et al. 2006, Li et al. 2007). *Il2* gene silencing in T_{REG}, discovered early as being mediated directly by FOXP3 was later shown to be mediated through interaction of the forkhead DNA-binding region with NFAT at the *Il2* promoter (Bettelli et al. 2005, Wu et al. 2006).

The regulation of FOXP3 itself is under transcriptional control of a number of transcription factors. These transcription factors bind three conserved non-coding sequences (CNS1-3) in the FOXP3 promoter, thereby directing FOXP3 transcription (Zheng et al. 2010). For instance, it has been shown that nuclear factor κ B (NF κ B) family member REL binding to CNS3 is required for transcription of FOXP3 (Ruan et al. 2009). This binding results in a permissive transcriptional state of the FOXP3 gene, allowing the binding of NFAT, cyclic AMP (cAMP)-responsive element binding (CREB) and SMADs to bind CNS1 and CNS2 (Tone et al. 2008). In addition, binding of runt-related transcription factor 1-core binding factor β (RUNX1-CBF β) complex to CNS2 have been shown to be crucial for the maintenance of stable FOXP3 expression in T_{REG} (Kitoh et al. 2009). Prerequisite binding of REL to CNS3, dependent on TCR signalling support the notion of activation-induced generation of T_{REG} in the periphery. In addition, CNS1 has also been implicated in the generation of peripheral T_{REG} generation (Samstein et al. 2012).

Importantly in the context of human T_{REG}, FOXP3 can be transiently expressed following TCR stimulation in conventional, non-T_{REG} cells (Allan et al. 2007, Tran et al. 2007, Wang et al. 2007, Miyao et al. 2012). However, these CD4⁺FOXP3⁺ cells typically do not exhibit other typical T_{REG} markers, and lack suppressive capacity. Studying the methylation pattern of conventional T cells with induced FOXP3 expression reveal a partially methylated FOXP3 locus, in contrast to FOXP3⁺ T_{REG} cells where complete demethylation is seen (Floess et al. 2007, Polansky et al. 2008, Ohkura et al. 2012). Another feature distinguishing human T_{REG} from their murine counterpart is the existence of different FOXP3 isoforms in human. Both FOXP3 lacking exon 2 and exon 7 exists; FOXP3 Δ Ex2 and FOXP3 Δ Ex7, respectively (Allan et al. 2005, Smith et al. 2006). There has been speculations about the role of FOXP3 Δ Ex2 in

relation to T_H17 cell differentiation (Zhou et al. 2008), although the relevance of this remains unclear.

1.2.2.2 The T_{REG} phenotype

The original definition of T_{REG} in mice and human as being CD4⁺CD25⁺ and CD4⁺CD25^{high}, respectively, is still a widely used way of characterisation using flow cytometry. Originally seen in mice, where CD25 expression alone is sufficient for T_{REG} characterisation, the T_{REG} population displays lower expression of CD4 (Fontenot et al. 2003). This has also been shown in human T_{REG}; functional T_{REG} display intermediate CD4 expression and thus are CDdim⁺CD25^{high} (Baecher-Allan et al. 2001, Baecher-Allan et al. 2006, Mjosberg et al. 2009).

One of the most specific characteristics of the T_{REG} phenotype is FOXP3 expression, which is also a pseudo-functional marker, as it has been shown that FOXP3 expression correlates with suppressive capacity (Venken et al. 2008, Miyara et al. 2009). Although transient expression of FOXP3 in non-T_{REG} occurs upon activation, a combination of FOXP3 and other markers of T_{REG} constitute an accurate definition for T_{REG} often used for flow cytometry, although the intra-nuclear localisation of FOXP3 renders it unsuitable for sorting viable cells. In mice, FOXP3 appears to be an even more specific marker of T_{REG}. More careful examination of the FOXP3⁺ T_{REG} has shown that this population may be further divided by CD45RA expression, a marker typically expressed on naïve T cells. Activated CD45RA⁻FOXP3^{high} T_{REG} rapidly suppress conventional T cell proliferation in the presence of stimuli, while resting CD45RA⁺FOXP3^{int} T_{REG} are of lower suppressive capacity and require a prolonged exposure to stimulation before suppression becomes evident (Miyara et al. 2009, Haseda et al. 2013). In a similar manner, HLA-DR, an activation marker of conventional T cells, may also be used to divide the T_{REG} population. HLA-DR⁺ T_{REG} rely on rapid contact dependent suppression, while HLA-DR⁻ T_{REG} act through expression of IL-10 and IL-4 in a slower fashion (Baecher-Allan et al. 2006).

CTLA-4 was one of the first markers of T_{REG} both in human and mouse. In addition to being a functional mediator of T_{REG} suppression, it is constitutively expressed in high levels on the cell surface of a large proportion of T_{REG} (Birebent et al. 2004) and under direct FOXP3 transcriptional control (Wu et al. 2006).

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Another functional surface marker T_{REG} is CD39. CD39, in conjunction with CD73, performs a two-step enzymatic cleavage of adenosine triphosphate (ATP) to adenosine. CD39, when used together with other markers, may be used to identify T_{REG} cells, with the defined population being highly suppressive (Borsellino et al. 2007, Deaglio et al. 2007).

CD127 (the IL-7 receptor α -chain) expression is under control of FOXP3-mediated transcriptional repression. The expression of CD127 is thus inversely correlated to FOXP3 expression, and T_{REG} may be, and frequently is, identified as being CD127^{low} (Liu et al. 2006). CD127 is also negatively correlated with suppressive function, opposite to FOXP3 (Venken et al. 2008).

Similarly to the expression of CD45RA and CD45R0 by naïve/resting and activated/memory T_{REG}, CD62L may be used to divide the T_{REG} population (Wing et al. 2002). CD62L, being a homing receptor for lymph node, is expressed on naïve CD4⁺ T cells, including T_{REG}. Upon activation, the CD62L expression diminishes (Tedder et al. 1990). GITR is expressed by T_{REG}, although the functional role of this molecule in immune regulation has been debated (McHugh et al. 2002). Initial observations showed that suppressed proliferation of effector T cells in T_{REG} co-cultures was reversed by GITR agonism, thus demonstrating a potentially anti-regulatory role of GITR signalling (Shimizu et al. 2002). It was later shown that in addition to this augmentation of conventional T cell responses, the same signal in T_{REG} caused an increase in suppressive responses (Stephens et al. 2004). The net result of this concomitant stimulation of both T_{REG} and conventional T cells has not been established.

1.2.2.3 Mechanism of suppression

The original observations of T_{REG} in both mice and human were of a population of CD4⁺ T cells expressing CD25 constitutively. CD25, as being a part of the heterodimeric IL-2 receptor, was naturally investigated for mechanistic potential in the suppression of conventional T cells. IL-2 is critical for maintenance and activation of effector T cells, and it was hypothesised that the high CD25 expression on T_{REG} would act as an IL-2 sink, consuming available IL-2 and thereby inhibiting activation and proliferation of effector T cells. This hypothesis was initially confirmed when it was demonstrated that addition of excess IL-2 in co-cultures restored proliferation. More recently, however, responder T cell IL-2 mRNA and proliferation was shown to be inhibited by T_{REG}, even in presence of a

surplus of IL-2 (Tran et al. 2009). Thus, the hypothesis of IL-2 deprivation as a mechanism of suppression appears to be rejected.

Murine T_{REG} are able to confer suppression by means of IL-10 secretion. In a conditional knock-out mouse, IL-10 was found to be redundant in systemic immune regulation, but crucial for suppression in colon and lungs (Rubtsov et al. 2008). In human, both ability and inability of IL-10 secretion has been reported (Levings et al. 2002, Birebent et al. 2004). Both membrane bound, inactive, and soluble TGF- β appears to be another non-redundant mechanism of suppression in human (Levings et al. 2002, Nakamura et al. 2004).

IL-35, a cytokine constituted by the IL-12 family proteins p35 and Epstein-Barr virus induced gene 3 (EBI3), was first described in 1997 (Devergne et al. 1997). Later, it was shown that IL-35 expression by T_{REG} in mice was an important mediator of suppression (Collison et al. 2007). Both *Il12*^{-/-} and *Ebi3*^{-/-} have demonstrated the importance of IL-35 in suppression in mice and also the ability of recombinant IL-35 to suppress immune responses (Olson et al. 2013). In addition, treatments of human conventional, FOXP3⁻ T cells with IL-35 cause these cells to start producing IL-35, demonstrating so called 'infectious tolerance' (Chaturvedi et al. 2011). This claim was later retracted due to miscalculations related to the anti-proliferative capacities of IL-35 (Chaturvedi et al. 2013). In conclusion, the importance of IL-35 is firmly established whereas its role in human tolerance is still debated.

CD39 and CD73, expressed in subsets of murine T_{REG}, are surface-bound ectonucleases catalysing a two-step conversion of ATP into adenosine which upon ligation to the A2a adenosine receptor conveys anti-inflammatory effects (Borsellino et al. 2007, Deaglio et al. 2007). Studies on human T_{REG}, however, revealed expression only of CD39, and only worked in conjunction with CD73 expressed on effector T cells, thereby the requiring presence of both conventional T cell and T_{REG} for successful inhibition (Doherty et al. 2012).

Apart from soluble factors secreted from or induced by T_{REG} mediating suppression, a number of mechanisms exert immune regulation in a contact-dependent manner. T_{REG} can induce apoptosis by at least two pathways. In the suppression of human effector CD8⁺ cytotoxic T cells, FOXP3⁺CD25^{high} T_{REG} induced apoptosis of the responder cells through the Fas/FasL pathway (Strauss et al. 2009). Similarly, a CD4⁺ T cells identified as T_{REG} were shown to express Granzyme B, with the capacity to induce apoptosis in target cells in a perforin-dependent manner (Grossman et al. 2004).

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CTLA-4 is a surface-bound molecule that in addition to its constitutive expression on T_{REG} also can be expressed by conventional T cells upon activation (Perkins et al. 1996). It is a CD28 homologue, binding CD80/CD86 on the surface of APC during antigen presentation, thus representing signal 2. In contrast to CD28 ligation, engagement of CTLA-4 with CD80/CD86 results in inhibitory, immunomodulatory signalling (Linsley et al. 1994) in both the receiving APC and the T cell. Studies in CTLA-4^{-/-} mice highlight its importance in immune regulation; these mice rapidly develop lymphoproliferative disease with multi-organ lymphocytic infiltration and tissue damage (Tivol et al. 1995, Waterhouse et al. 1995). In CTLA-4^{-/-} mice, the lack of CTLA-4 expression by activated conventional T cells may be of importance, other studies have shown that a similar pathology can be accomplished by selectively abrogating CTLA-4 expression in T_{REG} (Wing et al. 2008, Jain et al. 2010). On the APC side, CTLA-4 ligation results in the induction of indolamine-2, 3-dioxygenase (IDO), and enzyme that cleaves tryptophan to kynurenine, which have direct immunosuppressive effects (Munn et al. 2004).

1.2.3 B and NK cells

B cells are instrumental in the adaptive immune response. Originating in the bone marrow, immature B cells migrate to secondary lymphoid organs where they may become Ig-producing plasma cells. In lymph nodes, initial activation is dependent of specificity of unrefined membrane-bound IgM towards presented antigens. B cell activation is enhanced by activated T cells, expressing CD40L binding to CD40 on the naïve B cell. After recognition most B cells undergo maturation and clonal expansion in the B cell zone of the lymphoid tissue, a process dependent on interaction with follicular DC and T_{FH} cells. During this maturation process, Ig isotype switching and somatic hypermutation takes place. The final isotype of the produced antibody is dependent on the cytokine milieu during maturation. T_H1-associated cytokines result in the production of IgG₁ and IgG₃ antibodies, while T_H2-cytokines lead to an IgE isotype switch. T_H17 responses, associated with IL-21 production, leads to class switching to IgG and IgA isotypes (LeBien et al. 2008, Pistoia et al. 2009, Pieper et al. 2013). Somatic hypermutation is a process where the affinity of the Ig is increased by random alterations in the antigen-binding, light chain peptide sequence. After a resolved immune response, long-lasting immunity is upheld by long-lived CD27⁺ memory B cells, able to rapidly respond upon antigen re-exposure. The most direct form of B cell

immunity is mediated through opsonisation of pathogens with subsequent phagocytosis. Opsonisation is also an important route for activation of complement. CD19 is a marker for most types of B cells, while CD20 recognises mature B cells (LeBien et al. 2008, Pieper et al. 2013). Regulatory B cells, or B_{REG}, recognised by CD25 expression, are a population for which interest has grown recently. They have the ability to inhibit both B and T cell mediated immunity and are characterised by secretion of IL-10 (Kessel et al. 2012).

Natural killer cells, or NK cells, constitute a bridge between innate and adaptive immunity. Although mainly known for their cytotoxic functions, especially in virus infection and immunosurveillance of malignancy, regulatory NK cells have the ability to shape adaptive immunity. Classically, NK cells are defined as part of innate immunity, based on the lack of receptors bearing antigen-specificity (Vivier et al. 2011). All NK cells are CD3⁻, distinguishing them from T cells. Based on surface expression of CD56, NK cells can be divided into cytotoxic and regulatory populations, where the cytotoxic population is recognised as CD56^{dim} while regulatory NK cells are CD56^{bright} (Poli et al. 2009). These populations display different migratory patterns; cytotoxic NK cells are homing to sites of inflammation whereas CD56^{bright} preferentially locate to lymphoid organs where they exert their regulatory functions (Campbell et al. 2001). NK cells are also great cytokine producers, among which IFN- γ are the most prominent, but IL-10 and GM-CSF are also secreted upon stimulation (Fehniger et al. 1999).

1.3 MS immunology

1.3.1 Overview of MS pathophysiology

Much of what today is known about the initiation, development and sustenance of MS is based on studies of an animal model resembling MS; experimental autoimmune encephalomyelitis (EAE). EAE develops in mice after injection of myelin-derived peptides in complete Freund's adjuvant. Depending on the strain of the animal different peptides may be used. Commonly, peptide sequences from proteolipid protein (PLP), myelin basic protein (MBP) or myelin oligodendrocyte glycoprotein (MOG) are used. Depending on the strain of rodent and the peptide used, chronic, acute and relapsing-remitting variants of EAE can be achieved (Stromnes et al. 2006).

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EAE is primarily mediated by CD4⁺ T cells. Naïve T cells are primed in the periphery and migrate to the CNS where a second round of activation occurs via MHC class II expressed on tissue-resident and migrated APC. This leads to breakdown of the blood-brain barrier (BBB), permitting entrance of immune cells, and demyelination and axonal damage ensues (Fletcher et al. 2010). In adoptive transfer experiments, transfer of CD4⁺ T cells from a mouse with EAE to a healthy mouse leads to disease development, highlighting the importance of CD4⁺ T cells (Stromnes et al. 2006).

In human, the seclusion and unassailable nature of the CNS have prevented studies of the organ-specific immune response, other than post-mortem. The central role of CD4⁺ T cells in human disease is supported by genetic studies showing that a large portion of disease genes are associated with CD4⁺ T cell function and homeostasis (Dyment et al. 2004, Lincoln et al. 2005, IMSCG 2007, Lundmark et al. 2007, Ramagopalan et al. 2007, IMSCG 2011). B cells are also implicated in MS. The presence of oligoclonal bands in CSF is indicative of intrathecal antibody production, and histopathological studies have shown prominent deposition of immunoglobulins and complement activation in active lesions (Lucchinetti et al. 2000, Breij et al. 2008), even though the cellular infiltrate mainly is composed of CD3⁺ T cells and macrophages (Lucchinetti et al. 2000).

Similar to EAE, MS is thought to be initiated peripherally (Fig. 3). Traditionally, antigen-presentation and clone expansion was thought to take place in classical secondary lymphoid organs including lymph nodes and spleen. More recently, both the gastrointestinal and the respiratory tract have been implicated as important in the priming of encephalitogenic, auto-reactive T cells in rodents (Berer et al. 2011, Odoardi et al. 2012). Migration of encephalitogenic cells into the CNS marks the second major event in MS pathogenesis. In normal circumstances, the BBB constitutes an obstacle for cell migration. The migration of primed, auto-reactive cells is dependent on the interaction between integrins on the surface of activated lymphocytes with adhesion molecules expressed on BBB endothelial cells (Engelhardt et al. 2012). An alternate route of entry of the choroid plexus by auto-reactive T_H17 cells has also been proposed, although the requirement for lymphocyte integrin expression persists (Steffen et al. 1996, Reboldi et al. 2009). Once inside the CNS, a second round of activation takes place; tissue resident microglia expressing MHC class II molecules has been implicated as mediators of re-activation signals (Carson et al. 1998). In addition, migrated peripheral cells including macrophages are also thought to play a role. After CD4⁺ T cell activation, a combined immune response involving activated microglia, macrophages,

CD8⁺ cytotoxic T cells, antibodies and activation of complement mediates tissue destruction. The demyelination, leading to conductive block of neuronal signalling, causing clinical symptoms, has been convincingly shown (Lucchinetti et al. 2000, Breij et al. 2008); a process involving damage to oligodendrocytes. Following the acute, demyelinating process, axonal loss and neuron destruction ensues (Trapp et al. 1998).

Figure 3. Hypothetical elements of neuroinflammation. Naïve CD4⁺ T cells (Tc) are activated peripherally (1.), developing into Th1 or Th17 cells. B cells (Bc) are activated. After traversal over the BBB, Tc most likely are activated again by MHC class II-expressing cells. Production of cytokines and chemokines drives the inflammation. CD8⁺ T cells, macrophages (MΦ) and microglia are thought to be important cellular components in the process of myelin and axon destruction, directly or indirectly through damage to oligodendrocytes (ODC). Complement and antibodies are likely to contribute as well.

1.3.2 The role of CD4⁺ T cells in MS/EAE

1.3.2.1 Th1 and Th17 cells

For a long time inflammation in EAE and MS was thought to be driven by Th1 cells. A major reason for this was the prevention of EAE seen in *Il12p40*^{-/-} mice, lacking IL-12, known to be important for Th1 differentiation. In contrast, *Ifng*^{-/-} developed aggressive disease with total penetrance (Segal et al. 1998). Early attempts of treating RR-MS with IFN-γ resulted in relapses and clinical deterioration (Panitch et al. 1987). Later it was shown that *Il12p35*^{-/-} mice were susceptible to EAE (Gran et al. 2002). IL-12 is composed of two subunits; p35 and p40. However, other combinations of IL-12 family proteins results in other heterodimeric

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cytokines. IL-23 for instance, important in T_H17 differentiation, is composed of IL-12p40 coupled with IL-23p19. *Il23p19*^{-/-} mice were rescued from EAE development, similar to *Il12p40*^{-/-} strains (Cua et al. 2003). It was also demonstrated that transfer of PLP-specific T cells differentiated under IL-23 could induce EAE while IL-12-differentiated cells could not. Administration of blocking anti-IL-17 antibodies was shown to abrogate disease while blocking of IFN- γ exacerbated EAE (Langrish et al. 2005). While these studies dismissed a role for T_H1-associated immunity altogether, more recent observations are less clear. In a transfer experiments with *in vitro* polarised, highly purified T_H1 and T_H17 clones, IFN- γ was crucial for disease induction; IFN- γ ⁺ T_H1 induced aggressive disease, while T_H17 cells either caused a mild EAE or failed to induce disease. It was found that T_H17 cells with EAE-inducing capacity were IFN- γ ⁺ as well as IL-17⁺ (O'Connor et al. 2008). In another study on EAE induced by adoptive transfer by encephalitogenic T_H1 clones devoid of IL-17, CD4⁺ T cells producing IL-17 was found in the CNS of affected animals, originating from the host (Lees et al. 2008).

In MS, microarray studies of brain lesions have identified overexpression of both IFN- γ and IL-17 (Lock et al. 2002, Mycko et al. 2003). IL-17⁺ cells, including CD4⁺ and CD8⁺ T cells and glial cells, have been identified in active MS lesions (Tzartos et al. 2008). In an elegant experiment using human BBB endothelial cells and a transwell system, both IL-17⁺ and IFN- γ ⁺ CD4⁺ T cells displayed capacity for transmigration. IL-17⁺IFN- γ ⁺ cells differentiated under IL-23 showed increased migratory potential compared to single-positive cells (Kebir et al. 2009). In peripheral blood, there was an increase of circulating CD4⁺IL-17⁺ cells in active MS compared to healthy subjects and MS in remission. Also, the amount of double-positive IL-17⁺IFN- γ ⁺ cells were greatly increased in both active MS and MS in remission compared to controls (Durelli et al. 2009).

In conclusion, experiments from rodents reveal the importance of autoreactive CD4⁺ T cell clones, be it T_H1 or T_H17 cells. The outcome of immunisation is probably influenced not only on the peptide sequence, but also on the strain used for EAE induction. In MS, the situation is equally complex, and the role of IFN- γ and IL-17 in pathogenesis of disease needs to be further clarified.

1.3.2.2 T_{REG}

Experiments in EAE showcase the ability of T_{REG} to modulate immune responses *in vivo*. In an early hallmark study, transfer of extracted $CD4^+CD25^+$ T_{REG} cells was shown to limit EAE severity in a MOG₃₅₋₅₅ induced disease in C57BL/6 mice, a classical model system. T_{REG} cells were also shown to inhibit proliferation and IFN- γ production by MOG₃₅₋₅₅-specific effector T cells *in vitro* (Kohm et al. 2002). The prevention of disease by $CD4^+CD25^+$ cells appeared to be mediated by IL-10, as shown in a similar system with *Il10*^{-/-} mice. In addition, treatment of animals with an anti-CD25 antibody prior to induction aggravated disease (Zhang et al. 2004). TGF- β was also implicated in FOXP3⁻ T_{REG} mediated EAE abrogation as it was shown that parasite-associated bystander activation of T_{REG} producing TGF- β , but not IL-10, could prevent MOG-induced EAE (Walsh et al. 2009). In a self-limiting EAE model, clinical recovery correlated with the proportion of T_{REG} within the CNS. The T_{REG} , also competent suppressors *in vitro*, were characterised as FOXP3⁺CTLA-4⁺IL-10⁺ (McGeachy et al. 2005).

In MS, the influence of a possible T_{REG} defect has been discussed. The number of T_{REG} appears to be unaltered in peripheral blood (Putheti et al. 2004, Huan et al. 2005) although decreased FOXP3 expression has been observed (Huan et al. 2005). However, increased numbers of $CD4^+CD25^+$ T_{REG} were observed in CSF of patients, although the definition of T_{REG} as $CD25^+$ rather than $CD25^{high}$ leaves a possibility for contamination of activated effector T cells (Feger et al. 2007). Even though the frequency of T_{REG} in MS is unchanged, a lowered capacity of suppression has been observed in several studies (Viglietta et al. 2004, Haas et al. 2005, Kumar et al. 2006, Venken et al. 2008), although the use of a $CD4^+CD25^+$ definition may account for some of the results (Viglietta et al. 2004). Although studied extensively, the nature of the suppressive dysfunction has not yet been fully explained and should be further investigated.

1.3.3 The role of B and NK cells in MS/EAE

In early transfer experiments for EAE, serum transfer alone was insufficient to induce disease, thereby proving that antibodies were redundant for induction. However, B cells and antibody production have been shown to be important in the pathogenesis of disease (Lington et al. 1988, Lyons et al. 1999). Studies of B_{REG} cells in EAE have revealed protective functions of this population. Depletion of IL-10-producing B_{REG} aggravates disease, as does the transfer of

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IL-10⁻ B cells. On the other hand, transfer of IL-10-producing B cells to affected animals could control disease progression (Fillatreau et al. 2002, Matsushita et al. 2008). In RR-MS, B_{REG} have not been extensively studied, but it has been shown that IL-10 production of B cells is reduced during relapse (Knippenberg et al. 2011). Interestingly, a recent study on RR-MS patients demonstrated the presence of CD19⁺CD25⁺FOXP3⁺ B cells in blood and CSF, displaying suppressive capacity (de Andres et al. 2014), suggesting that FOXP3 signifies suppressor function in B cells.

NK cells have also been studied in EAE, although not as extensively as B and T cells. Interestingly, the role of NK cells in rodents appears to be protective rather than deleterious, presumably through regulation of T cell responses. In a PLP-induced mouse model, depletion of NK cells led to increased disease activity, and it was speculated that NK cells were involved in the control of autoreactive T cell clones (Xu et al. 2005). Another group showed that by preventing NK cell entry into the CNS by selective chemokine receptor knockout, aggressive EAE developed, indicating *in situ* regulation of immune responses (Huang et al. 2006). Similarly to the EAE situation, NK cells are suspected to contribute to immune regulation and suppression in MS. Of relevance for this thesis, CD56^{bright} regulatory NK cells were increased after immunomodulatory treatment. In two studies of effects of IFN- β , the proportion of CD56^{bright} NK cells increased after treatment, concurrent with clinical improvement (Saraste et al. 2007, Vandenbark et al. 2009) while a decrease was seen in the CD56^{dim} cytotoxic population (Saraste et al. 2007). Additionally, in a study of the humanised anti-CD25 antibody daclizumab, expansion of CD56^{bright} NK cells was observed (Bielekova et al. 2006). Although only providing indirect evidence, these studies support the protective role of regulatory NK cells.

1.3.3 High-throughput analysis of MS

The advent of high-throughput techniques has led to the possibility of studying global changes in MS. Microarray permits the measurement of a high number of mRNA transcripts from the selected tissue, which in the case of MS often consist of different lymphocyte populations in peripheral blood. In a systematic review, the combined data from six previously conducted gene expression studies was included. Pathway analysis identified signalling pathways for glucocorticoids, IL-6, IL-17, IL-2 and IL-15 as enriched for differentially expressed (DE)

genes from $\geq$ two independent studies (Kempainen et al. 2011). This said, another main finding of the study was the poor overlap of DE genes of only 6%; a great majority of DE genes were only identified in a single study. More recently, a similar approach was used to identify genes and pathways using data collected from experiments of CNS tissue. In lesion sections from MS patients, pathways involving axonogenesis and protein catabolic processes were enriched for DE genes. Gene-set enrichment analysis revealed enrichment of immune related pathways (Raddatz et al. 2014), in accordance with earlier studies (Lock et al. 2002, Mycko et al. 2003). Investigation of peripheral blood mononuclear cells (PBMC) revealed 380 DE genes; principal component analysis could clearly distinguish MS patients (RR-, SP- and PP-) from healthy subjects. Pathway analysis of DE genes showed enrichment for “immunological disorder” and “inflammatory disease” (Ratzer et al. 2013). In another investigation of PBMC in relapse and remission, 530 DE transcripts were found. T cell and non-T cell associated genes were inferred by assessment of the contribution of these populations to the total RNA content used for microarray. Gene ontology revealed immune pathways as overrepresented (Lindsey et al. 2011). A study of CD4⁺ T cell transcription profiles of CIS patients, employing machine learning models was successful in prediction of conversion to overt RR-MS. Interestingly, down-regulation of pathways involving immune responses were observed, suggested to represent a physiological immune response challenged with autoimmune events (Corvol et al. 2008).

In summary, global gene expression arrays for investigation of MS immunity constitute a valuable tool for a deeper understanding of pathogenesis of disease. Although there is a profound disparity in the reported DE genes, possibly based on the heterogeneity of individuals, tools like pathway analysis, GSEA and network-based analysis are valuable in the face of overwhelming data. In addition, novel mathematical concepts, at least for biologists, in the field of machine learning are currently being employed in transcriptomics.

1.4 Immunomodulatory treatment in MS

1.4.1 Natalizumab

Introduced on the market in 2006, natalizumab is a biological drug designed specifically for the treatment of MS. It is a humanised monoclonal antibody directed against CD49d (α_4 -integrin) which is parts of the heterodimers VLA-4 ($\alpha_4\beta_1$) and $\alpha_4\beta_7$, important for

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extravasation of leukocytes. The antibody is of the IgG₄ subclass, and administered to patients intravenously once every four weeks. Competitive binding of the natalizumab to the α_4 -integrin on the leukocyte surface prevents binding to VCAM-1 and MAdCAM-1, thereby inhibiting adhesion and subsequent diapedesis. VCAM-1 is expressed by endothelium throughout the circulation while MAdCAM-1 is preferentially expressed on endothelium in the gastrointestinal tract. Prevention of leukocyte entry into the CNS by blockade of VLA-4-VCAM-1 ligation is the hypothesised mode of action of natalizumab in the context of MS, although additional mechanisms may play a role.

In the early 1990's, the anti-migratory effects of VLA-4 blockade was demonstrated in mice, where an increase of blood lymphocytes was also observed (Issekutz 1991). Later it was shown that administration of an anti- α_4 monoclonal antibody could prevent disease onset in an adoptive transfer EAE model, in addition to preventing lymphocyte migration in rats (Yednock et al. 1992). Another EAE model employing guinea pigs showed that anti- α_4 antibodies not only could prevent disease onset for longer periods of time given sufficient plasma concentrations, but also reverse acute disease (Kent et al. 1995). In a phase II study in RR-MS, natalizumab was proven to be effective in reducing the number of relapses compared to placebo (Miller et al. 2003). In 2006, results from a study comprising 942 patients, natalizumab showed an annualised relapse rate reduction of 68% (Polman et al. 2006), thereby eclipsing the first line treatments of IFN- β and glatiramer acetate (GA). In addition, a reduction of lesion load on MRI was observed.

As mentioned, the beneficial effects of natalizumab are mainly attributable to the prevention of lymphocyte migration into the CNS. In MS, this notion has been proven through several lines of evidence. Following natalizumab treatment, there is a marked decline in the number of leukocytes intrathecally, both when measured in CSF (Stuve et al. 2006, Khademi et al. 2009) and in perivascular spaces post-mortem (del Pilar Martin et al. 2008). Interestingly, anti-VLA-4 antibodies may confer additional effects apart from the limitation of lymphocyte extravasation. VLA-4 is important in the formation of the immunological synapse, formed between lymphocyte and APC during antigen presentation (Mittelbrunn et al. 2004), providing co-stimulatory signals to T cells (Sato et al. 1995). Studies in rodents have also revealed that administration of VLA-4 antibodies both *in vitro* and *in vivo* may affect the activation and differentiation of CD4⁺ T cells (Sato et al. 1995, Coito et al. 2000). Changes in gene expression profiles noted in peripheral leukocytes *ex vivo* (Lindberg et al. 2008) also exemplifies a feature of VLA-4 blockade not attributable to adhesion to endothelial cells.

One major drawback of natalizumab treatment is the risk of activation of JC virus, resulting in progressive multifocal leukoencephalopathy (PML), an aggressive neurological disease with high lethality, seen in patients treated with immunosuppressive drugs (Tan et al. 2010). However, since the observations that the risk of PML development is correlated to the presence of anti-JC antibodies (Bloomgren et al. 2012), this risk can now be minimised.

1.4.2 IFN- β and Glatirameracetate

IFN- β and GA constitutes first line treatments in RR-MS, both reducing the annualised relapse rate with approximately 30% (Martinelli Boneschi et al. 2003, Rudick et al. 2007). GA, constituted of a random mix of myelin-protein derived peptide sequences was thought to induce tolerance of immune cells via so called bystander suppression. Similarly, T_{REG} were speculated to be induced as a result of the same process or by itself. For instance, increased FOXP3 expression as a result of GA exposure has been demonstrated. In addition, *in vitro* studies demonstrated the capacity to skew differentiation of naïve CD4⁺ T cells towards a protective T_H2 phenotype (Racke et al. 2010). Similarly to GA, the effects of IFN- β are largely unknown. It has been shown that treatment leads to decreased antigen presentation and subsequent activation of T cells by down-regulation of MHC class II on APC and interference with CD80/CD86 and CD28, respectively. Furthermore, IFN- β decreases the permeability of the BBB through down regulating matrix metallo-proteinases, important in BBB breakdown (Dhib-Jalbut et al. 2010).

2. AIMS

The general aim of this thesis is to increase our understanding of immune mechanisms in RR-MS by investigating lymphocytes, in particular CD4⁺ T cells and T_{REG}. Two factors affecting CD4⁺ T cell function are investigated; T_{REG} and natalizumab.

The specific aims of each paper were:

- I To explore the balance of circulating CD4⁺ T cell subsets in RR-MS using T_H-associated transcription factors in whole blood.

- II To assess the function and phenotype of T_{REG} in RR-MS. To achieve this, T_{REG} were analysed for phenotypic and functional characteristics using flow cytometry and global gene expression was analysed by microarray analysis.

- III To increase our understanding of immunological mechanisms of natalizumab treatment in RR-MS by evaluating changes in peripheral lymphocyte population and functional responses of circulating T cells after one year of treatment.

- IV To investigate if multiple diseases, including MS, involve a module of pleiotropic genes and if these genes may be potential candidates as therapeutic targets or biomarkers. An additional aim was to evaluate if natalizumab therapy responses in RR-MS could be predicted on the basis of expression profiling of CD4⁺ T cells.

3. MATERIAL AND METHODS

3.1 Study subjects

In Paper I, patients were included at the Department of Neurology at Linköping University Hospital and Karolinska University Hospital, Stockholm. All MS patients included in Paper II-IV were included at the Department of Neurology at Linköping University Hospital. Inclusion criteria for patients were a confirmed diagnosis of RR-MS, according to the McDonald criteria (McDonald et al. 2001). Exclusion criteria for all subjects were presence of infection or inflammatory conditions other than MS at the time of inclusion. For controls, immunomodulatory treatment led to exclusion. In total, 81 patients were included, with 23 patients participating in more than one paper, as shown in Table I. In Paper IV patients with seasonal allergic rhinitis (SAR) were included at the Department of Pediatrics at Queen Silvia Children Hospital, Gothenburg. SAR was defined as a positive seasonal history and a positive skin prick test or a positive ImmunoCap Rapid (Phadia; Thermo Fisher Scientific, Allerød, Denmark) to birch and/or grass pollen.

3.1.1 Paper I

33 patients (18 male, 15 female) with RR-MS were recruited. Median age at inclusion was 35 years (range 26-71 years) and median EDSS was 2.0 (range 0.0-7.0). 12 patients (36%) had been treated with IFN- β and nine patients (27%) had experienced a relapse within the three last months prior to inclusion. 20 healthy controls were included (median age 46 years, range 21-62, 13 female and 7 male). Controls were recruited among the staff at the Blood Center at Linköping University Hospital. All patients and controls complied with the inclusion/exclusion criteria stated above.

3.1.2 Paper II

27 patients (nine male and 18 female; median age 40, range 26-57 years) with RR-MS were included. At the time of sampling the median EDSS was 2.0 (range 0.0-5.5) and median MSSS was 2.0 (range 0.2-6.9). 13 patients (48%) were on immunomodulatory treatment; 11 on IFN- β , one on GA and one on intravenous immunoglobulin (IVIg). As a control group, 29 healthy subjects were recruited at Linköping University and Linköping University Hospital

(median age 31, range 25-63 years, 22 female and 7 male). All patients and controls fulfilled the inclusion/exclusion criteria.

3.1.3 Paper III

40 patients with RR-MS (22 male, 18 female; median age 40, range 22-62 years) were included before natalizumab treatment was initiated. New samples were collected after one year of treatment. Median EDSS at inclusion was 2.5 (range 0.0-7.0) and MSSS was 3.8 (range 0.2-8.6). A total of 30 patients had received immunomodulatory treatment within three months prior to inclusion (25 IFN- β , four GA, one IvIg) while 10 patients had not. As controls for the lymphocyte response assay, 23 healthy subjects were recruited at the Department of Clinical Immunology and Transfusion Medicine, median age 45 years (range 35-59, 21 female and two male). All patients and controls fulfilled inclusion and exclusion criteria.

3.1.4 Paper IV

16 patients with RR-MS were included into high-responder (HR, $n = 8$) and low-responder (LR, $n = 8$) groups. The patients were recruited among patients receiving natalizumab treatment, using frozen cells from the same cohort as in Paper III. LR and HR were identified after a three year follow-up period (except two patients followed for one and two years, respectively); patients with sub-adequate response to natalizumab (defined as presence of clinical relapse) were classified as LR. The annualised relapse-rate for LR was 0.65 ± 0.26 (mean and SD) and 0 for all in the HR group. The HR were selected as to be matched for age, sex and EDSS. All samples were obtained and frozen before initiation of treatment, thus representing the untreated state for both LR and HR. The LR and HR had comparable age distribution (LR mean 37.3 ± 5.8 (SD) and HR 33.9 ± 6.2 years), EDSS score (LR median 2.5, range 0.0-7.0, HR 2.0 range 1.0-5.5), disease duration (LR mean 9.1 ± 6.6 years, HR 9.9 ± 6.6 years) and sex distribution (seven male and one female for both LR and HR). In addition, 16 SAR patients were included in a similar manner; LR and HR were defined for glucocorticoid (GC) treatment (11 female, five male; mean age 35.6 ± 2.3 years).

Table I. Overview of RR-MS patients in Paper I-IV. $n = 81$.

Year of birth	Sex	I	II	III	IV	Year of birth	Sex	I	II	III	IV
1937	f	x				1970	f			x	
1945	m			x		1970	f			x	
1955	m		x			1971	m			x	x
1955	f	x				1971	m	x		x	x
1955	f		x			1972	f		x		
1957	f			x		1973	f			x	
1959	f		x			1973	m		x		
1959	f	x				1973	m	x		x	x
1959	m		x	x		1973	f	x			
1960	f			x		1973	f			x	
1961	f		x			1973	m	x			
1961	m			x	x	1974	f	x			
1962	f	x		x		1974	f	x			
1963	m			x		1974	m		x	x	x
1963	f		x			1975	f	x		x	x
1963	f			x		1975	m		x		
1963	f	x				1975	m	x		x	x
1963	m	x		x	x	1975	m	x		x	x
1964	f		x			1976	m			x	x
1964	m			x		1976	m	x		x	
1964	m	x		x	x	1976	m		x		
1965	f		x			1977	f		x		
1965	f	x				1978	f	x			
1965	f	x				1978	m	x			
1965	f			x		1978	m	x			
1965	m	x	x	x	x	1979	m			x	
1965	f			x		1979	f		x		
1966	m			x	x	1979	f	x			
1966	f	x	x			1980	f	x	x		
1967	m	x		x	x	1980	f			x	
1967	m	x				1981	m	x			
1967	f			x	x	1982	m	x			
1967	f	x				1982	f		x		
1967	f			x		1982	m		x		
1968	m	x		x		1982	m	x		x	x
1969	f		x			1982	m			x	
1970	f		x			1986	f		x		
1970	f			x		1986	f			x	
1970	f			x		1987	f		x	x	
1970	f		x								

x: marking participation if patient in Paper I-IV, respectively. m: male, f: female.

3.2 Brief summary of procedures used in Paper I-IV

Clinical scoring and characterisation of patients and controls were employed in Paper I-IV.

In Paper I, CD4⁺ T cell transcription factors were analysed in whole blood, using reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). Furthermore, T cells and classically defined T_{REG} were analysed with 3-color flow cytometry.

In Paper II, T_{REG} populations were investigated in depth. Fluorescence-activated cell sorting (FACS) was employed to isolate different populations of T_{REG}, preceded by isolation of PBMC and subsequent immunomagnetic separation of CD4⁺ T cells. T_{REG} were cultured and suppressive capacity of these cells was investigated using flow cytometry analysis of proliferation by carboxyfluorescein succinimidyl ester (CFSE) and activation by CD69 expression. In addition, phenotyping of T_{REG} was performed using 6-color flow cytometry. Microarray analysis was used to investigate potential differences between T_{REG} in patients and controls. Gene set enrichment analysis (GSEA) was performed to aid interpretation of results.

Paper III-IV focuses on the influence of natalizumab on lymphocytes. In Paper III, lymphocyte populations of patients were investigated using 6-color flow cytometry before and after one year of treatment. Furthermore, the lymphocyte responsiveness was assessed using a whole blood antigen-stimulation culturing assay.

In Paper IV, a systems biology approach was employed to identify pleiotropic genes in a number of diseases, including MS. GWAS data and publically accessed gene expression data was used, and using pathway and network analysis disease-specific and pleiotropic network modules were identified. In addition, microarray-based gene expression profiling was performed to assess adequate or suboptimal response to natalizumab treatment in MS patients and glucocorticoid treatment in patients with allergy.

3.3 Assessment of disease severity (Paper I-IV)

Evaluation of disease course and assessment of immunomodulatory treatment was made by use of medical records and the Swedish MS Registry. All included patients were clinically examined by a neurologist. EDSS score was used to assess disability and in paper I-III, MSSS was used as a measurement of disease severity. Additionally, in Paper III, SDMT and MSIS-

29 was also employed. The MSIS-29 questionnaire was distributed to patients at the time of natalizumab infusions. SDMT was carried out by a nurse, also at the initiation of treatment.

3.4 CSF parameters (Paper III)

Routine CSF analyses including counts of poly- and mononuclear cells, IgG index, albumin ratio and the occurrence of oligoclonal bands under isoelectric focusing were performed at the Department of Clinical Chemistry at Linköping University Hospital. The albumin ratio represents a rough estimation of BBB integrity (Eq. 1). IgG index represents a measurement of intrathecal antibody production, and accounts both for plasma IgG levels and BBB integrity (Eq. 2). In conjunction with presence of oligoclonal bands, it constitutes the CSF hallmarks of intrathecal inflammation.

$$albumin\ ratio = \frac{albumin_{CSF}(mg/L)}{albumin_S(g/L)} \quad Eq. 1$$

$$IgG\ index = \frac{IgG_{CSF}(mg/L)/IgG_S(g/L)}{albumin\ ratio} \quad Eq. 2$$

S: serum

3.5 Isolation of cells

3.5.1 PBMC (Paper II, IV)

Whole blood was collected in sodium-heparin vacutainer tubes. PBMC were isolated from the blood using Lymphoprep (AxisShield, Oslo, Norway) gradient centrifugation for 30 min at 400 x g at room temperature within 4 hours of collection. During centrifugation, diluted blood will be layered on top and erythrocytes, due to their high density, are pelleted at the bottom. Granulocytes will shrink due to high osmolarity of the solution and cluster with the erythrocytes. Lymphocytes and monocytes (PBMC) are trapped in the interphase between the Lymphoprep and the diluted blood. PBMC were collected and subsequently washed thrice in Hank's balanced salt solution (HBSS) for 10 min at 400 x g at 7°C. After washing, cells were

either resuspended in PBS supplemented with ethylenediaminetetraacetic acid (EDTA) for immunomagnetic sorting (Paper II) or in freezing medium consisting of 10% dimethyl sulfoxide, 50% fetal calf serum (FCS) and 40% Iscove's modified Dulbecco's medium (IMDM) for cryogenic preservation (Paper IV).

3.5.2 Immunomagnetic sorting (Paper II, IV)

CD4⁺ T cell Isolation kit II (Miltenyi Biotec, Lund, Sweden) was used to isolate CD4⁺ cells from PBMC. After Lymphoprep isolation of PBMC, cells were resuspended in PBS with 2 mM EDTA supplemented with 0.5% FCS. With this method, untouched CD4⁺ T cells are separated through labelling of non-CD4⁺ T cells including CD8⁺ T cells, monocytes, neutrophils, eosinophils, B cells, NK cells, dendritic cells, γ/δ T cells and erythroid cells. The labelling cocktail of biotin-conjugated antibodies include antibodies specific for CD8, CD14, CD15, CD16, CD19, CD36, CD56, CD123, TCR γ/δ and CD235a. Cells were labelled using this cocktail for 15 min at 7°C in the dark, followed by incubation with anti-biotin antibodies conjugated with magnetic microbeads. Using a magnetic column, labelled cells are retained and CD4⁺ T cells pass through. Typically, a purity of > 95% of CD4⁺ T cells is achieved, as measured with flow cytometry. After sorting, cells were activated in culture (paper IV) or labelled for flow cytometry cell sorting (Paper II).

3.5.3 Flow cytometry cell sorting (paper II)

FACS is used to obtain highly purified populations from cells in suspension. The main principles are those applying to flow cytometry protein expression analysis, explained below. Briefly, this technique permits analysis of individual cells passing lasers at an interrogation point in a stream in single file. In FACS the stream with the cells then pass through a nozzle (70 or 100 μ m in diameter) where energy with a specific frequency is applied causing the stream to be broken up into droplets. The distance between the end of the stream and the first droplet is monitored; the software uses this information to determine which droplet contains which cell, with corresponding recorded fluorescence signals measured at the interrogation point. Based on the scatter and/or fluorescence properties of a cell it may be sorted or rejected. Sorting of droplets occurs downstream by utilising deflection plates on both sides of

the droplet stream. Rejected, unsorted cells are not deflected and continue to the waste unit. Up to four separate populations may be sorted simultaneously.

In addition to selection of scatter/fluorescence properties (through gating, further explained below), the desired purity/yield of sorted populations may be specified. The sorting of cells, based on the sorting of droplets, is based on the probability of the droplet of interest containing the specified cell. In principal, a purity-based sort will tend to exclude cells with low probability of containing a cell while yield-based sorting will include the same. The yield of the final population will also be determined by the cell event rate (in turn determined by the flow rate); at higher event rates more droplets will be discarded in purity-based sorting. The principals of flow cytometry cell sorting are shown in Fig. 4.

Figure 4. Principals of FACS Aria sorting. 1. Flow cell. 2. Laser interrogation point. 3. Nozzle. 4. Gap and Drop 1 measurement. 5. Deflection plates. 6. Collecting tubes. 7. Waste.

3.5.3.1 Sorting of activated and resting T_{REG} (Paper II)

Cells were pre-sorted using PBMC gradient centrifugation and $CD4^+$ T cell immunomagnetic isolation to create more optimal sorting conditions (in addition to economic advantages). Before labelling, cells were resuspended in PBS / 2% FCS to a concentration of 10^7 cells/mL. Cells were labelled with anti- $CD4$ PE-Cy7, anti- $CD25$ APC and anti- $CD45RA$ Horizon V450 (all from BD Biosciences, San Jose, USA; Table II). Prior to sorting the cells were washed and resuspended in PBS / 2% FCS and stored on ice. The final concentration of cells for sorting was approximately in the range of $10\text{--}14 \times 10^7$ / mL, suitable for sorting with a $100\text{ }\mu\text{m}$ nozzle. A FACS Aria with the FACSDiva software was used for sorting. The bigger nozzle was chosen to minimize stress on cells since the $70\text{ }\mu\text{m}$ nozzle results in a higher stream pressure. Sorting was made for purity to avoid contamination of the sorted populations. Four cell populations were sorted; resting, activated and classically defined T_{REG} and responder T cells (T_{RESP}). First, lymphocytes were selected based on FSC/SSC characteristics. $CD4^+$ T cells were then defined using

expression of CD4. Purity of CD4⁺ T cells (after immunomagnetical negative selection) was typically > 95%. From here, a CD4/CD25 plot was used to define the T_{RESP} (as CD4⁺CD25^{low}) and classically defined T_{REG} (cT_{REG}; CD4^{dim}CD25^{high}) (Fig. 5a). Resting and activated T_{REG} were defined by CD45RA and CD25 expression; activated T_{REG} were CD4⁺CD25^{high}CD45RA⁻ (aT_{REG}) while resting T_{REG} were CD4⁺CD25^{int}CD45RA⁺ (rT_{REG}; Fig. 5b). The purity of sorted populations was analysed for all included subjects; a re-analysis of already sorted cells from each population was performed. Typically, the purities were >97% for T_{RESP}, >98% for classically defined T_{REG}, >95% for resting T_{REG} and >98% for activated T_{REG}. Cells were sorted into 1.0 mL of culture medium in polypropylene tubes pre-coated with FCS. After sorting, additional culture medium was added and cells were put on ice until culturing or mRNA extraction. Cells destined for microarray analysis were pelleted and resuspended in 100 μ L TRI Reagent (MRC, Cincinnati, USA) and frozen at -70°C until RNA extraction.

Figure 5. Defining gates of responder T cells (T_{RESP}; **a**), classically defined T_{REG} (cT_{REG}; **a**), activated T_{REG} (aT_{REG}; **b**) and resting T_{REG} (rT_{REG}; **b**).

3.6 CD4⁺ T cell cultures (Paper II and IV)

CD4⁺ T-cell cultures were set in Paper II and IV. In Paper II highly purified, FACSaria sorted cells in different combinations were used, while immunomagnetically sorted CD4⁺ T cells were used in Paper IV. In both settings, IMDM supplemented with 5% FCS (Sigma Aldrich, Stockholm, Sweden), L-glutamine 292 mg/mL (Sigma Aldrich), sodium bicarbonate

3.024 mg/mL (Sigma Aldrich), penicillin 50 IE/mL (Cambrex, New Jersey, USA), streptomycin 50 µg/mL (Cambrex) and 100x non-essential amino acids (Gibco BRL, Paisley, Scotland; UK) was used..

3.6.1 T_{RESP} : T_{REG} co-cultures (Paper II)

In Paper II, flat-bottomed 96-well polystyrene plates were used. The plates were pre-coated with anti-CD3 and anti-CD28 antibodies, mimicking TCR stimulation. Both antibodies were added in 0.5 µg/mL and incubated for 2 h at 37°C with subsequent washing with PBS. Two different culturing conditions were used to assess T_{REG} suppression; 18 h for rapid T cell activation assessed through CD69 expression and five days for CFSE proliferation assay. The capacity of T_{REG} to suppress these two events was evaluated through the co-culturing of T_{REG} together with T_{RESP} at different ratios. A successful suppression by T_{REG} would be revealed by a decreased expression of CD69 in the case of activation and a decreased proliferative fraction, as measured by CFSE dilution.

In both the activation and proliferation assay, two sets of controls were used. Unlabelled, unstimulated T_{RESP} were used to define background fluorescence cells. In addition, labelled, stimulated T_{RESP} were analysed for CD69 expression or CFSE dilution to establish non-suppressed activation and proliferation. In CFSE, an additional control was used; unstimulated T_{RESP} labelled with CFSE was used to set the peak corresponding to no proliferation in the CFSE histogram. In both suppression assays T_{RESP} were co-cultured with different populations of T_{REG} at different ratios. For the CD69 activation assay, 5.0×10^4 T_{RESP} were cultured together with 2.5×10^4 (2:1 ratio) or 1.25×10^4 (4:1 ratio) T_{REG}. Classically defined T_{REG}, resting T_{REG} and activated T_{REG} were used in co-cultures. For assessment of suppression of proliferation, 2.5×10^4 T_{RESP} were co-cultured with 1.25×10^4 (2:1 ratio) or 6.25×10^3 (4:1 ratio) T_{REG} cells. Here, activated and resting T_{REG} were used. Cells were incubated 18 h or five days at 37°C humidified air with 5% CO₂. To be able to distinguish T_{RESP} from T_{REG}, all T_{RESP} were labelled with CFSE after FACSaria sorting. Briefly, cells were resuspended in 1.0 mL PBS. CFSE was then added to a final concentration of 0.1 µM. Staining was quenched by addition and washing with 10 mL PBS / 5% FCS thrice. After culturing, CD69 expression or CFSE dilution were analysed by flow cytometry, as described below.

3.6.2 Activation of cells in presence of natalizumab or glucocorticoids (Paper IV)

For cultures in Paper IV, flat-bottomed 24-well polystyrene plates were used. CD4⁺ T cells typically with a purity >95%, were acquired through immunomagnetic selection. 1.0×10^6 cells were set per well. For each individual one unstimulated culture and two stimulated cultures were set. Stimulation was achieved by anti-CD3 and anti-CD28 antibodies at 0.1 µg/mL, pre-bound to the designated wells. In one of the stimulated cultures, anti-VLA4 (CD49d) antibodies (BiogenIdec, Cambridge, USA) were added, diluted in PBS to a final concentration in culture of 25 µg/mL. Cells were incubated 48 h in humidified air with 5% CO₂ at 37°C. After culturing, a small portion of the cells were analysed for CD4, CD49d and CD69 expression with flow cytometry (described below). The remaining cells were pelleted with subsequent addition of 350 µL TRIreagent (MRC) and frozen at -70°C for later extraction of mRNA. Samples from SAR patients were activated by addition of allergen extract (100 µg/mL; ALK-Abelló A/S Hørsholm, Denmark), with or without hydrocortisone (10^{-7} M; Sigma-Aldrich). In contrast to MS samples, fresh whole blood was used to isolate PBMC by using Ficoll-Hypaque. PBMC cultures were then incubated for seven days at 37°C and 5% CO₂. CD4⁺ T cells were selected after culturing using immunomagnetic selection (Miltenyi Biotec). RNA was extracted using the Agilent Absolutely RNA Microprep Kit (Agilent Technologies, Santa Clara, USA) in accordance to the supplied instructions. Samples were frozen at -70°C until microarray analysis.

3.7 Whole blood cultures (Paper III)

For assessment of whole blood lymphocyte responsiveness we employed a modified version of the previously described FASCIA method (Svahn et al. 2003). Peripheral blood was drawn into sodium-heparin vacutainer tubes. 50 µL whole blood was diluted 1:10 in culturing medium consisting of Roswell Park Memorial Institute (RPMI) 1640 (Gibco BRL) supplemented with L-glutamine 584 µg/mL (Sigma Aldrich), penicillin 200 IE/mL and streptomycin 200 µg/mL (both from Cambrex). Cultures were stimulated with different antigens and mitogens to evaluate lymphocytes responses. The following antigens and mitogens were used; Influenza antigen 1:1000 (Vaxigrip; Sanofi Pasteur, Solna, Sweden),

purified protein derivate (PPD) 10 µg/mL (SSI, Copenhagen, Denmark), a mix of cytomegalovirus (CMV) peptides 0.125 µg (BD Biosciences), tetanus toxin 5.7 Lf/mL (SSI), phytohaemagglutinin (PHA) 5 µg/mL (Sigma Aldrich), pokeweed mitogen (PWM) 10 µg/mL (Sigma Aldrich) or myelin basic protein (MBP) 100 µg/mL (Sigma Aldrich). In addition, cultures without antigen or mitogen were used as background controls. Cultures were incubated at 37°C in humidified air with 5% CO₂ for seven days. After culturing, lymphocyte responses were analysed with flow cytometry as described below.

3.8 Flow cytometry: General principles

Flow cytometry is a method to count different particles in suspension, be it cells, beads or other bodies. Technical refinements have increased the precision and speed of analysis and at present, several thousands of particles can be analysed every second. Particles in suspension are forced into a stream of particles in single file by exploitation of Bernoulli's law of laminar flow; pressure gradients created by the different flow rates of a stream force particles to align in the center where the rate of flow is the highest. This permits data to be collected from each individual cell.

At the point of measurement the cells will pass focused lasers. Basic measurements include the light scatter properties of the cell. The forward-scatter (FSC) is proportional to the size and the refractive index of the cells and the FSC detector is located in line with the incident laser. Side-scatter (SSC) signals correspond to the granularity of cells and are measured perpendicularly to the incident laser. FSC and SSC characteristics in conjunction may be used for identification of cell populations. Lymphocytes are relatively small and non-granular, while monocytes are both larger (FSC) and display a higher granularity (SSC). As the name implies granulocytes are highly granular. In addition, since FSC signal is affected by the refractive index of the cell, dead cells may roughly be distinguished based on low FSC and high SSC signals.

Analysis of surface and intracellular protein expression are made possible through the use of monoclonal fluorochrome-conjugated antibodies specific for protein amino acid sequences or

Figure 6. Overview of flow cytometry. PMT: photomultiplier tube. ADC: analog to digital converter. R, G, B: red, green and blue fluorescence detectors.

other antigens. Lasers of different wavelengths are used to excite the fluorochromes on cells expressing the antigen for which antibodies are specific for. An excited fluorochrome emits light of a longer wavelength than the laser that excited it, a property explained by the energy loss and termed Stoke's shift. Emitted fluorescence is measured at a 90° angle of the exciting laser, similar to SSC measurement. Since a single cell may be stained with several fluorochromes, light of different wavelengths are split using dichroic mirrors and fluorescence signals recorded separately. Fig. 6 provides an overview of the method.

Due to the fact that fluorochromes have relatively broad emission spectra after excitation, or a spectrum with more than one peak, fluorescence tends to 'leak' into multiple detectors, a phenomenon called spectral overlap. The leakage of a fluorochrome into the wrong channel can be quantified and compensated for by subtraction of a percentage of the leaking signals from the signal of interest.

3.8.1 Analysis of flow cytometry data

Scatter and fluorescence signals are recorded for each cell passing the interrogation point. Normally, scatter signals are displayed on a linear scale while fluorescence intensity is logarithmic. All analyses are performed in specifically designed software packages. When analysing lymphocytes, cells with FSC and SSC characteristics of lymphocytes are first selected. This is done by selecting the cells that match the desired characteristics. Typically, when two parameters are plotted against each other in a scatter plot, cells may be selected through drawing boundaries on the scatter plot; the bounded area in the plot is a gate. Gated cells may then be separately depicted on new scatter plots or histograms. This permits accessible analysis of protein expression in several dimensions as well as clear definitions of subpopulations. To distinguish which cells are negative and positive for a certain marker requires the use of either a cell population that is known to lack the marker, or by using isotype controls. An isotype control is an antibody of the same isotype (preferably from the same company) as the fluorescent antibody but directed against an irrelevant antigen that is not present in the context of investigation. By labelling a cell population expressing the marker with an isotype control, the background fluorescence of the cell and the antibody is depicted, and the upper boundary of fluorescence of this population may be used to set the lower boundary of positive fluorescence when assessing the same population labelled with a fluorochrome-conjugated antibody of the relevant specificity.

Typically, protein expression is presented as a percentage, *i.e.* the fraction of a gated population with a positive staining for a certain antigen. However, when analysing *ex vivo* samples in particular from whole blood, the absolute number of cells per volume is of interest. The flow cytometer will keep track of how many events are analysed per second, but due to fluctuations in the event rate and the flow rate, there might be uncertainty associated with this measurement. One way of obtaining the absolute event number collected by the software is the usage of beads. A fixed number of beads are added to a fixed volume of the sample to be analysed. Post-analysis, the beads are gated, and the number of beads collected will be directly correlated to the volume of the sample that has been collected. In this way, the definitive cell number in the original sample volume may be calculated from the event count of cells in the sample.

3.8.2 Carboxyfluorescein succinimidyl ester for cytoplasmic labelling

Carboxyfluorescein diacetate succinimidyl ester, CF(DA)SE is a membrane-permeable molecule. After passage over the plasma membrane, the acetate groups are cleaved by cytoplasmic esterases, and the molecule, now named CFSE, becomes fluorescent (emitting green fluorescence, measured in the fluorescein isothiocyanate (FITC) channel) and non-permeable, thereby trapping it inside the cell. The penetration of the outer plasma membrane occurs readily, and no permeabilising agents are required for passage. The main application of CFSE is tracking of cell division by flow cytometry. By diffusion, CFSE is evenly distributed in the cytoplasm of the cell, ensuring its equal division between the two daughter cells upon mitosis. Flow cytometry is used to track different populations of cells based on their CFSE content, using the original non-replicated population as negative control. Subsequent cell divisions are seen upon plotting the CFSE content in one-dimensional histograms or two-dimensional scatter-plots (Fig. 7). In Paper II, CFSE is used for assessment of proliferation in a suppression assay. In addition, CFSE staining of cells before culturing was used as a tracking agent for the responder population in co-cultures.

Figure 7. Gating strategy of CFSE^{dim} cells. Cells that have undergone proliferation (centre panel; CFSE^{dim}) may be readily gated in a CFSE histogram by use of CFSE⁻ cells (top left panel) and CFSE⁺ unstimulated cells (top right panel). The right panel shows cell divisions of cells using a 2D scatter plot. In this example, three cell divisions have taken place.

3.8.3 Classically defined T_{REG} analysis with 3-color flow cytometry (Paper I)

Whole blood was drawn into EDTA vacutainer tubes. Cells were labelled with the designated antibodies for 15 min at room temperature in the dark (antibodies are listed in Table II). Red blood cells were lysed through addition of FACS lysing solution (BD Biosciences) for an additional 15 min at room temperature in the dark. Absolute cell numbers were calculated by using TrueCount™ tubes (BD Biosciences). Classically defined CD4^{dim}CD25^{high} T_{REG} were analysed using 3-color flow cytometry. First, lymphocytes were gated by FSC/SSC characteristics. Next, CD4⁺ T cells were selected through gating of CD3⁺CD4⁺ cells. T_{REG} were gated in the CD4⁺ T cell population; the CD4^{dim}CD25^{high} T_{REG} were selected in a plot of CD4 and CD25. As comparison, CD4⁺CD25⁺ cells, corresponding to T_{REG} as defined in rodents, were also analysed. All gates were set using isotype controls for CD3, CD4 and CD25. All data was collected on a FACSCanto II system employing the FACSDiva software (BD Biosciences), and analysis was done in FACSDiva software.

3.8.4 6-color flow cytometry phenotyping and analysis of T_{REG} suppression (Paper II)

Different subpopulations of T_{REG} were investigated. For the phenotype analysis two separate samples were analysed per individual, each from 100 µL whole blood taken from sodium-heparin vacutainer tubes. Initially, antibodies for surface markers were added for 30 min at 4°C in the dark. After labelling, erythrocytes were lysed through addition of 0.8% NH₄Cl for 15 min at 4°C in the dark, followed by washing of cells twice. For staining of intracellular markers, cells were permeabilised and fixated using an intracellular staining kit (eBiosciences, San Diego, USA). After adding antibodies to intracellular markers, cells were incubated for 30 min at 4°C in the dark. After washing, the samples were kept on ice until data collection.

CD4⁺ T cells were selected by first gating lymphocytes using FSC/SSC characteristics with subsequent gating of CD3⁺CD4⁺ cells. Three subpopulations of T_{REG} were defined. Classical T_{REG} were defined as CD4^{dim}CD25^{high}. Resting and activated T_{REG} were defined through expression of CD45RA and CD25; activated T_{REG} were CD45RA⁺CD25^{high} while resting T_{REG} were CD45RA⁺CD25^{int}. These three populations were then further analysed for of CD39

and FOXP3 expression. In addition, Helios was analysed since this transcription factor initially was suggested to distinguish between thymically and peripherally derived T_{REG}.

After co-culturing (described above), the suppressive capacity of T_{REG} were analysed through flow cytometric assessment of CD69 (activation) and CFSE (proliferation) in 18 h and five days co-cultures, respectively. In all cultures, T_{RESP} were stained with CFSE pre-culturing to distinguish this population from CFSE⁻ T_{REG}. CFSE⁺ T_{RESP} were gated for CD69, using an unstimulated control culture to set the CD69⁺ gate. The CFSE⁺ gate was set using the CFSE⁻ T_{REG} in the co-culture. For proliferation, cells not proliferating were defined as CFSE^{high}. Proliferating cells were CFSE^{dim} and defined as cells having a CFSE expression between CFSE^{high} (non-proliferating cells) and CFSE⁻ (unlabelled) cells. Suppression of T_{RESP} activation was assessed through analysis of CD69 expression of T_{RESP} in co-cultures compared to CD69 expression in uninhibited cultures (*i.e.* stimulated T_{RESP} without T_{REG}). A suppressive index (SI) was calculated (Eq. 3). Similar to the activation assay, five day suppression of proliferation of T_{RESP} was evaluated by comparison between the fraction of T_{RESP} that were CFSE^{dim} in presence and absence of T_{REG}. As for the activation suppression assay, a SI for proliferation was then calculated from the CFSE^{dim} fractions (Eq. 4). All antibodies used in Paper II are listed in Table II.

$$SI = 1 - \frac{\%CD69^{+}_{T_{resp}+T_{reg}}}{\%CD69^{+}_{T_{resp}}} \quad Eq. 3$$

$$SI = 1 - \frac{\%CFSE^{dim}_{T_{resp}+T_{reg}}}{\%CFSE^{dim}_{T_{resp}}} \quad Eq. 4$$

3.8.5 Flow cytometry for analysis of lymphocyte subpopulations and lymphocyte responses (Paper III)

6-color flow cytometry was used for assessment of lymphocytes subpopulations. In total, 17 different markers were used in five different tubes. TrueCountTM tubes (BD Biosciences) were used for absolute cell numbers of the main lymphocyte populations; CD4⁺ and CD8⁺ T cells, CD19⁺ B cells, CD3⁻CD16/CD56⁺ NK cells. Absolute cell numbers for were then calculated in relation to the main populations. Whole blood staining of cells was done through addition

of antibodies for 15 min at room temperature in the dark with lysing of erythrocytes using FACS lysing solution (BD Biosciences).

In all tubes, lymphocytes were first gated by FSC/SSC properties. Definitions of other populations were based on the presence of discrete cell populations or by using other populations as negative or positive control populations. T cells were defined as CD3⁺, with subsequent definition of CD4⁺ and CD8⁺ T cells. B cells and NK cells were defined as CD3⁻CD19⁺ and CD3⁻CD16/CD56⁺, respectively. In the second tube, senescent T cells were assessed through expression of CD28, CD56 and CD57, gated separately for CD3⁺CD4⁺ and CD3⁺CD8⁺ populations. CD28⁻ cells were gated for both T cell populations. The gate for CD28⁻ cells was set by examination of CD28 expression on CD3⁻ cells. CD28⁻ T cell populations were then investigated for CD56 and CD57 expression. In this tube, the proportion of total NKT, CD3⁺CD56⁺, cells as well as CD4 and CD8 NKT cells, defined as CD3⁺CD4⁺CD56⁺ and CD3⁺CD8⁺CD56⁺, respectively, were also assessed. In the third tube, activation of CD4, CD8 and NK cells was investigated through expression of CD69 and HLA-DR. In addition, NK cells were analysed for proportions of CD56^{dim} and CD56^{bright} cells. In tube four, classically defined T_{REG} were gated as CD4^{dim}CD25^{high}, and total CD25⁺ cells was also gated for both CD4⁺ and CD8⁺ cells, respectively. In addition, OX40L expression was gated for both T cell populations. In the last tube, B cells were defined as CD19⁺. Memory B cells were gated as CD19⁺CD27⁺, and B cells with regulatory potential were defined as CD19⁺CD25⁺. For setting of CD25 and CD27 gates, polymorphonuclear cells were used to as a negative population. Specifications of all antibodies used are listed in Table II.

Assessment of lymphocyte responses were made on seven day whole blood cultures, stimulated with antigens or mitogens (outlines above). After harvesting of cultures, cells were labelled with CD3, CD4, CD8 and CD108 antibodies for 15 min at room temperature in the dark (Table II). After subsequent lysis of erythrocytes using a 0.8% NH₄Cl solution, data was collected on a FACSCanto II machine employing the FACSDiva software (BD Biosciences). Data analysis was done using the FACSDiva and Kaluza (Beckman Coulter) software.

Lymphocyte and lymphoblast gate was set based on FSC/SSC characteristics in unstimulated cultures (containing only cells and culturing medium). The lymphoblast gate was based on the lymphocyte gate; cells with higher FSC and SSC signals were defined as blasts (although very few lymphoblasts were present in unstimulated samples). Lymphoblasts were further gated

into CD4⁺ and CD8⁺ T cells (CD3⁺CD4⁺ and CD3⁺CD8⁺ gates, respectively); the fraction of CD108⁺ cells in these two populations was also investigated. Absolute cell numbers were assessed by using beads from TrueCount™ tubes (BD Biosciences). Given the comparable (and low) grade of cell death in cultures, absolute cell numbers in unstimulated cultures after seven days was used to estimate and compare available cell numbers in blood between patients and controls. The mean number of lymphoblasts in the control cultures was subtracted from the mean number of lymphoblasts in the stimulated cultures to account for baseline activation of cells. For each stimulus the numbers of CD4⁺, CD8⁺ and activated CD108⁺ cells were compared between patients and controls.

To explore the responsiveness of lymphocytes in cultures before and after one year of treatment with natalizumab, the fraction of cells responding to different stimuli was calculated. Responding cells were defined as the ratio of the amount of lymphoblasts, that is cells that have responded and been activated by the stimulus, and total lymphocytes, *i.e.* non-activated lymphocytes and lymphoblasts. Again, unstimulated cultures were used to set the lymphocyte and lymphoblasts gates. This ratio is presented as the percentage of lymphoblasts of total lymphocytes. Primarily, we were interested in the effect of natalizumab treatment on lymphocyte responsiveness. However, we also included controls in this analysis, and for simplicity the responsiveness of control responses to the different stimuli was set to 100%.

3.8.6 3-color flow cytometry for analysis of CD4⁺ purity and activation level (Paper IV)

After culturing for 48 h, anti-CD3 and anti-CD28 stimulated CD4⁺ cells with or without natalizumab were labelled with CD4 and CD69 antibodies as previously described (labeling post-culturing in Paper II). Flow cytometry was carried out to control purity and activation of cells. Lymphocytes were gated on FSC/SSC properties, and CD4⁺ cells were gated subsequently. The purity of CD4⁺ cells were typically >96% after culturing. Level of activation was assessed by CD69 expression, and the expression of stimulated cells was compared to unstimulated cultures. Fluorochromes used are listed in Table II.

Table II. Fluorochromes used in Paper I-IV

Marker	Fluorochrome	Clone	Paper
CD16/CD56	PE	B73.1/NCAM16.2	III
CD19	APC	SJ25C1	III
	PE-Cy7	SJ25C1	III
CD25	FITC	2A3	I, III
	APC	MA251	II
	APC	2A3	III
CD27	PE	L128	III
CD28	PE	L293	III
CD3	PerCP	SK7	III
	APC-Cy7	SK7	II
	FITC	SK7	III
CD39	PE	TU66	II
CD4	PE	SK3	III
	PE-Cy7	SK3	II, III, IV
CD45	PerCP	2D1	III
CD45RA	Horizon V450	H100	II
	FITC	H100	II
CD5	PerCP-Cy5.5	L17F12	III
CD56	APC	NCAM16.2	III
CD57	FITC	HNK-1	III
CD69	PE	L78	III
	APC-Cy7	FN50	II, IV
CD8	APC-Cy7	SK3	III
FOXP3	FITC	PCH101	II
Helios	Horizon V450	22F6	II
HLA-DR	FITC	L243	III
	APC-Cy7	L243	III
OX40L	PE	Ik1	III

PE: phycoerythrin, APC: allophycocyanin, PerCP: peridinin chlorophyll, FITC: fluorescein isothiocyanate, ^a: Except for anti-FOXP3 FITC (eBioscience) and anti-Helios Horizon V450 (BioLegend), all antibodies were purchased from BD Biosciences.

3.9 mRNA expression analysis

3.9.1 Extraction of messenger RNA (mRNA) (Paper I, II, IV)

In Paper I, mRNA was extracted from whole blood using the PAXGene system (PreAnalytix GmbH, Hombrechtikon, Switzerland). 2.5 mL blood was drawn into a vacutainer tube containing cell lysing and mRNA stabilisation agents, thereby providing total cellular mRNA content directly *ex vivo*, without any manipulation of cells. Samples were frozen at -70°C until extraction of mRNA. The extraction procedure involves a column based system and was carried out according to the provided instructions. Samples were treated with DNase to avoid DNA contamination before cDNA synthesis. All samples were eluted in 40 µL of RNase-free water and mRNA quality and quantity was assessed with spectrophotometry (NanoDrop Technologies, Wilmington, USA). Prior to cDNA synthesis mRNA concentrations were diluted to ensure consistent mRNA amount used in reverse transcription. Analysis of mRNA expression is outlined below.

In Paper II and IV, we used microarray technology to analyse global gene expression. In Paper II, highly purified, flow cytometry sorted T_{REG} were analysed and in Paper IV, immunomagnetically sorted CD4⁺ cells were used. For mRNA extraction, TRI Reagent was used (MRC) in accordance with the manufacturer's instructions. Before extraction of mRNA, cell pellets were resuspended in TRI Reagent to ensure cell lysis and RNA stabilisation due to guanidine thiocyanate being a constituent of the reagent. Phenol ensures the separation of RNA from DNA and protein during extraction. To facilitate the purification of RNA during extraction, GlycoBlue (Life Technologies, Stockholm, Sweden) was added to the samples. In Paper II, 1.0-2.0 x 10⁵ T_{REG} were used for extraction. Here, 100 µL of TRI Reagent was used, and all volumes in the protocol were adapted accordingly. In Paper IV, mRNA was extracted from 0.9-1.1 x 10⁶ CD4⁺ cells, using 350 µL TRI Reagent. Before RNA amplification and labelling for microarray (described below), concentrations across samples were standardised.

3.9.2 Quantitative polymerase chain reaction (RT-qPCR) (Paper I)

In RT-qPCR, also called real-time RT-PCR, the amount of mRNA is quantified by fluorescence signals. After extraction from a sample, the mRNA is first reversely transcribed (RT in RT-qPCR) to complementary DNA (cDNA). The detection and quantification of cDNA, of which the amount is directly proportional to the amount of mRNA in the original sample, is based on the use of fluorochrome-labelled, gene specific probes, spatially located between the forward and reverse primers. In addition to the 5' fluorochrome, a quencher is bound to the 3' end. During a single round of amplification, the probe binds the cDNA and during polymerase activity, the fluorochrome at the 5' end is cleaved, increasing the distance to the quencher. A laser is then used to excite the fluorochromes in the sample and the subsequent fluorescence emission is proportional to the amount of unbound fluorochrome, which in turn is proportional to the amount of amplified material. During the qPCR reaction, amplification is progressing through three phases; an initial phase with increasing amplification, an exponential phase in which the rate of increase of amplification is linear and a plateau phase where the rate of amplification is decreasing and finally comes to a halt. Fluorescence is measured in all cycles (in all three phases), but quantification is based on assessment of fluorescence signals in the exponential phase. Gene expression is quantified using a serially diluted standard curve. To ensure specificity of quantification, the sequence of either primer or the probe is spanning an exon boundary, thereby preventing binding to DNA sequences. In addition, a negative control (water) is used to detect DNA/mRNA contamination of samples. Assessment of RNA quantity in the sample is made through the use of a reference, or house-keeping, gene; a gene with stable, constitutive expression in all cells. In the context of this thesis, *18S*, a subunit of human rRNA, fulfils these criteria. Gene expression is presented as a ratio of expression of the gene of interest divided by the expression of the reference gene.

In paper I, mRNA was obtained from 2.5 mL whole blood samples as described above. A random hexamer method was used for reverse transcription of cDNA (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, Foster City, USA). Thermal cycling was done using the Mastercycler Ep (Eppendorf, Hamburg, Germany). Amplification of cDNA was performed in a 25 μ L reaction with 1.0 μ L template. Samples were amplified for 40

Table III. Primer and probe sequences (5'→3')[†]

Gene		Nucleotide sequence
<i>FOXP3</i>	Forward	GTGGCCCGGATGTGAGAA
	Reverse	GCTGCTCCAGAGACTGTACCATCT
	Probe	CCTCA <u>AG</u> CACTGCCAGGCGGAC
<i>GATA3</i>	Forward	CAAAATGAACGGACAGAACCG
	Reverse	GCTCTCTGGCTGCAG <u>ACA</u>
	Probe	CCCCTCATTAAGCCCAAGCGAAGG
<i>18S</i>	Forward	CGGCTACCACATCCAAGGAA
	Reverse	GCTGGAATTACGCGGGCT
	Probe	GAGGGCAAGTCTGGTGCCAGCA

[†]: Exon-exon boundary is denoted by bold, underlined nucleotides.

cycles of 10 s at 95°C followed by 1 min at 60°C. All samples were analysed in duplicates and expression was calculated as the mean of these two measurements. A variation coefficient of < 0.15 was determined as acceptable. Samples not fulfilling this criterion were re-analysed. Gene expression of samples that displayed sub-threshold cDNA quantities in three subsequent, separate duplicate measurements were set to zero. For quantification, a five-point serially diluted standard curve was used, derived from PBMC *in vitro* cultures stimulated with PHA. The quantified genes were *TBX21*, *GATA3*, *RORC*, *FOXP3* and *EBI3*. Human rRNA *18S* expression was used as reference of gene expression. The variation coefficient of all assessed genes was < 8%. Primers and 3'-FAM/5'-TAMRA labelled probes for *GATA3*, *FOXP3* and *18S* were developed in-house using the PrimerExpress 3.0 software (Applied Biosystems); HPLC-purified oligonucleotide primers and probes were bought from MedProbe (Oslo, Norway; sequences are listed in Table III). All in-house mRNA amplicons included at least one exon-exon boundary to ensure mRNA specificity. Primer and probes for *TBX21*, *RORC* and *EBI3* were bought from Applied Biosystems (Taqman[®] Gene Expression assays, Hs00203436_m1, Hs01076112_m1 and Hs01057148_m1, respectively).

3.9.3 Microarray analysis of mRNA expression (Paper II, IV)

Microarray technology permits the assessment of global RNA expression analysis. Amplified RNA is hybridised with high specificity onto a chip onto which complementary RNA strands have been synthesised. Measurement of RNA expression in the sample is based on

fluorescence signals emitted from the sample RNA after laser excitation. During sample preparation, the sample RNA is first converted to cDNA which is amplified. After amplification, a T7 RNA polymerase is used to convert cDNA back to cRNA. The T7 RNA polymerase incorporates Cy3-coupled cytosine into the cRNA, effectively labelling the cRNA, which is then purified. After purification, the quantity and quality of cRNA needs to be assessed to control that adequate amplification and conversion reactions have occurred. In addition, the net fluorescence in each sample is measured to ensure sufficient fluorescence signals during scanning. Samples are then fragmented before hybridisation, creating short, labelled RNA sequences. The chip consists of a matrix (array) of short, specific synthesised RNA sequences, chosen from thousands of different RNA sequences, covering the whole transcriptome. Labelled, fragmented RNA is incubated on the chip under rotation at temperatures optimal for RNA:RNA hybridisation and the sample RNA is bound complementary to the chip probes followed by washing. During scanning, the chip is targeted by a laser and the resulting fluorescent emission is registered with high precision, allowing measurement of per-probe fluorescence. Every RNA sequence in the transcriptome is represented on the chip by several probes; the expression of target RNA is calculated as an average of the fluorescence corresponding to replicate probes.

The Agilent Sureprint G3 Human Gene Expression 8x60k (Agilent Technologies) was used for microarray analysis. In both Paper II and IV, RNA concentrations were standardised before microarray sample preparation ensued. In short, RNA was first converted to cDNA and amplified. The cDNA was then converted to cRNA and labelled with fluorescence (Cy3). The cRNA was then purified using a column based assay (Qiagen, Hilden, Germany). RNA quality and quantity and fluorescence total intensity was assessed using spectrophotometry (NanoDrop Technologies). Fragmentation of labelled cRNA was carried out using a fragmentation buffer (Agilent Technologies). The fragmented cRNA was finally hybridised onto the microarray slides for 17 h at 65°C under constant rotation. After washing, data was collected using an Agilent G2565BA scanner running the Agilent Feature Extraction software (both from Agilent Technologies). Raw data was exported for data analysis in R 3.0.2.

In Paper II, highly purified resting and activated T_{REG} were used for RNA extraction. In total, 10 samples, five MS patients and five controls, with sufficient RNA concentrations were included. For Paper IV, 32 MS samples and 32 SAR samples were included for analysis; eight LR and eight HR to natalizumab or GC treatment, respectively. For each of these groups, cells

with and without natalizumab and GC, respectively, were analysed. TRI Reagent or Agilent Absolutely RNA MicroPrep was used for RNA extraction.

3.10 Statistics and bioinformatics

The software used for statistical comparisons in Papers I-IV was the following: SPSS 20.0 Software (SPSS Inc., Chicago, USA) (Paper I-III), GraphPad 5 (GraphPad Software, La Jolla, USA) (Paper II), R 3.0.2 (Paper II, IV) and MatLab (MathWorks, Natick, USA) (Paper IV).

3.10.1 Paper I

Differences in transcription factor gene expression were calculated using Mann-Whitney's test and Kruskal-Wallis' test. Flow cytometry data was compared using independent samples *t* test. Differences were considered significant for *p*-values ≤ 0.05 .

3.10.2 Paper II

3.10.2.1 Statistical analysis

For demographic data, Mann Whitney's test and Student's *t* test were used to compare study subjects. Unpaired *t* test was used to compare flow cytometric data between controls and patients. Stratified MS patients were compared to controls using one-way ANOVA with Tukey's post-hoc test. One-way ANOVA with Tukey's post-hoc test was also used to compare different T_{REG} populations. *p*-values ≤ 0.05 were considered statistically significant, and *p*-values ≤ 0.1 were considered as a trend.

3.10.2.2 Microarray gene expression analysis

For microarray analysis, the R package Limma was used (Smyth 2005). Raw data were corrected for background and normalised using quantile normalisation. All probe replicates were averaged. Calculation of DE genes between patients and controls was performed by linear model fitting (using the function *lmFit* in R 3.0.2) employing empirical Bayes statistics. False discovery rate (FDR) according to Benjamini-Hochberg was used to control for multiple comparisons.

3.10.2.3 Gene set enrichment analysis (GSEA) of T_{REG} gene expression data

GSEA is a method for evaluating subtle trends in high-throughput data (Mootha et al. 2003, Subramanian et al. 2005). Instead of analysing expression data between two groups gene by gene, GSEA analyses the collective over- or under-expression of pre-defined gene-sets between groups. The analysis is based on comparisons of enrichment distributions and random distributions permuted from the original data; deviation from the zero is assessed by Kolmogorov-Smirnoff statistics. The permutations involve either shuffling of group labels or gene labels, and are typically presented as a mean distribution of 1000 permutations. The enrichment score (ES) is calculated for all gene sets and normalised for gene set size (NES; normalised enrichment score). FDR statistics are commonly employed for evaluating differences between NES of enrichment. In explorative analyses, and FDR q -value < 0.25 is recommended to discriminant differentially enriched gene sets (Subramanian et al. 2005).

GSEA was employed in Paper II to detect subtle differences in mRNA expression data between groups. For analysis the GSEA analysis script for R 3.0.2 was used, downloaded from the Broad Institute GSEA web page (<http://www.broadinstitute.org/gsea/>). Expression data from T_{REG} from MS patients was compared to controls. Mean NES from a 1000 permutations were used to generate a random null distribution, and an FDR $q < 0.25$ was used to identify enriched pathways.

3.10.3 Paper III

Clinical and CSF variables were compared using Wilcoxon signed rank test. Flow cytometry data patients and controls as well as lymphocyte activation data were compared using paired t tests. Pearson's correlation was used to examine possible correlations between flow cytometry data and patient clinical variables. For analysis of lymphocyte activation assay data between controls and patients before and after treatment we employed a one-way ANOVA with Tukey's post-hoc test for multiple comparisons. Here, p -values ≤ 0.05 denoted significance. In all other tests, p -values ≤ 0.01 were considered as significant and p -values ≤ 0.05 marked a tendency.

3.10.4 Paper IV

3.10.4.1 Public databases

For construction of disease-specific and pleiotropic networks, the STRING protein-protein interaction (PPI) v 9.03 was used (Szklarczyk et al. 2011). To assess robustness of the pleiotropic module, five separate databases were used (latest versions as of 15 November 2013), concerning different aspects of the human interactome; HPRD (Peri et al. 2004), Reactome (Milacic et al. 2012), Intact (Yu et al. 2011), HI2 (CCSB Interactome database; <http://interactome.dfci.harvard.edu/>) (Yu et al. 2011) and a high confidence database (Wang et al. 2012). For each of these databases, the shortest paths between all pairs of genes in the pleiotropic module also present in the largest connected component were calculated. As null distribution, an equal number of randomly selected genes from the largest connected component were selected, and shortest paths were calculated. This randomisation procedure was repeated a 1000 times, and mean values of the shortest paths were used to calculate *p*-values using Wilcoxon's test.

Enrichment of pathways of disease-associated genes from GWAS data was tested using all pathways in three separate databases, downloaded 15 November 2013: Kyoto Encyclopaedia of Genes and Genomes (KEGG) (Kanehisa 2013), Ingenuity Pathway Analysis (IPA; <http://www.ingenuity.com/>) and Gene Ontology (GO) (Ashburner et al. 2000). Analysis of mouse knockout phenotypes was performed by downloading phenotype information from the Mammalian Phenotype database (31 January 2013) (<ftp://ftp.informatics.jax.org/pub/reports/index.html>). Pathway enrichment for these databases were calculated by Fischer's exact test, as described below. For further GWAS analysis in MS, data from 25 000 individuals with MS and controls was acquired (IMSGC 2011).

Analysis of therapeutic targets and potential biomarkers was based on the IPA database, where all therapeutic targets and genes annotated as markers for disease or prognosis were used. For correlation analysis of expression of pleiotropic genes and drug responses in cancer cell lines, data were downloaded from the Genomics of Drug Sensitivity project (Garnett et al. 2012). A database comprising PPIs with predicted therapeutic potential was also included (Sugaya et al. 2012).

3.10.4.2 Network construction and analysis of gene expression data

Disease-specific networks were constructed using publically available microarray analyses for the following eight diseases: MS, systemic lupus erythematosus, rheumatoid arthritis and allergy, acute myeloid leukaemia, adult T cell leukaemia, chronic lymphocytic leukaemia and hypereosinophilic syndrome. All included arrays were downloaded from the Gene Expression Omnibus database on 31 December 2012. Inclusion criteria were 1) expression profiling of CD4⁺ T cells from patients and controls, 2) at least five samples per group and 3) untreated samples (both groups).

PPI networks were constructed using a modification of a previously described method (Barrenas et al. 2012). Maximal cliques (complete sub-networks not contained by other cliques) were extracted from the STRING PPI database. Each clique was assigned a weight calculated as the sum of $-\log(p\text{-values})$ of all the genes in the clique where the p -values were extracted from differential expression analysis for the different diseases, respectively. To determine significance of these disease-associated sub-networks, randomised null distributions of clique weight were generated. The p -values of differential expression were randomised and clique weight was re-calculated; this process was repeated 10 000 times, giving a null distribution of weights for each clique. Significance was calculated by comparing the fraction of randomised permutation cliques with a weight higher than the original, real weight.

The disease-specific modules were then used to find intersecting elements of these, a sub-network containing shared genes included in multiple disease-specific modules. This shared-gene, or pleiotropic, network was constructed by identifying genes present in all eight disease-specific modules.

A null distribution for overlapping genes was created; the above described method of construction of disease-specific modules was used 100 times with randomised p -values to create 100 different disease-modules per disease. The number of genes present in all eight randomised networks was recorded for 10^6 permutations and the significance level of the true, original pleiotropic module was estimated by the fraction of these permutations resulting in an equal or higher number of genes with overlap in the selected eight modules. In addition, these

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100 random disease-specific sets were also used to identify genes represented in more disease modules than expected by chance.

3.10.4.3 Statistical analysis

Calculations of differential expression of genes in microarray expression data (for construction of disease-specific modules and treatment exposure effects for SAR and MS) were done using the R package Limma (Smyth 2005). For gene expression data from MS and SAR, batch effects were corrected by COMBAT with phenotype and stimulation as potential covariates before differential expression analysis (Johnson et al. 2007). Cancer cell line gene expression data was normalised using the RMA algorithm (Irizarry et al. 2003). Non-annotated probes were removed from correlation analysis (described below).

Classification of patients/controls (from disease-specific gene expression data) and prediction of treatment response to GC and natalizumab (using the experimental gene expression data) by LASSO was performed by the MatLab function *lassoglm*. This function performed LASSO regularisation of a logistic model. Results were also investigated by testing parameter fits using leave-one-out cross-validation.

Enrichment of a set of genes, for example the enrichment of pleiotropic genes in GWAS disease-associated genes, was investigated through Fischer's exact test. As a measurement of effect size, fold enrichment was used. Here we define fold enrichment as the ratio between the observed frequency divided by the size of a gene set with a certain feature and the frequency of that feature divided by the number of all annotated genes. For instance, when investigating enrichment of the pleiotropic module in GWAS data, the fold enrichment was calculated by taking the ratio of the number of GWAS disease-associated genes found in the pleiotropic module (53 GWAS-genes divided by 158 gene in the pleiotropic module) and the number of total GWAS disease-associated genes in the human genome (2 298 GWAS-genes in total divided by the number of annotated human genes, 22 500). This results in a fold enrichment = $(53 / 158) / (2\,298 / 22\,500) = 3.3$.

In addition, gene set enrichment for GWAS disease-associated genes, cancer susceptibility genes, mouse knockout phenotypes, potential therapeutic targets and biomarkers was tested for connectivity bias. This was performed by random sampling of 158 genes (the number of genes in the pleiotropic module) from the STRING network with the same median and

minimal degree as the pleiotropic module; this process was repeated 10^6 times, generating a null distribution of connectivity, and compared with the true pleiotropic module.

To determine if a set of genes was differentially expressed in experimental MS and SAR gene expression analyses, the mean of the squared t statistics (t^2) was used. To assess statistical significance, a permutation test was performed, using 10^6 permutations.

Correlation of pleiotropic genes and drug responses using in cancer cell line gene expression data was done through calculation of the Pearson correlation coefficient (PCC). The null distribution comprised 1000 sets of 158 genes randomly selected from the cancer cell line library expression data. Calculation of the PCC was also used to validate the definition of the pleiotropic module using GWAS disease-associated and cancer susceptibility genes and their occurrence in the disease-specific modules. In addition, PCC was used to investigate the effect of GC and natalizumab treatment of genes in the pleiotropic module.

4. RESULTS AND DISCUSSION

4.1 Lymphocyte populations in whole blood (Paper I)

In Paper I, mRNA expression of T_H subset specific transcription factors was analysed to investigate the immune deviation of $CD4^+$ T cells in RR-MS. For extraction of mRNA we employed the PAXGene system in which mRNA is stabilised and preserved instantly, thus providing an *in vivo*-like imprint of systemic immune deviation. Although using whole blood as a medium, the specificity of chief $CD4^+$ T cell transcriptional regulators *TBX21* (T_{H1}), *GATA3* (T_{H2}), *RORC* (T_{H17}) and *FOXP3* (T_{REG}) ensures that $CD4^+$ cells are assessed. Additionally, *EBI3* was analysed, suspected to be involved in immunoregulatory processes. In all analyses, RR-MS patients were compared to healthy controls.

When comparing patients to controls, quantitative analysis of mRNA transcriptional expression revealed a decreased expression of T_H2 associated *GATA3* and T_{REG} associated *FOXP3* in RR-MS compared to healthy controls (Fig. 8b, d). However, no significant

Figure 8. Expression of transcription factors and *EBI3* in patients with MS and controls (ctrl). **a:** *TBX21* (T_{H1}). **b:** *GATA3* (T_{H2}). **c:** *RORC* (T_{H17}). **d:** *FOXP3* (T_{REG}). **e:** *EBI3*. Bars show median and 25th and 75th percentile. *: $p < 0.05$. **: $p < 0.01$.

Figure 9. Expression of *GATA3* (a), *FOXP3* (b) and *EBI3* (c) expression in patients with (IFN+) and without (IFN-) IFN- β treatment and healthy controls (ctrl). Boxes show median and 25th/75th percentile, whiskers show 10th and 90th percentile. †: $p < 0.1$. *: $p < 0.05$.

differences in T_H1 and T_H17 associated *TBX21* and *RORC* expression were observed between MS and controls (Fig. 8a, c). Immunoregulatory *EBI3* showed a lower expression in RR-MS patients (Fig. 8e). All expression values were normalised to expression of human rRNA *18S* expression. *18S* expression levels were also assessed and found to be similarly expressed between groups, therefore not contributing to the observed differences.

Out of the total 33 RR-MS patients included; 12 patients were treated with IFN- β at the time of sampling while 21 were untreated. To avoid confounding effects of IFN- β on treatment, the material was stratified into IFN- and IFN+ groups, and expression of the transcription factors and *EBI3* was reanalysed using these new groups. Comparisons between the IFN+ and IFN- groups revealed no significant differences. When comparing IFN- patients to healthy controls, similar patterns of expression were observed as when comparing all patients to controls, *i.e.* lower expression of *GATA3*, *FOXP3* and *EBI3* (Fig. 9) while *TBX21* and *RORC* were similarly expressed (data not shown). These findings were not surprising; irrespective of an effect of IFN- β , untreated patients (constituting a majority of patients) would reflect the original patterns seen. When comparing the 12 IFN+ patients to the controls, *EBI3* expression was significantly lower in patients while *GATA3* and *FOXP3* tended to be decreased ($p < 0.1$) (Fig. 9). Again, no differences were observed for *TBX21* and *RORC* expression (data not shown). Although the differences observed for IFN- patients compared to controls were non-significant for *GATA3* and *FOXP3*, the patterns in data was consistent with the hypothesis that IFN-treatment would not influence transcription factor expression and in extension the balance of CD4⁺ T cells systemically. This notion was also supported by the smaller sample size in the IFN+ group, which could explain that the difference was not statistically significant. Stratification of patients was also performed on the occurrence of relapse within

three months prior to sampling. Analysis of these data revealed similar results to those of IFN- β stratification, with lower *GATA3*, *FOXP3* and *EBI3* expression in both groups compared to controls and comparable expression between patients and controls of *TBX21* and *RORC* (data not shown). These results suggest that possible differences in pre-relapse conditions are not sufficient to influence the deviation of systemic CD4⁺ T cell immunity.

EBI3, which was decreased in patients (regardless of stratification), encodes a protein associated with immune regulation. In mice, it was shown that IL-35, a heterodimeric protein consisting of *EBI3* and *IL-12p35*, was critical for the suppressive function of a particular population of T_{REG} (Collison et al. 2007). In human, a possible role of IL-35 secretion by T_{REG} is still debated (Bardel et al. 2008, Chaturvedi et al. 2011, Chaturvedi et al. 2013). If important for immune regulation, our finding of decreased expression in blood might be of interest. A defect in IL-35 production might deregulate encephalitogenic CD4⁺ T cells.

In addition to direct comparisons of CD4⁺ T cell transcription factors, we also analysed the balance of pairs of reciprocally opposing transcription factors. *TBX21* and *GATA3* are regarded to inhibit the expression of each other (Zheng et al. 1997, Ouyang et al. 1998, Szabo et al. 2000), respectively, as are *FOXP3* and *RORC* (Ichiyama et al. 2008, Zhou et al. 2008). This implies that by comparing two opposing transcription factors in relation to each other may reveal overall patterns in CD4⁺ T cell deviation. Therefore the ratios of *TBX21/GATA3* and *RORC/FOXP3* were investigated in patients and healthy controls. In RR-MS patients, the *RORC/FOXP3* ratio was significantly increased, representing a relative overweight of *RORC* due to under-expression of *FOXP3*. However, when investigating the ratio of *TBX21/GATA3*, no significant difference between the groups was observed (Fig. 10).

Whether the increased *RORC/FOXP3* expression ratio in RR-MS represents an assessment of per-cell concurrent expression of transcription factors or the occurrence of separate CD4⁺ T cell subsets (*i.e.* T_H17 and T_{REG}) with lineage-specific expression, this finding highlights the importance of *FOXP3* and T_{REG} function. T_H17 cells have been suggested to be instrumental in the pathogenesis of MS as shown by studies in both mice and humans (Gran et al. 2002, Cua et al. 2003, Tzartos et al. 2008). Regarding *FOXP3* expression in RR-MS observations of

Figure 10. *TBX21/GATA3* (top) and *RORC/FOXP3* expression fold change in MS and healthy controls. Bars and whiskers show median and 25th/75th percentiles.

both decreased (Huan et al. 2005) and increased expression exist (Haas et al. 2005, Ito et al. 2008). Speculatively, a defect in the T_{REG} population in MS could result in uninhibited T_H17 responses, aggravating neuroinflammation. Furthermore, the similar *TBX21/GATA3* expression ratio in patients and controls may suggest that, although *GATA3* expression was decreased in simple group comparison, T_H1 and T_H2 cells are still in balance systemically in RR-MS. T_H1 cells was long thought to be centrally responsible for MS pathology (Lassmann et al. 2004), in humans highlighted by the observation that IFN- γ aggravated disease (Panitch et al. 1987). Taken together, the lack of suppressive T_H2 and T_{REG} associated expression is in line the notion that both T_H1- and T_H17-associated responses play a role in CNS inflammation, supported by the previously suggested role of IL-17⁺IFN- γ ⁺ cells (Durelli et al. 2009, Kebir et al. 2009).

4.2 T_{REG} in MS

4.2.1 Frequency of T_{REG} in RR-MS (Paper I, II)

Figure 11. Typical picture of gating of T_{REG} cells, defined as CD25^{hi} (upper right quadrant (Q2)), CD4^{dim}CD25^{hi} (bold square) and total CD4⁺ cells (squared line). Cells were gated from CD3⁺ cells.

In addition to assessment of CD4⁺ T cell transcription factors, 3-color flow cytometry was used in Paper I to investigate the frequency of CD4⁺ T cells and classically defined T_{REG}. Lymphocytes (defined through FSC/SSC), CD3⁺ and CD3⁺CD4⁺ cells were first compared between controls and patients. When analysing relative frequency of cells, MS patients were found to have an increased proportion of lymphocytes and a decreased proportion of CD4⁺ T cells as compared with controls. Importantly, these populations did not differ in the absolute number of cells when comparing RR-MS patients to controls.

Gating of T_{REG} was based on the classical definition of human T_{REG} as being CD4^{dim}CD25^{hi} (Baecher-Allan et al. 2001, Baecher-Allan et al. 2006, Mjosberg et al. 2009) (Fig. 11). These cells have previously been shown to be FOXP3⁺ and CD127^{low} and display suppressive function (Mjosberg et al. 2009). The frequency of CD4^{dim}CD25^{hi} T_{REG} did not differ between patients and controls and was found to constitute approximately 1.8% of the CD4⁺ T cells population, in line with previous findings (Baecher-Allan et al. 2001). Taken together with the decreased *FOXP3* expression observed in RR-MS, these data may imply a per-cell decrease in functionally competent CD4^{dim}CD25^{hi} T_{REG} contributing to systemic *FOXP3* expression. Similarly, in Paper II, we also assessed CD4^{dim}CD25^{hi} T_{REG} in patients and controls without finding any difference in the percentage of these cells in blood. Our observations are supported by others also failing to detect differences in the size of the peripheral T_{REG} pool (Putheti et al. 2004, Huan et al. 2005).

4.2.2 Regulatory T cells in MS: analysis of subpopulations (Paper II)

The finding of decreased *FOXP3* expression in Paper I prompted a more careful investigation of T_{REG} cells in RR-MS. Again, patients with RR-MS were compared to healthy individuals. In this study, we defined three distinct T_{REG} populations; classically defined T_{REG},

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characterised as $CD4^{dim}CD25^{high}$ as in Paper I (cT_{REG}), resting T_{REG} defined as $CD4^{+}CD25^{int}CD45RA^{+}$ (rT_{REG}) and activated, $CD4^{+}CD25^{high}CD45RA^{-}$, T_{REG} (aT_{REG}) (Fig. 12a, b). These three populations were analysed and further characterised using 6-color flow cytometry of whole blood from patients and controls.

Initial analysis revealed that CD45RA may be used to readily discriminate between the two distinct T_{REG} populations of rT_{REG} and aT_{REG} (Fig. 12a). Back-gating of rT_{REG} and aT_{REG} showed that the cT_{REG} gate encompasses a population mainly constituted of $CD4^{+}CD25^{high}CD45RA^{-}$ cells, which was expected due to the use of CD25 as one of the discriminating variables in the definition of all three populations. However, there was a slight overlap in CD25 of rT_{REG} and aT_{REG} (as seen in Fig. 12b), resulting in “contamination” by rT_{REG} in the cT_{REG} gate. Typically, around 80% of cT_{REG} cells are aT_{REG} while the remaining cells are rT_{REG} . When comparing the frequency of cT_{REG} , rT_{REG} and aT_{REG} between patients

Figure 12. a, b: Definitions of T_{REG} populations; activated $CD4^{+}CD45RA^{-}CD25^{high}$ T_{REG} (aT_{REG} , a), resting $CD4^{+}CD45RA^{+}CD25^{int}$ T_{REG} (rT_{REG} , a) and classically defined $CD4^{dim}CD25^{high}$ T_{REG} (cT_{REG} , b). Conventional responder T cells (T_{resp} , b) used in co-culture experiments were defined as $CD4^{+}CD25^{-}$. c, d: Gates for FOXP3 (c) and CD39 (d). The plots shown include all $CD4^{+}$ cells.

and controls, no significant differences in the size of T_{REG} populations were present, in line with the previous findings from Paper I, but here also involving subsets of T_{REG}.

We also investigated the frequency of CD4⁺ T cells expressing the functional T_{REG} markers FOXP3 and CD39 (according to the gates shown in Fig. 12c, d). Neither the FOXP3⁺ nor the CD39⁺ populations (expressed as % of CD4⁺ cells) were present at different frequencies in RR-MS compared to controls.

The expression of CD39 and FOXP3 was also investigated within T_{REG} subpopulations, using the gates shown in Fig. 12. For FOXP3, aT_{REG} was the subpopulation exhibiting the highest expression, around 80-85%. Furthermore, cT_{REG} also showed a high FOXP3 expression, an expected finding due to the high interdependence of the definition of the cT_{REG} and aT_{REG} populations. rT_{REG} displayed the lowest FOXP levels, significantly lower than both cT_{REG} and aT_{REG} when comparing frequencies across all study participants (patients *and* controls).

Comparing the FOXP3 expression between controls and patients in all three T_{REG} subpopulations failed to reveal any significant differences (Fig. 13a), although a tendency ($p < 0.1$) of lower expression was shown for MS patients in the rT_{REG} population. A similar pattern of expression was seen for CD39, being significantly higher than both cT_{REG} and rT_{REG}; cT_{REG} CD39 expression was lower than that of aT_{REG} but significantly higher than rT_{REG} CD39 expression. Also, no significant differences were seen when comparing patients to controls for CD39 expression within the separate populations (Fig. 13b). Helios, a member of the Ikaros family of transcription factors, has been proposed as a discriminator between thymically derived, natural T_{REG} and peripherally induced T_{REG} (Thornton et al. 2010).

Analysis of Helios expression in a subset of the included patients and controls showed that, as was shown for FOXP3, aT_{REG} and cT_{REG} expressed Helios to the highest degree, significantly higher than the rT_{REG} (Fig. 13c). These data contradict the notion of Helios as a marker for thymic T_{REG}. Thymic emigrant T_{REG} are thought to be CD45RA⁺, as they are naïve and non-activated. As the cells mature peripherally, including switching to an activated/memory phenotype CD45RA is down-regulated in preference for CD45R0 with a concomitant switch from Helios⁺ to Helios⁻. In contrast, we observe the highest Helios expression in CD45RA⁻ aT_{REG} cells, displaying an activated phenotype. Thus, we support the notion, previously proposed by others (Gottschalk et al. 2012, Himmel et al. 2013), that Helios expression correlates with activation in human T_{REG}, rather than origin.

Figure 13. Expression of FOXP3 (a), CD39 (b) and Helios (c) in different T_{REG} populations. MS: patients with MS; ctrl: healthy controls. Data was analysed with a one-way ANOVA employing Tukey's post-hoc test. Bars and whiskers show mean and SEM. *: $p < 0.05$. **: $p < 0.01$. ***: $p < 0.005$.

Fig. 14 depicts typical FOXP3 and CD39 expression, respectively, in the three T_{REG} subpopulations. For FOXP3, it may be observed that cT_{REG} constitutes a population with intermediate FOXP3 expression (Fig. 14a), compared to aT_{REG} and rT_{REG}, while all three populations display higher expression than T_{RESP}. For CD39, however, the expression in the aT_{REG} and rT_{REG} populations is more diverse, and analysis of cT_{REG} show that CD39 expression in this population follows a more binary distribution, corresponding to the separate resting and activated populations, respectively (Fig. 14b).

Figure 14. Expression of FOXP3 (a) and CD39 (b) in cT_{REG}, aT_{REG} and rT_{REG} demonstrating the constitution of the cT_{REG} population and difference in expression of aT_{REG} and rT_{REG}. Black: T_{RESP}, teal: cT_{REG}, purple: rT_{REG}, green: aT_{REG}.

Figure 15. Suppression of responder T cell CD69 expression in 18 h co-cultures with cT_{REG} (a), rT_{REG} (b) and aT_{REG} (c). Y-axis show suppressive index, where 0 corresponds to no suppression. MS patients (filled squares) compared to controls (empty squares). Boxes show mean values and whiskers show standard errors. SI: suppressive index

4.2.3 T_{REG} functional deficiency in MS: activation and proliferation (Paper 11)

It has previously been shown that T_{REG} in MS display impaired suppressive capabilities (Viglietta et al. 2004, Haas et al. 2005, Kumar et al. 2006, Venken et al. 2008, Baecher-Allan et al. 2011), permitting continued activation and effector function of non-inhibited effector populations. Therefore co-culturing experiments were performed to confirm this hypothesis, using the above defined T_{REG} subpopulations. While earlier observations were based on the suppression of proliferation of effector cells using H³-thymidine or CFSE proliferation assays, we also wanted to study suppression of activation. To achieve this, an 18 h activation-suppression assay was developed. After investigating several different rapidly induced activation markers of T cells, we chose CD69, an activation marker which is one of the earliest to be upregulated following T cell stimulation (Rangel et al. 2004). CD69 exhibited stable expression in repeated measurements of individuals repeatedly stimulated with anti-CD3/anti-CD28 antibodies. In addition, CD69⁺ cells can be readily identified using flow cytometry. In order to study the activation and proliferation of cells, sufficient stimulation of the responder population is required. We therefore performed a titration assay of anti-CD3/anti-CD28 monoclonal antibodies (mimicking TCR-associated activation of cells) with CD69 as the readout. 0.5 µg/mL of both anti-CD3/anti-CD28 antibodies were chosen, inducing a reasonable activation level of approximately 40-70% after 18 h. Since we also wanted to study proliferation, a CFSE-based proliferation assay was developed alongside the activation assay. Cells were here cultured for five days with the same anti-CD3/anti-CD28 concentrations.

Figure 16. Suppression of responder T cell proliferation co-cultures with rT_{REG} (a) and aT_{REG} (b). Y-axis show suppressive index, where 0 corresponds to no suppression. CFSE dilution was used to assess suppression of proliferation. MS patients (filled squares) compared to controls (empty squares). Boxes show mean values and whiskers show standard errors. SI: suppressive index. *: $p < 0.05$.

From whole blood, PBMC were extracted, followed by immunomagnetic negative selection of CD4⁺ cells which were labelled for flow cytometry sorting. CD4⁺CD25⁻ effector T cells ('responder' T cells; T_{RESP}; Fig. 12b) and the different T_{REG} populations were sorted with high purity and used for co-culturing assays where T_{RESP} was cultured alone (to establish baseline activation/proliferation) together with varying amounts of T_{REG}. For activation-induced CD69 expression, a dose-response relationship was observed in co-cultures of T_{RESP} and all tested T_{REG} populations, *i.e.* activation was inversely correlated to the size of the fraction of T_{REG} in the co-culture (Fig. 15a-c). There were no significant differences when comparing the suppressive function of different T_{REG} populations from patients to controls. In five day co-cultures assessing suppression of proliferation a similar pattern was observed (Fig. 16a, b).

However, the suppressive capacity of aT_{REG} from RR-MS patients was found to be significantly lower than that of controls in co-cultures of 1:0.25 T_{RESP}:aT_{REG} ratio (25 000 T_{RESP} and 12 500 aT_{REG}) (Fig. 16b). Interestingly, co-cultures with aT_{REG} from patients resulted in a higher proliferation of T_{RESP} than in cultures of T_{RESP} alone. A representative example of CFSE dilution of a patient and a control is shown in Fig. 17. Previous studies displaying a diminished anti-proliferative capacity of T_{REG} in RR-MS have not distinguished between activated and resting phenotypes. However, it seems plausible that such a reduction of suppression would be present in the activated pool of T_{REG}, representing the actuators of T_{REG} suppression. On the other hand, we failed to reveal a similar defect in the higher T_{RESP}:aT_{REG} ratio (more T_{REG} in relation to T_{RESP}). Physiologically, the proportion of T_{REG} would be expected to constitute a minority of the whole CD4⁺ T cell population (that is, both

Figure 17. Typical data showing the CFSE^{dim} fraction in aT_{REG} co-cultures from a MS patient and a healthy subject, compared to the uninhibited proliferation seen in responder T cells single-culture (Tresp 1:0).

effector and regulatory T cells). When comparing suppression of proliferation between aT_{REG} and rT_{REG}, even if not significant, a pattern of lower suppressive capacity may be identified even for aT_{REG} at the higher T_{RESP}:T_{REG} ratio. We speculate that the comparable suppressive function of aT_{REG} at this higher ratio between patients and controls may be an effect contributable to the sheer increase in numbers in this setting. A defective T_{REG} function could still be present but may be overcome by the non-physiological high proportion of T_{REG} at the time of effector T cell stimulation. It was furthermore shown by Michel and colleagues that by employing CD127^{high} cells from sorted CD4⁺CD25^{high} T_{REG}, the previously observed anti-proliferative dysfunction could be restored, *i.e.* the sorting strategy often employed was flawed due to contamination of non-T_{REG} cells in a presumed pure T_{REG} population (Michel et al. 2008). Although CD127 expression was not used in our study to define T_{REG}, it has been shown that both the rT_{REG} and aT_{REG} populations are enriched for CD127^{low} cells (Miyara et al. 2009). Due to the high FOXP3 expression of resting and activated T_{REG}, this is expected since CD127 is transcriptionally repressed by FOXP3 (Liu et al. 2006). The discrepancy seen between early (CD69) and late (proliferation) stimulatory responses is interesting, and may implicate that a putative T_{REG} dysfunction affect these cellular processes in different ways and/or on different levels. Given the nature of MS, it would be expected that defects affecting the temporally later process of proliferation would be more important and the setting of chronic neuroinflammation.

4.2.4 Global gene expression of T_{REG} in MS: a role for chemokines? (Paper II)

To further study the functional defect we found in T_{REG} in MS, we performed microarray analysis of highly purified T_{REG} cells from patients and controls. The sorted T_{REG} consisted of pooled aT_{REG} and rT_{REG}. Initially, we performed standard microarray analysis to find genes with significant differential expression between patients and controls. The model was corrected for multiple comparisons using FDR according to Benjamini-Hochberg with a $q < 0.05$ considered statistically significant. Based on these comparisons, no single genes were found to be significantly differentially expressed between the groups. Owing to the limited sample size (only five individuals in each group) and the large number of genes this might have been expected. On the other hand, as is the case in many high-throughput applications on human samples, differences between the samples might be too low to be detectable with these rather strict statistical criteria. Based on these notions, we used a different strategy for the next step in the analysis.

The major determinant of the correction of multiple testing in the FDR post-hoc test is the number of comparisons made. To counteract this we sought to reduce the number of entries of interest in the microarray probe set. Of all the probes present on a microarray a large proportion is constituted of non-mRNA probes. To include only probes of interest, we employed three filtering steps to reduce the number of probes available for analysis. First, probes which across all arrays exhibited a very low signal (≤ 1.5 times background fluorescence of the chip) were excluded. Second, since the mRNA transcriptome was the focus of the study, we chose to exclude probes not belonging to this category. Excluded probes were belonging to one of the following categories; long intergenic non-coding RNA, mitochondrial RNA, ribosomal RNA, microRNA and miscRNA. Furthermore, mRNA probes representing unannotated genes were excluded. We also employed a third strategy to increase the specificity of the compared set; using publically available whole-genome CpG methylation datasets (E-GEOD-49667, GSE49667) we were able to identify a number of genes (albeit very few) hypermethylated in CD4⁺ T cells, compared to non-CD4⁺ cells. We hypothesised that these genes would be of low relevance in the T_{REG} transcriptome, although one might discuss the process of the possible differentiation of naïve CD4⁺ T cells into induced T_{REG}. In total, out of the ~59 000 probes on the chip, ~18 000 were included for analysis using these strategies.

Using the same analysis strategy as described above, the new probe set was compared between T_{REG} from patients and controls. Even using this heavily reduced set we failed to detect any statistically significant differentially expressed genes (with $FDR\ q < 0.05$).

Instead, using a systems biology approach, we employed gene set enrichment analysis (GSEA) to detect patterns in data (Mootha et al. 2003, Subramanian et al. 2005). In GSEA, fold-changes of groups of genes in the same direction are investigated for enrichment in pre-defined gene sets. We chose to investigate gene ontology (GO) gene sets for enrichment. Gene sets with a size of over 15 and less than 500 genes were chosen, excluding small and extremely large generic sets; a total of 890 GO gene sets were included for the analysis. A random distribution was calculated using 1000 permutations of the phenotype labels (*i.e.* MS or controls) for each set. Since GO gene sets were used replicate gene symbols were averaged prior to GSEA, resulting in a total of 13406 genes. In MS, one gene set was found to be enriched compared to controls; ‘Chemokine signaling pathway’ (GO:0070098). In this set 10 genes were found to contribute to the core enrichment profile, all displaying a positive fold-change in MS versus controls when comparing the original gene expression data (Table IV and Fig. 18a). Six gene sets were found to be enriched in controls, *i.e.* displaying a negative

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fold-change when comparing MS to controls, which was confirmed in the original microarray analysis data. The identified gene sets were mainly involved in mitosis and proliferation (GO:0005819, GO:0000922, GO:0007059, GO:0005769, GO:0003968, GO:0000819) (Table IV and Fig. 18b).

The use of microarray as an assessment of global transcription is of tremendous use in biology, and the amount of microarray assays performed each year have increased exponentially just in the last few years. In this study, we used microarray aiming to detect differentially expressed genes in purified T_{REG} samples from patients and controls. One major restriction we encountered was the limitation of material available for the assay. First, we only wanted to include patients that were non-treated, an inclusion criterion that severely limited the number of patients available for inclusion. Second, the scarcity of aT_{REG} and rT_{REG} in whole blood samples of included individuals constituted another limitation; for successful microarray analysis a certain amount of extracted RNA is required. Due to this, only five patients and five controls were included in the final study, where shortcomings of the efficiency of the RNA extraction method used played a major role. The failure to identify

Table IV. Enriched pathways in GSEA

Gene set	NES	FDR
Enriched in MS		
Chemokine receptor binding	1.760	0.228
Enriched in controls		
Spindle	-1.922	0.071
Spindle pole	-1.867	0.114
Chromosome segregation	-1.926	0.134
Early endosome	-1.769	0.223
RNA polymerase activity	-1.732	0.237
Sister chromatid segregation	-1.780	0.247

NES: normalised enrichment score; FDR: false discovery rate.

differentially expressed genes in the original analysis of gene expression data was explainable, and not completely unexpected. Also, the use of whole blood as the studied tissue might not be completely representative of T_{REG} selected for suppression of autoreactive effector T cell clones giving rise to disease development and/or progression. These encephalitogenic T cells, along with suppressing T_{REG} would be expected to be found inside the CNS.

Despite the shortcomings discussed, the use of GSEA as a systems biology approach was able to display enrichment of groups of genes, rather than the single comparisons traditionally used for analysis of global gene expression data. Interestingly, the only gene set enriched, 'Chemokines signalling pathway', in MS was distinctly related to immune regulation and function. The genes contributing to the core enrichment profile included 10 chemokine ligands. Chemokines are important in controlling and directing the immune response through chemotactic recruitment of specific cell populations (Charo et al. 2006). Indeed, even T_{REG} have been found to express chemokines, including CCL2 through CCL5 in human (Himmel et al. 2011) and T_{REG} secretion CCL3 through CCL5 have been demonstrated in mice (Nguyen et al. 2011). The remaining chemokines enriched here have to our knowledge not been previously reported as being part of the T_{REG} secretory repertoire. In this study, CCL3, CCL4 and CCL5 were all constituents of the core enrichment profile. These chemokines are traditionally associated with recruitment of neutrophils, monocytes and T cells to the site of inflammation (Castellino et al. 2006, Charo et al. 2006, Chou et al. 2010). CXCL14 and CCL27 are involved in macrophage and T cell homing to the skin but have not been implicated in neuroinflammatory disease (Kurth et al. 2001, Sigmundsdottir et al. 2007). However, CXCL14 is abundantly expressed in the CNS of mice, although the significance of this finding is still unclear (Hara et al. 2012). C-X3-C motif ligand (CX3CL) 1 has on the other hand been implicated in EAE in which it was found to be involved in NK cell migratory patterns (Huang et al. 2006). CCL19 is a chemokine directing the migration of naïve lymphocyte populations and dendritic cells to secondary lymphoid organs through binding of CCR7 (Mori et al. 2001). The roles of CCL15 and CCL16 are currently less well defined. CXCL4, associated with coagulation has recently been implicated in Th17 differentiation (Shi et al. 2014). Even though not studied extensively, the possible role of chemokine secretion of T_{REG} at the site of inflammation is intriguing. By secretion of different classes of chemokines, T_{REG} may influence the suppression of immune reactions through the recruitment of cells either contributing to or inhibiting inflammation. Although highly speculative, an increase in

chemokine secretion of T_{REG} in MS could lead to recruitment of populations of immune cells aggravating inflammation.

In T_{REG} of healthy controls, many of the enriched pathways may be identified as important in mitosis, including genes regulating the mitotic spindle and segregation of chromosomes (Table IV). T_{REG} are known to be hypoproliferative *in vitro* but can be induced to divide under certain conditions *in vivo* (Takahashi et al. 1998, Walker et al. 2003). Again highly speculative, it would be of interest to investigate if the T_{REG} from MS patients compared to controls, display a lower tendency to proliferate, perhaps denoting an “exhausted” phenotype.

4.2.5 An alternate explanation to decreased T_{REG} function (Paper III)

While some putative defect in the T_{REG} population’s ability to control effector T cell responses is suspected to play a role in MS pathology, based on the observed unsuppressed proliferation that is evident in co-culturing assays, other explanations to this phenomenon could be considered. One obvious possibility is an activated state of effector T cell populations that is refractive to T_{REG}-mediated suppression, as has been shown in mice in the settings of type 1 diabetes (D’Alise et al. 2008). Although not extensively studied in MS, it has been shown that the T_{REG} dysfunction is primarily affecting the ability of human T_{REG} to suppress Tbet⁺ T_H1 cells (Frisullo et al. 2009).

In Paper III, we used a lymphocyte responsiveness assay was used to investigate the responses of CD4⁺ and CD8⁺ T cells toward different antigen and mitogens in MS patients and controls. As described below, the main aim of this study was to investigate the effects of natalizumab treatment on peripheral lymphocyte populations. However, since the responsiveness assay included patients before treatment in addition to post-treatment and healthy subjects, parts of the results are discussed in the present context.

Figure 19. Gating strategy for identification of lymphoblasts. **a:** Gating of lymphocytes based on FSC/SSC properties in unstimulated cultures; the lymphoblasts were defined as cells with higher FSC and SSC than lymphocytes. **b:** Example of a stimulated culture (PWM), showing the relation between the lymphocyte and lymphoblast populations under stimulation.

The assay investigating lymphocyte responses was based on whole blood cultures, in which the numbers of responding lymphocytes were assessed through flow cytometry. Lymphocytes were gated through FSC/SSC characteristics of unstimulated cells. Lymphoblasts were then gated using the lymphocytes as a negative gate (Fig 19). When comparing patients before treatment with control subjects, it was apparent that lymphocyte responses toward some of the included antigens and/or mitogens were decreased in patients. Specifically, MS patients responded with a decreased frequency of lymphoblasts toward Influenza and CMV compared with controls (Fig. 20a, b). The observation for Influenza was reflected when assessing absolute cell numbers in T cell subpopulations; decreased numbers of lymphoblasts were found in the total $CD4^+$ population as well as in the activated $CD4^+CD108^+$ T cell population stimulated with Influenza in MS patients before treatment (Fig. 20c, d).

Figure 20. Conversion of lymphocytes to lymphoblasts after antigen stimulation in untreated MS patients (MS) compared to healthy controls (ctrl). **a, b:** Percentage of lymphoblasts (of all cells) in MS patients after stimulation with Influenza (**a**) and CMV. Bars represent mean. (**b**). **c, d:** Number of lymphoblasts in cultures stimulated with Influenza, depicting both total CD4⁺ (**c**) and activated CD4⁺CD108⁺ (**d**) cells. Bars and whiskers denote mean and SD. *: $p < 0.05$. **: $p < 0.01$. ***: $p < 0.005$.

Thus, we failed to find any indication that lymphocytes in MS (pre-treatment) were hyperresponsive to antigen stimulation. On the contrary, peripheral lymphocytes in MS showed signs of being hyporesponsive (further discussed below). This finding was based on studies of whole blood, but there is no reason to believe that lymphocyte-based studies would have given other results.

4.3 Natalizumab as a disruptor of lymphocyte function in MS (Paper III-IV)

Natalizumab has proven to be a valuable addition to the treatment arsenal used in RR-MS (Miller et al. 2003). In addition to the putative mechanism responsible for treatment effects, *i.e.* the sequestration of lymphocytes from the CNS, additional modes of action may be of importance. Interestingly, CD49d (α_4 -integrin), the target of natalizumab, has been implicated in co-stimulation and activation in addition to its importance as an adhesion molecule (Sato et

al. 1995, Coito et al. 2000). We were therefore interested in investigating the effects of natalizumab on lymphocytes, hypothesising that the effect of the treatment may not be solely attributable to its ability to regulate CNS lymphocyte entry. In Paper III we investigate the effect of natalizumab treatment on circulating lymphocyte populations while Paper IV investigates the occurrence of genes shared between diseases, specifically genes involved in the differentiation of CD4⁺ T cells. This is investigated using a systems biology approach employing network analysis. In addition, we investigated effects of treatment on transcriptomics of CD4⁺ T cells in MS and allergy *in vitro*.

4.3.1 Natalizumab effectively prevents lymphocyte entry into the CNS (Paper III)

In Paper III, peripheral blood of RR-MS patients was analysed using flow cytometry for phenotypic characterisation of lymphocyte subpopulations. In addition, a whole blood culturing assay was used to determine lymphocyte responses toward different antigens and mitogens.

In this observational study, we initially documented clinical parameters of patients, comparing these before vs. after treatment. At the one year follow-up, 34 patients had experienced no

Table V. Clinical and CSF data at baseline and one year of treatment

Clinical /CSF parameters	Baseline	Follow-up	<i>p</i>
EDSS	2.5 (0-7.0)	2.5 (0-8.0)	0.08
MSSS	3.82 (0.19-8.55)	3.20 (0.17-9.20)	<0.0005
MSIS-29			
physical	2.18 (1.00-4.75)	1.40 (1.00-4.20) ^a	<0.0005
psychological	2.11 (1.00-4.56)	1.44 (1.00-4.56) ^a	<0.0005
SDMT	48 (5-66)	50 (11-65) ^b	0.03
Total CSF wbc count	2.55 (0.2-28.0) ^c	1.1 (0.0-4.0) ^d	<0.0005
IgG index	0.92 (0.48-3.0) ^c	0.77 (0.45-2.4) ^d	<0.0005
Albumin ratio	4.4 (2.1-11.4) ^c	4.7 (1.8-10.1) ^d	0.3

Median values are given and range within parenthesis. n=40 unless stated otherwise. *p* refers to Wilcoxon signed rank test comparing baseline and follow-up. ^a n=37 because of lack of follow-up data. ^b n=38 because of lack of follow-up data. ^c n=38 since two patients refrained from lumbar puncture at baseline. ^d n=36 since four patients refrained from lumbar puncture at follow-up.

Abbreviations: EDSS=Expanded Disability Status Scale, MSSS=Multiple Sclerosis Severity Score, MSIS-29=Multiple Sclerosis Impact Scale 29, SDMT=Symbol Digit Modalities Test, wbc=white blood cell.

Figure 21. Principal component analysis (PCA) of gated flow cytometry data. **a:** Scree plot of principal components. **b:** Patients before (red) and after (blue) one year natalizumab treatment plotted against the three first principal components.

relapse, and the annualised relapse rate had decreased from 1.0 to 0.1 on treatment. Furthermore, MSSS decreased significantly as did MSIS-29 scores while SDMT increased. CSF parameters were also investigated, where the IgG index and total CSF white blood cell count decreased after treatment, as expected. Results regarding clinical and CSF parameters are summarised in Table V.

A flow cytometric panel encompassing a variety of markers was used for phenotyping of circulating lymphocytes before and after one year of treatment with natalizumab. Using an unbiased analysis of all gated data using principal component analysis (PCA), the three first components could describe 46% of the data variance (Fig. 21a). The two treatment groups (*i.e.* untreated and one year of treatment) were readily separable using the first three components (Fig. 21b).

The frequency of lymphocytes expressed as a fraction of total leukocytes increased significantly after treatment, an absolute increase of approximately 10-15% (Fig. 22a and Table VI). When analysing the composition and absolute numbers of the main lymphocyte populations ($CD3^+$, $CD4^+$ and $CD8^+$ T cells, NK cells and B cells), drastic changes were observed. Due to the fact that the lymphocyte population is composed of these discrete subpopulations it is possible to misinterpret the net changes when only taking proportions (%) into consideration. When assessing $CD3^+$ T cells, the relative percentage of this population

decreased when comparing one-year follow-up to pre-treatment (Fig. 22b) as was also true for CD4⁺ T cells (Fig. 22c, d). However, since the CD3⁺ T cell population is constituted mainly of CD4⁺ and CD8⁺ T cells, the decrease in the proportion of CD4⁺ T cells was indeed reflected by a concomitant increase in the proportion of CD8⁺ T cells. Both the proportion of NK cells and B cells increased after one year of treatment (Fig. 22c, d and Table VI). Even though the proportions of subpopulations are important we also assessed the absolute cell numbers (Table VI). As expected by the increase in percentage of total lymphocytes, the number of cells in all investigated populations increased. The largest increase was seen for NK cells and B cells, revealing an increase of 195% and 105%, respectively. Total CD3⁺ T cells showed an increase of 73%. These findings are in line with the putative mechanism of natalizumab treatment. Notably, however, is that the drastic increase in lymphocytes in peripheral blood cannot be solely explained by the prevention of BBB traversal; at a given moment in RR-MS in remission, the number of cells inside the CNS constitute a relatively small proportion of the total number of lymphocytes. Increases in lymphocyte populations, therefore, should be highly dependent on differentiated migratory patterns to other peripheral tissues. Natalizumab, preventing interaction of VLA-4 and VCAM-1, has been shown to

Table VI. Lymphocyte main populations in blood before and after treatment

Number (cells / μ L)	Baseline	Follow-up	Change	<i>p</i>
Lymphocytes	1989 $\pm$ 630	3889 $\pm$ 1163	+96 %	<0.0005
T cells	1501 $\pm$ 554	2591 $\pm$ 915	+73 %	<0.0005
CD4 ⁺	896 $\pm$ 285	1435 $\pm$ 397	+60 %	<0.0005
CD8 ⁺	502 $\pm$ 316	975 $\pm$ 599	+94 %	<0.0005
NK cells	277 $\pm$ 145	816 $\pm$ 248	+195 %	<0.0005
B cells	258 $\pm$ 182	528 $\pm$ 296	+105 %	<0.0005

Percentage of parent population (%)

Lymphocytes	27.7 $\pm$ 6.8	40.3 $\pm$ 7.0	+45 %	<0.0005
T cells	75.0 $\pm$ 7.0	66.1 $\pm$ 6.7	-12 %	<0.0005
CD4 ⁺	61.3 $\pm$ 9.7	56.6 $\pm$ 7.7	-8 %	<0.0005
CD8 ⁺	31.8 $\pm$ 8.4	35.9 $\pm$ 8.6	+13 %	<0.0005
NK cells	14.3 $\pm$ 6.6	21.4 $\pm$ 5.2	+50 %	<0.0005
B cells	12.5 $\pm$ 6.4	13.2 $\pm$ 5.1	+6 %	0.3

Mean $\pm$ SD, n=40 except for CD4⁺ where n=38. *p* values refers to paired samples t test comparing number of cells and percentage of parent population, respectively, at baseline and follow-up. Change refers to difference between baseline and follow-up mean, given in % of baseline values.

Figure 22. Overview of lymphocyte populations in patients before and after one year of natalizumab treatment. **a, b:** Distribution of lymphocytes (% of total leukocytes; **a**) and T cells (% of lymphocytes; **b**). Comparisons are pairwise. Bars denote mean values. **c, d:** Relative distribution of discrete lymphocyte subpopulations before (**c**) and after (**d**) natalizumab treatment.

greatly influence the migration of cells over endothelium in the gastrointestinal tract (Ghosh et al. 2012). Furthermore, studies demonstrating an increase in the mobilisation of hematopoietic stem cells in the bone marrow could also influence the changes seen after treatment (Bonig et al. 2008).

Interestingly, different populations of lymphocytes appear to express varying levels of VLA-4; B cells have been shown to have higher expression than T cells (Niino et al. 2006). In addition, the amount of natalizumab binding to CD3⁺CD56⁺ NK cells was shown to be the higher than binding to CD19⁺ B cells, which in turn surpassed the binding capacity of CD3⁺ T cells (Harrer et al. 2012). These observations are in line with our findings where NK cells showed the highest increase (195%), followed by B cells (105%) and T cells (73%).

4.3.2 Natalizumab effects on lymphocyte subpopulations (Paper III)

Different subpopulations of the main lymphocyte populations were also analysed before and after treatment. In all these analyses, the proportion of cells is presented; within a population the proportions of different subpopulations is of greater relevance than the absolute cell numbers since they give information about the balance of different *e.g.* CD4⁺ T cells in addition to displaying the net change of population size after treatment.

Figure 23. Changes in peripheral CD4⁺ and CD8⁺ T cell subpopulations, comparing baseline to one year follow-up of natalizumab treatment. Pairwise comparisons. Bars show mean values, whiskers denote SD.

4.3.2.1 CD4⁺ and CD8⁺ T cells

For CD4⁺ T cells, a decrease in activated CD4⁺CD25⁺ cells was observed, while activated CD4⁺CD69⁺ and CD8⁺HLA-DR⁺ cells tended to increase after treatment (Fig. 23a-c).

A decrease in the proportion of activated cells is unexpected; immune cells participating in the neuroinflammatory reaction and now excluded from the CNS would be expected to be activated.

Possibly, activated cells are not entirely sequestered in the CNS during uncontrolled (*i.e.* untreated) neuroinflammation, resulting in occurrence of these cells in the periphery. Upon prevention of CNS trafficking during natalizumab treatment, the composition of immune cells in the periphery is altered (as shown in this study), affecting the activation pattern of cells. The reason for partly discrepant results (CD25 versus CD69/HLA-DR) is unknown, but may be due to the markers' different kinetic profiles. Frequencies of both CD4⁺OX40L⁺ and CD8⁺OX40L⁺ T

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cells decreased after treatment (Fig. 23d, e). The OX40:OX40L interaction has been shown to be of importance in the survival and clonal expansion of T cells while also influencing T cell-mediated cytokine secretion (Croft et al. 2009). A role in the promotion of T_H2 -associated immune responses has also been proposed (Ohshima et al. 1998). More recently, OX40L expression on activated T cells in mice was shown to require stimulation in the presence of T_H1 -associated cytokines (Mendel et al. 2006). Decreased OX40L expression on cells may reflect a general decrease in effector T cell responsiveness in the periphery after treatment. Although highly speculatively, this decrease might also impact the $T_H1:T_H2$ balance in the periphery. In contrast, the tendencies of increased CD69 expression in the $CD4^+$ and $CD8^+$ populations, together with the increased responsiveness seen in culturing assays (described below) may indicate an increase in peripheral responsiveness, at least against some stimuli.

Classically defined $CD4^{dim}CD25^{bright}$ T_{REG} showed a non-significant tendency to decrease after treatment (Fig. 23f). T_{REG} have previously been shown to express relatively low levels of VLA-4 (Stenner et al. 2008), a finding supporting the observation in our study. We also observed a significant decrease in terminally differentiated, senescent $CD8^+CD28^-CD57^+$ cells after treatment (Fig. 23g) (Strioga et al. 2011). Given the assumption that cells participating in the aberrant immune response in MS are activated memory cells, a dilution of the peripheral senescent pool is to be expected.

Figure 24. NK cell populations before and after treatment with natalizumab. Pairwise comparisons. Bars show mean values, whiskers denote SD.

4.3.2.2 NK cells

In the CD3⁻CD56⁺ NK cell population, a tendency to a decreased proportion of recently activated, CD69⁺ NK cells was observed (Fig. 24a). Interestingly, the fraction of cytotoxic CD56^{dim} cells increased significantly in the periphery, reflected by a corresponding decrease in CD56^{bright} regulatory, cytokine-secreting cells (Fig. 24b, c). In our study, an increase in the proportion of cytotoxic CD56^{dim} NK cells could be explained by the preferential tissue residence of this population within the CNS during the autoimmune reaction; blockade of CNS patrolling and infiltration cells would thus result in a larger increase in CD56^{dim} cells in blood. A larger increase in blood of CD56^{dim} cells than CD56^{bright} cells would result in a relative increased proportion of the former of the total CD3⁻CD56⁺ NK cell pool (comprising both CD56^{bright} and CD56^{dim} cells) which indeed was the observation here. In addition, the increased proportion of cytotoxic NK cells in blood after treatment could be influenced by the difference in migratory patterns of these cells compared to regulatory CD56^{bright} cells. CD56^{bright} cells reside mainly in secondary lymphoid organs while CD56^{dim} cells migrate to inflammatory sites (Campbell et al. 2001, Poli et al. 2009).

4.3.2.3 B cells

There was a statistically significant increased fraction of both CD19⁺CD27⁺ memory and CD19⁺CD25⁺ regulatory B cells in blood after natalizumab treatment (Fig. 25). An increase in memory cells together with a concomitant decrease in naïve B cells after natalizumab treatment has been shown previously (Haas et al. 2011, Planas et al. 2012). Taken together with the large increase in the total number of B cells, this finding underlines the implications of the B cell relevance in MS immunology. Recently, CD25⁺ B cells expressing FOXP3 were found in the blood and CSF of patients with MS during relapse (de Andres et al. 2014), although the regulatory B cell phenotype is most often characterised by secretion of IL-10 (Kessel et al. 2012). An increase in proportion after treatment could lend support to an active role of regulatory B cells in MS neuroinflammation.

Figure 25. B cell populations before and after one-year natalizumab treatment. Pairwise comparisons. Bars show mean values, whiskers denote SD.

4.3.3 Additional effects of natalizumab: lymphocyte response assay (Paper III)

In addition to phenotyping of lymphocyte populations after treatment, a whole blood culturing assay was also performed. Adapted from the FASCIA method (Svahn et al. 2003), the readout is the number of lymphocytes developing into lymphoblasts under different stimuli. In addition, we assessed this development rate in CD4⁺ and CD8⁺ T cells with and without expression of the activation marker CD108. Although part of these results has been discussed above in relation to T_{REG}, some of those results will briefly be discussed here as well.

In the initial analysis we observed increased responses of CD4⁺ and CD8⁺ T cells when comparing patients before and after one year of treatment. Specifically, CD4⁺ showed a significantly increased response towards both Influenza and PPD (Fig. 26a, b). CD8⁺ cells responded with a significant increase in magnitude towards PPD, PWM and CMV (Fig. 26c-e). Furthermore, activated CD4⁺CD108⁺ cells reacted more promptly toward Influenza (Fig. 26f). To put these results into context, healthy controls were also included and the responses of controls were compared to patients

Figure 26. Antigen and mitogen responses in T cells before and after treatment. Pairwise comparisons between patients pre- and post-treatment.

before and after treatment. Interestingly, a pattern of decreased responsiveness of lymphocytes in MS was observed when comparing pre-treatment patients to controls (Fig. 27). Comparisons of controls to post-treatment patients, however, failed to reveal any significant differences, while a few of the investigated stimuli were increased in the post-treatment group compared to pre-treatment. These data are based on assessment of absolute cell numbers and thus could be influenced by a decreased cell count in pre-treatment samples since all cultures were set using a fixed blood volume. To investigate if this was the case we compared the number of lymphocytes after seven days in the negative control cultures (only containing RPMI without stimulating agent). Interestingly, there were no differences in the number of cells in comparison between the pre-treatment and control cultures while significantly increased cell numbers were seen, as expected, when comparing post-treatment to both controls and pre-treatment cultures. This finding suggests that the decreased responses seen in pre-treatment cultures is not explained by low cell numbers in culture and that the

Figure 27. Comparisons of T cell responsiveness of controls and patients pre- and post-treatment. For visualisation purposes, data are normalised to the average of the healthy controls for the respective antigens/mitogen. One-way ANOVA with Tukey's post-hoc test. * $p<0.05$, comparing controls to pre-treatment patients. ¶ $p<0.05$, comparing pre- to post-treatment patients. Bars represent mean values and whiskers SEM.

restored responses after treatment could be attributable to an increased proportion of responding cells in the culture. Comparable responses of post-treatment patients and controls indicate that more cells are required for an adequate net reactivity of lymphocytes after natalizumab treatment.

Figure 28. Fraction of activated lymphocytes in response to Influenza (a) and CMV (b). Groups compared with Kruskal-Wallis test, using Dunn's post-hoc test. Median and interquartile ranges are shown. * $p<0.05$, ** $p<0.01$, *** $p<0.005$.

To further evaluate function on a cell-by-cell basis, we analysed the fraction of lymphoblasts responding to stimuli (calculated as the percentage of lymphoblasts in relation to all lymphocytes). The fraction responding to Influenza and CMV was lower in pre-treatment patients than controls. Post-treatment patients tended to have a larger lymphoblast fraction compared to pre-treatment values while displaying levels comparable to controls (Fig. 28). Thus, the increased responsiveness seen in patients post-treatment might in part be attributable to a component of increased per-cell responsiveness, in addition to an increase in lymphocyte cell numbers. A recent study also investigated patient responses before and after

natalizumab treatment and no functional difference could be identified in response to MBP and tetanus (Bornsen et al. 2012). However, older studies have observed that MS is associated with a decrease in T cell responses toward anti-CD3 (Brod et al. 1994), PWM (Walker et al. 1979) and viral antigens (Walker et al. 1979, Ilonen et al. 1981).

4.4 MS is sharing disease associated genes with other diseases (Paper IV)

Paper IV aimed to identify so called pleiotropic genes; genes common to a large number of diseases. The occurrence of pleiotropic genes might give important clues to disease pathogenesis, with implications for treatment and prognosis of disease progression. In addition, an experimental study of CD4⁺ T cells was conducted to investigate the influence of GC and natalizumab treatment *in vitro* for patients with SAR and MS, respectively. These responses were also used in an attempt to identify high- and low-responders (HRs and LR, respectively) to treatment in these two diseases.

4.4.1 Enrichment of CD4⁺ T cell differentiation genes in GWAS data

In a first step to identify shared disease genes, publically available GWAS data was analysed for identification of a cell type common to multiple diseases. Genes influencing risk of acquiring disease were analysed using pathway analysis. In total, the final dataset comprised 256 different diseases with 2 298 gene loci harbouring potentially associated SNPs were included. Interestingly, the CD4⁺ T_H cell differentiation pathway was found to be the most significantly enriched for genes identified in the GWAS analysis. Notably, the original GWAS dataset was not restricted to typical immune-mediated diseases but also included a variety of diseases not generally assumed to be associated with CD4⁺ T cells. The T_H differentiation pathway with identified GWAS genes is depicted in Fig. 29.

addition, 446 genes with known somatic cancer mutations were included (henceforth described as cancer susceptibility genes); significant enrichment of T_H differentiation pathway was found here as well. Lastly, 4 613 genes with known disease annotation from the Online Mendelian Inheritance of Man (OMIM) were also enriched for the T_H pathway. This validation resulted in the conclusion that genes in the $CD4^+$ T_H cell differentiation pathway were indeed of importance in a broad variety of diseases including those not traditionally associated with aberrant immune reactions. Accordingly, further analyses were restricted to gene expression data of $CD4^+$ T cells.

4.4.2 Disease-specific PPI networks identifies a pleiotropic module

In order to identify pleiotropic genes, publically available gene expression datasets of $CD4^+$ T cells were analysed in eight separate diseases. Typical immune-mediated diseases included MS, systemic lupus erythematosus, rheumatoid arthritis and allergy. In addition, four malignant or proliferative diseases were included; acute myeloid leukaemia, adult T cell leukaemia, chronic lymphocytic leukaemia and hypereosinophilic syndrome. For all diseases, gene expression data from patients was compared to healthy controls. As validation, we investigated if patients and controls could be distinguished by their original $CD4^+$ gene expression data using the LASSO approach, which was the case for all included diseases (Fig. 30).

Figure 30. Classification of patients/controls by $CD4^+$ T cell gene expression data using LASSO. RA: rheumatoid arthritis, CLL: chronic lymphocytic leukaemia, HES: hypereosinophilic syndrome, ATL: adult T cell leukaemia, AML: acute myeloid leukaemia, SLE: systemic lupus erythematosus, MS: multiple sclerosis, A: allergy.

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The initial gene expression analysis identified genes with differential expression between patients and controls. Next, these genes were mapped onto the human protein-protein interaction (PPI) network. If the identified genes were chosen by random, one would not expect any patterns in this mapping. However, analysis revealed that genes for each disease formed distinct modules that partially overlapped. Thus, the resulting inferred network consisted of eight, separate disease modules and a pleiotropic module resulting from the overlap of the disease modules. Comparing to a randomised null-distribution using 10^6 permutations, the occurrence of a random module with as high an overlap as the pleiotropic module was found to be highly improbable. The disease modules contained in the range of 1 200 to 1 900 genes each, while the pleiotropic module contained 158 highly and significantly interconnected genes. In total, 7 144 connections between the genes constituting the pleiotropic module were present, illustrating its high connectivity.

Since different PPI databases have different inclusion criteria we also examined the pleiotropic module in five separate, other databases covering different aspects of the human interactome. Shortest paths between 158 random genes in these networks were (from a null distribution) compared to the shortest paths of the pleiotropic module; in all cases, the average shortest path length was lower for the pleiotropic module.

Even though the data used to infer the pleiotropic module was derived from CD4⁺ T cells, pathway analysis of the constituting genes showed that these genes not only were partaking in pathways associated with inflammation, but also multiple other disease-associated pathways. Due to the high interconnectivity and pleiotropy of pathways enriched for genes in the pleiotropic module, mouse knockout data was analysed to investigate whether the pleiotropic genes were connected to disease susceptibility. Indeed, using a database of mouse knockouts, the pleiotropic genes were found to be associated with disease to a high degree when targeted for knockout.

There was a high, significant enrichment for GWAS disease-associated and cancer susceptibility genes in the pleiotropic module, that is, a higher fraction of genes associated with disease (GWAS) or cancer susceptibility was found to be within the pleiotropic than expected by chance. Fig. 31 shows the pleiotropic genes and correlation analysis of pleiotropy of genes and GWAS/cancer association. To further validate that the pleiotropic module indeed was pleiotropic for diseases other than immune-related disorders and cancer, we queried the

Figure 31. a: The pleiotropic module was enriched for GWAS disease-associated and cancer susceptibility genes. The inner circle represents nucleoplasmic genes and the outer circle genes outside of the nucleoplasm (according to GO). Therapeutic targets are marked with squares, biomarkers with tilted squares and hexagons represent both. Node colours are blue for GWAS disease-associated genes, yellow for cancer susceptibility genes and green for both. The 7 144 interactions between nodes are not shown for simplicity. **b:** correlation of the fraction of GWAS disease-associated and cancer susceptibility genes in modules versus the number of disease-specific modules. PCC: Pearson's correlation coefficient.

GWAS data again, this time removing any diseases that were not *a priori* classified as immune-related or cancer (comprising of 1 437 of the original 2 298 disease-associated gene loci). Even for these diseases/traits, the pleiotropic module was significantly enriched (19 of the pleiotropic genes were associated with disease).

To validate that the definition of the pleiotropic module, derived from gene expression data, was not responsible for the enrichment, we investigated the distribution of GWAS disease-associated and cancer susceptibility genes in the different disease-specific modules. There was a positive significant correlation of fraction of GWAS and cancer genes and the pleiotropy of these genes (*i.e.* the number of diseases-specific modules these genes were part of). Thus, when mapping the general GWAS and cancer susceptibility genes onto the disease-specific modules, a higher fraction of these genes were present in higher number of disease-specific modules, thereby showing that the enrichment seen was not dependent on the definition of the pleiotropic module (Fig. 31b).

In order to increase statistical power, the 158 pleiotropic genes were also analysed for enrichment in original MS GWAS data comprising approximately 25 000 individuals (IMSGC 2011). Identification of the original significant SNPs was based on genome-wide

significance levels. In this new, large GWAS data set, we defined a gene loci significant if it contained at least one SNP with a nominal p -value <0.05 . Using this method, we could confirm that pleiotropic genes were enriched for MS SNPs using p -values in the range of 10^{-5} to 0.05. By investigating all SNPs ($n=4\,990$), 150 SNPs covering 51 of the pleiotropic genes showed association with disease.

4.4.3 Potential therapeutic targets and biomarkers are enriched for pleiotropic genes

After the identification and validation of the pleiotropic module's link to disease development, we then investigated if the pleiotropic module was enriched for known diagnostic markers ($n=1\,177$) and/or therapeutic targets ($n=404$) in human diseases (Knox et al. 2011). Indeed, there was a significant enrichment both regarding markers and targets. In addition, with the hypothesis that functional therapeutic targets are likely to be functionally related, we analysed a database comprising of predicted PPIs with therapeutic potential (Sugaya et al. 2012). Interestingly, examination of druggable PPIs revealed 173 novel therapeutic target candidates. To further explore the relevance of the pleiotropic module in the context of treatment, gene expression data of 100 cancer cell lines exposed to 100 different drugs were assessed (Garnett et al. 2012), representing a wide variety of tissue types. Several drugs were shown to be correlated to gene expression changes in cell lines, supporting the notion that genes in the pleiotropic module could provide novel therapeutic targets.

To summarise, using GWAS data from a large number of diseases/traits as the point of origin, we were able to identify the $CD4^+$ T_H cell differentiation pathway as being important, a finding we were able to replicate in different settings. Using this knowledge, we used gene expression data from $CD4^+$ T cells from different non-immune- (proliferative/malignant) and immune-related complex diseases (including MS). Initial analyses showed that the classification of patients and controls was possible using only publically available gene expression data from patients with these diseases and healthy controls. Disease-specific modules were then constructed using network-based analysis. We then defined, based on the overlap of disease-specific modules, a network of highly interconnected genes (in a PPI network) common to many diseases. This pleiotropic module, that withstood several controls for confounding, contained a diversity of genes. Some were related to $CD4^+$ T cell differentiation and activation, including *GATA3*, *NFκB*, *STAT1* and *STAT3*, but also genes not

traditionally associated with immunity including the breast cancer-related *BRCA1*. However, a recent study has shown a possible role for *BRCA1* in regulating inflammation (Teoh et al. 2013). Recent observations from GWAS studies have suggested important etiologic potential for pleiotropic genes and proteins (Wellcome Trust Case Control 2007, Cotsapas et al. 2011), thus supporting the current findings. However, an important consideration when using GWAS data for analysis is the potential role of epigenetics influencing susceptibility to complex diseases (Feil et al. 2011). The use of gene expression data, which constitute the net result of genotype and epigenetic modifications, overcomes this limitation. The pleiotropic module was also used to identify novel therapeutic candidates.

4.5 Stratification of treatment outcome using pleiotropic or disease-specific gene expression (Paper IV)

Since the pleiotropic module was enriched for therapeutic targets, we speculated that differences in the expression of genes either in the pleiotropic or the disease-specific modules might be used to stratify patients for response to therapy. Patients with MS and seasonal allergic rhinitis (SAR) were investigated for treatment outcome. Low- and high-responders (LR and HR, respectively) in relation to treatment were identified. For MS patients, PBMC from LR and HR had previously been collected and stored in liquid N₂ before initiation of treatment. After thawing, CD4⁺ T cells were isolated using immunomagnetic selection. In SAR, PBMC cultures were set, and CD4⁺ T cell enrichment was performed after activation by allergen. Cells were moderately activated *in vitro*, using low-dose anti-CD3/CD28 antibodies and allergen in MS and SAR, respectively, in the presence or absence of natalizumab (MS) or glucocorticoids (SAR). Gene expression in CD4⁺ T cells from both LR and HR, with or without *in vitro* treatment was analysed with microarray.

Gene expression of CD4⁺ T cells activated in the presence of the treatment was compared to expression of cells activated in medium only. The differentially expressed genes were analysed for ability to separate LR from HR. For SAR, the pleiotropic genes that were differentially expressed (with/without GC treatment) were able to separate patients responding to therapy from low-responders. In contrast, patients responding to natalizumab

Figure 32: Classification of treatment response based on pleiotropic or disease-specific genes. **a:** GC exposure to SAR and natalizumab exposure to MS CD4⁺ T cells *in vitro*. X-axis: number of modules of common to genes affected by drug exposure; Y-axis: squared t statistics (t^2) of genes affected by drug exposure. **b:** Classification of LR/HR by gene expression using LASSO.

treatment were separable from low-responders using the genes specific for MS. Again using the gene expression results from *in vitro* cultures with or without GC/natalizumab, the likelihood of therapy response in multiple disease modules was investigated. For SAR, there was a significantly increased likelihood for response in several disease-specific modules, while no such relationship was found for natalizumab. As seen in Fig 32, genes affected by GC treatment tended to be part of multiple disease-specific modules, while a similar relationship for natalizumab could not be found. This finding was somewhat expected due to the previous finding that HR to GC was separable from LR using the pleiotropic module while natalizumab used the MS-specific module for separation. In addition, GC is known to influence a wide range of genes in immune cells while natalizumab constitutes a more specific therapy (Zhao et al. 2012).

Next, we tried to correctly classify HR and LR blindly using gene expression data from CD4⁺ T cells with or without treatment. Genes with differential expression were selected. Due to the high number of genes responding to GC in different disease-specific modules, we restricted the selection for GC to genes within at least two disease modules. Using LASSO, most patients were correctly classified as HR or LR. Analysis of the classifier used for the MS and SAR, respectively, revealed differences of the features used for classification. For GC, the GC pathway (from IPA) was used as classifier. In contrast, the natalizumab classifier utilised genes dispersed in different pathways in the MS-specific disease module. The main difference

for classifying MS LR and HR was instead the increased tendency of natalizumab to perturb the gene expression of LR to a higher degree than HR. That is, gene expression of LR was affected to a higher degree than gene expression of HR when exposing cells to natalizumab *in vitro*. This finding was highly unexpected. The shown *in vitro* effect of natalizumab to influence gene expression would be expected to affect responders to therapy to a higher degree than low-responders if the treatment effect outside prevention of BBB passage was one of the main modes of action. However, it is plausible that HR are refractive to natalizumab effects other than the prevention of CNS trafficking. In contrast the induction of changes in gene expression of LR, possibly due to genotype, baseline gene expression or epigenetic status, could affect pathways that might influence the activation or tendency to induce deteriorating immune responses in CD4⁺ T cells. This notion was not explored and further analyses are warranted.

5. CONCLUDING REMARKS

The pathogenesis of MS has been unknown since the discovery of the disease. Although several theories have been proposed and tested, the exact mechanisms have not yet been elucidated, although accumulated evidence from both humans and mice support a role for CD4⁺ T cells being central in the development and progression of disease. Encephalitogenic T_H cells, self-reactive towards a putative antigen coordinate the immune response, resulting in tissue damage and the emergence of clinical symptoms. Both T_H1 and T_H17 have been proposed as the main candidates, having the ability of transmitting disease in mice and encountered in the CNS of humans. Similarly, a role for T_{REG} in disease has been proposed based on the finding that T_{REG} in MS appears to be functionally deficient when compared to cells from healthy individuals. Treatment strategies of RR-MS are based on immune regulatory mechanisms, and natalizumab, targeting lymphocytes and disrupting migratory patterns, is an example of this. In addition to the primary mechanism, secondary effects of natalizumab have been proposed.

In Paper I we show an imbalance in mRNA expression of CD4⁺ T cell lineage specific transcription factors in whole blood in RR-MS. *TBX21* and *RORC*, specific for T_H1 and T_H17 cells, respectively, were similarly expressed in MS and controls. However, the T_H2 and T_{REG} associated *GATA3* and *FOXP3* were decreased in RR-MS. In addition, immune regulatory *EBI3* showed decreased expression. Given the reciprocally inhibitory nature of T_H populations, a decreased expression of T_H2 and T_{REG} transcription factors may permit differentiation of encephalitogenic T_H1 and T_H17 cells. The size of the classically defined T_{REG} population in peripheral blood was similar in MS and controls.

When further investigating of three populations of T_{REG} in MS in Paper II (classically defined, activated and resting T_{REG}) we found that FOXP3, CD39 and Helios were similarly expressed in MS and controls. However, we clearly showed that activated T_{REG} expresses all three markers to a higher degree than resting T_{REG}, both in patients and controls. Classically defined T_{REG} found to comprise mainly activated T_{REG}, also expressed FOXP3, CD39 and Helios to a higher degree than resting T_{REG}. When assessing the functional properties of T_{REG} we found resting T_{REG} to be functionally competent in suppressing responder T cells both regarding short-term activation and proliferation. However, although being able to suppress short-term activation of responder T cells, activated T_{REG} in MS failed to suppress proliferation of

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responder cells, in contrast to the suppression noted by T_{REG} from healthy subjects. The decreased suppressive capacity of T_{REG} in MS has been shown previously although activated and resting T_{REG} have never been separately assessed. We hypothesise that the activated T_{REG} population might be responsible for the previously observed defect, given the fact that the classically defined T_{REG}, which was often used in previous studies, population mainly consists of activated T_{REG}. Alternatively, a decreased suppressive capacity might be attributable to a refractory activation state of conventional T cells in MS. However, in Paper III, when investigating lymphocytes in whole blood cultures from MS patients stimulated with different antigens and mitogens, cells from patients appeared to be hyporesponsive when compared to controls. This applied both to lymphocytes and to CD4⁺ T cells for selected stimuli.

To further explore the nature of the functional defect of T_{REG} in MS in Paper II, we performed gene expression analysis using microarray. Gene set enrichment analysis revealed a collective over-expression of chemokine signalling genes. Even so far relatively unexplored in T_{REG} biology, a defect in chemokine signalling might possibly affect the migratory patterns of cells involved in the local immune response.

In Paper III, we studied the effects of natalizumab on lymphocyte population before and after one year of treatment. In line with the putative mechanism of action of natalizumab, there was a marked increase in all lymphocyte populations after treatment. The increase was most prominent in the NK and B cell compartments, although still substantial among T cells. Further analysis of NK cells revealed an increased proportion of cytotoxic CD56^{dim} NK cells with a concomitant decrease in regulatory CD56^{bright} cells. This finding could be the result of an increased propensity of cytotoxic CD56^{dim} cells to reside in the CNS during neuroinflammation. In the B cell compartment, there was an increase both in the proportion of regulatory CD25⁺ B cells and CD27⁺ memory B cells. This implies a sequestration of both of these cell types at the site of inflammation, demonstrating the reactionary nature of the immune response, with both pro- and anti-inflammatory components being present.

Furthermore, as mentioned above, the responsiveness of lymphocytes towards antigens and mitogens was assessed and we could show a decreased responsiveness in the CD4⁺ T cell compartment before treatment which was restored after treatment. Interestingly, this effect could not solely explained by the increase of lymphocyte numbers in peripheral blood, suggesting effects of natalizumab not trivially explained by its putative action of cell migration.

In Paper IV, the importance of CD4⁺ T cells in human disease was highlighted. Using public GWAS data from a large number of diseases, we showed an enrichment of genes involved in CD4⁺ T cell differentiation in genes associated with diseases. Interestingly, GWAS data comprised diseases not classically associated with the immune system. Using public gene expression data from CD4⁺ T cells in eight human diseases, including malignant/hyperproliferative disorders we were able to separate patients from controls using a logistic model. Using a network-based approach, PPI networks were constructed for each of the eight diseases were constructed based on the genes differentially expressed between patients and controls. Using the overlap of these eight modules a pleiotropic module was inferred. This highly interconnected module was found to be enriched for a potential therapeutic targets and biomarkers. These findings underline the importance of CD4⁺ T cells, not only in immune-related diseases. In addition, the pleiotropic module comprises transcription factors and other genes with great impact on differentiation and phenotype, thereby constituting interesting targets for future therapies.

We also investigated the pleiotropic module in the context of therapy prediction. MS and SAR patients with known response to therapy with natalizumab and GC, respectively, were selected. After *in vitro* treatment, we tried to classify low- and high-responders based on gene expression data alone. In MS, classification was successful, although, unexpectedly, gene expression differential expression was shown to be primarily associated with high-responders. In addition, in MS the genes affected by treatment tended to localise in the disease-specific module rather than the pleiotropic module.

To summarise, the presented findings are relevant not only in the context of expanding the knowledge on CD4⁺ T cells but also bears clinical significance with regard to future identification of biomarkers and development of novel therapies. The composition and balance of the CD4⁺ T cell compartment appear to be important, possibly containing clues to the permissive state of encephalitogenic T_H1 and/or T_H17 cells. In addition, the transcriptional profile of CD4⁺ T cells appears to be important even in non-immune related disorders, displaying the importance of the immune system in general human pathology.

Since their discovery T_{REG} have been shown to be of great importance in adaptive immunity although their role in treatment of autoimmune disease has yet to be firmly established. Even so, the possibility of using autologous T_{REG} to treat disease is intriguing, and is currently being studied. Our findings provide new avenues to explore in the field of T_{REG} biology as the

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role of chemokines has not yet been fully established. Furthermore, as evident by previous work in the field of T_{REG} biology, the definition of the studied population is of key importance. Here, the use of activated and resting T_{REG} definition may provide a more coherent picture to the sometimes discordant findings in T_{REG} research.

The influence of natalizumab on immunity is not yet fully elucidated. As demonstrated, the use of bioinformatics and transcriptomics may be one way to better identify patients who might benefit from natalizumab treatment, in addition to identify new possible biomarkers and therapy targets. Furthermore, peripheral immunity might be improved, while the effects inside the CNS are, as reported previously, dominated by a lack of immune surveillance and an increased risk of PML development

In conclusion, the results presented in this thesis indicate a role for dysregulation of CD4⁺ T cells in the pathogenesis of MS. Furthermore, the importance of CD4⁺ T cells in other diseases is also implicated. Finally, treatment with natalizumab confers profound effects on the composition of the peripheral lymphocyte compartment.

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