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Institutsdirektor: Prof. Dr. med. Bernhard Fleischer

Immunophenotypical and functional characterization of CD56^{bright} NK cells in multiple sclerosis

Dissertation

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Vorgelegt von:

Angélica Cuapio Gómez

aus Mexiko

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Prüfungsausschuss, 2 Gutachter/in: Dr. M. Fauser

Prüfungsausschuss, 3 Gutachter/in: PD Dr. T. Magnus

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Project aim and objectives

$CD56^{bright}$ NK cells are a lymphocytic subset with suspected immunoregulatory properties. In multiple sclerosis (MS), treatment with daclizumab results in an increase of $CD56^{bright}$ NK cells which correlates with a reduction in brain inflammation and improvement of clinical symptomatology in most patients. In addition to daclizumab, treatment with IFNβ-1a also induces an increase of this particular cell population.

To understand the role of $CD56^{bright}$ NK cells in the pathogenesis of MS, we aimed to investigate the phenotypical and functional properties of these cells in healthy donors and MS patients either untreated or treated with anti-CD25 monoclonal antibodies, IFNβ-1a and natalizumab.

Specific objectives

1. To determine the effect of treatment on the relative frequency of T and NK cell subsets in peripheral blood of MS patients treated with anti-CD25 mAb, IFNβ-1a and natalizumab.

2. To phenotypically characterize NK cells in MS patients using cell surface markers.

3. To assess intracellular cytokine production.

4. To determine the mechanism and dynamics of $CD56^{bright}$ expansion.

1. Introduction

1.1 Multiple Sclerosis

Multiple sclerosis (MS) is a disabling disease of the central nervous system (CNS) that constitutes the most common cause of neurological disability in young adults between 20 and 40 years. MS is an inflammatory T-cell-mediated autoimmune disease characterized by the breakdown of the nerve insulating myelin sheath and axonal degeneration. The neurological disturbances translate mainly into progressive accumulation of motor, cognitive, sensory, autonomic and emotional impairment. Several therapies for MS exist, although there is currently no cure.

1.1.1 Epidemiology of MS

MS affects women approximately twice as often as men and its incidence and prevalence vary geographically (Ebers 2008). The epidemiology of MS has been characterized by a north-to-south gradient. Areas with high frequency include Europe, southern Canada, northern United States, New Zealand, and southeast Australia (figure 1). In these areas the prevalence is more than 100 per 100,000. The highest reported rate of 300 per 100,000 is in the Orkney Islands, Scotland. The prevalence rate in Germany was estimated as 149 per 100 000 in 2008 (www.who.int).

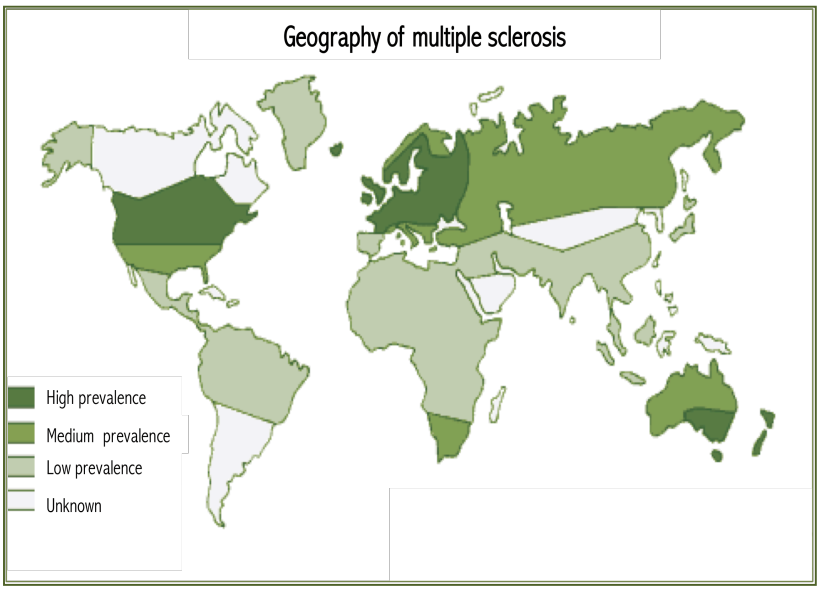

Figure 1. Epidemiology of MS
The five continents are depicted to show areas of high frequency (>60/100,000), medium prevalence of multiple sclerosis (5-60/100,000), those with low rates (<5/100,000), and areas of undetermined prevalence. Some regions are uncharted and these colors are only intended to provide an impression of the geographical trends.

1.1.2 The genetic factor

Studies in genetic epidemiology confirm that the environment acts at a population level indicating epigenetic modifications to germline susceptibility. Autoimmune diseases such as multiple sclerosis MS have a multiple genetic background (Ramagopalan et al. 2008). First-degree relatives of affected MS individuals have an approximately 2%–5% higher risk to develop MS. Studies of monozygotic twins suggest that 25–30% of MS risk is genetically determined and the risk rapidly drops to 3–5% with dizygotic twins, supporting the complex genetic susceptibility to MS and the importance of other factors (Ebers 2005).

1.1.2.1 MHC and non-MHC–related genes

Epidemiological and genetic studies have consistently identified associations with the major histocompatibility complex (MHC) class II alleles in several autoimmune diseases. The HLA-DR2 haplotype (DRB1*1501, DRB5*0101 and DQB1*0602) exerts the strongest effect on genetic risk for MS (Oksenberg et al. 2008 and Caillier, 2008). However, the precise contributions of its individual alleles and their modes of action remain poorly understood, due in part to the strong linkage disequilibrium in this region. For almost three decades the influence of non-HLA genes remained elusive, but recent Genome-Wide Association Studies in MS patients (MS-GWAS) have identified approximately 50 non-MHC risk loci for MS susceptibility (Sawcer et al. 2011). A large proportion of the associated genes in MS are involved in immune system processes such as 1) cell cycle and activation; 2) chemotaxis, adhesion and transendothelial migration; and 3) intracellular transport mechanisms (Infante-Duarte et al. 2005). Several genes are also associated with other autoimmune diseases like rheumatoid arthritis (RA) (CLEC16A, IL2RA, IL7RA, IRF5) or type I diabetes mellitus (CLEC16A, IL2RA, CD226) (Stahl et al. 2010).

1.1.3 The environmental factors

Epidemiological studies point to Epstein–Barr virus infection, sunlight exposure, vitamin D levels and dietary fatty acids as strong non genetic factors related to MS (Munger et al. 2006, Islam et al. 2007 and Fujinami et al. 2006). The time of the year at birth has also been implicated and individuals born in May have a higher risk for MS than those born in November, suggesting the gestational or neonatal environment or climate as a risk to develop the disease (Willer et al. 2005).

1.2 Clinical presentation and diagnosis

Around 80% of MS patients start their symptomatology with a clinically isolated syndrome (CIS). CIS describes a neurologic episode that lasts at least 24 hours and is caused by inflammation/demyelination in one or more sites in the CNS. The episode is monofocal when symptoms and signs are derived from only one clinical lesion. A multifocal episode is characterized by more than one lesion in CNS (Compston and Coles, 2008).

Eighty percent of the MS patients develop the relapsing-remitting form of the disease (RRMS), which is characterized by unpredictable relapses followed by relative quiet periods (remission) with no signs of disease activity. Gradually, the recovery of each episode is incomplete and the accumulation of symptoms persists. Around 65% of these patients evolve to a secondary progressive phase (SPMS), which develop a progressive neurological decline between each acute attack without any remission period. Around 10-15% of MS patients make their debut with a primary progressive subtype of the disease, the primary progressive form (PPMS), which is characterized by a progression of the disability from onset, without or only occasional and minor remissions and improvements (Fig. 2) (Compston and Coles, 2008).

The course of the disease is not predictable, but there are some disease-related factors that add some predictive value for a favorable prognosis, such as female gender, early age at the onset of disease (younger than 40), first attack consisting of optic neuritis or other sensory symptoms and non progressive disability 5 years after onset. The negative prognosis is seen in those patients with difficulty when walking or sustained impairment in coordination after the resolution of the first relapse, and those patients with a large number of lesions detected by magnetic resonance imaging (MRI) at the time of diagnosis (Tremlett et al. 2006).

The Kurtzke expanded disability status score (EDSS) is the most common used instrument to measure clinical disability in MS, quantifying disability in eight functional systems (pyramidal, cerebellar, brain stem, sensory, bowel and bladder, visual, cerebral and other)(Kurtzke 1983). This index uses numbers in a scale ranging from 0 for normal examination and function, to 10 for death due to MS. Scores from 0 to 4.5 refer to fully ambulatory patients, whereas values from 5.0 to 9.5 are emphasized on the impairment in deambulation. The average time spent with an EDSS score of 1 is 4 years, while the average for an EDSS score of 4 to 5 is 1.2 years and a score of 6 is 3 years (Weir et al. 2002).

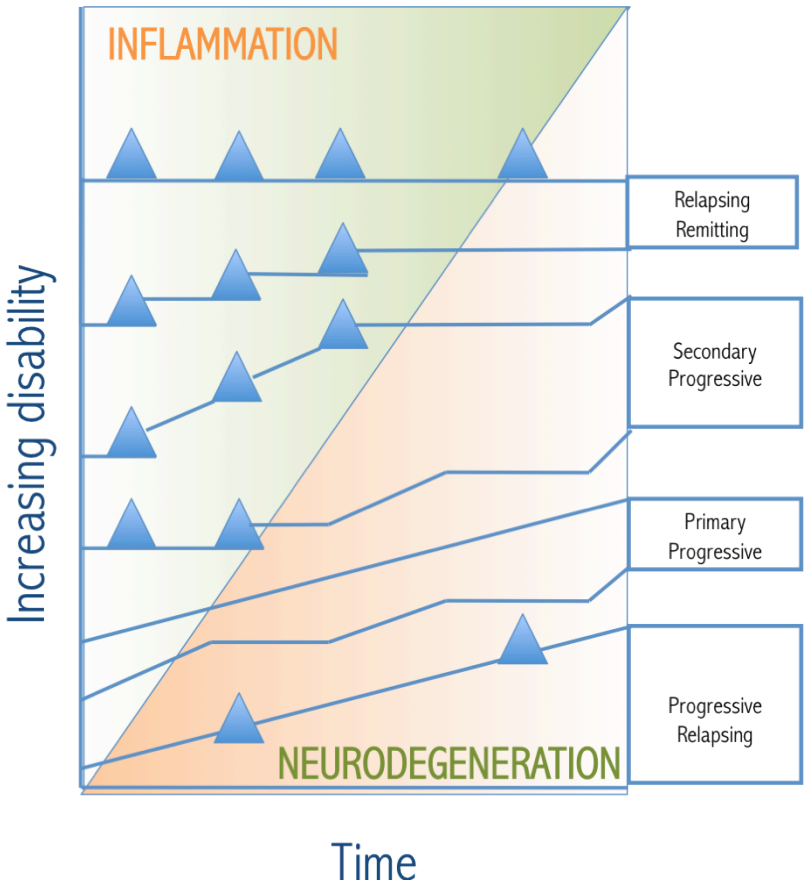

Figure 2. Graphical representation of MS progression
In MS, the prototypical course of the disease correlates to time and accumulating disability

The principle of diagnosis is to detect special and temporal dissemination of lesions in CNS. Clinical evidence is supplemented by laboratory tests such as CSF analysis and evoked potentials. The current diagnostic criteria for MS are the McDonald criteria, which includes MRI evidence. MS diagnosis in an early stage of the disease is important as early intervention is beneficial for long-term disease evolution (Hurwitz 2009) (Fig. 3).

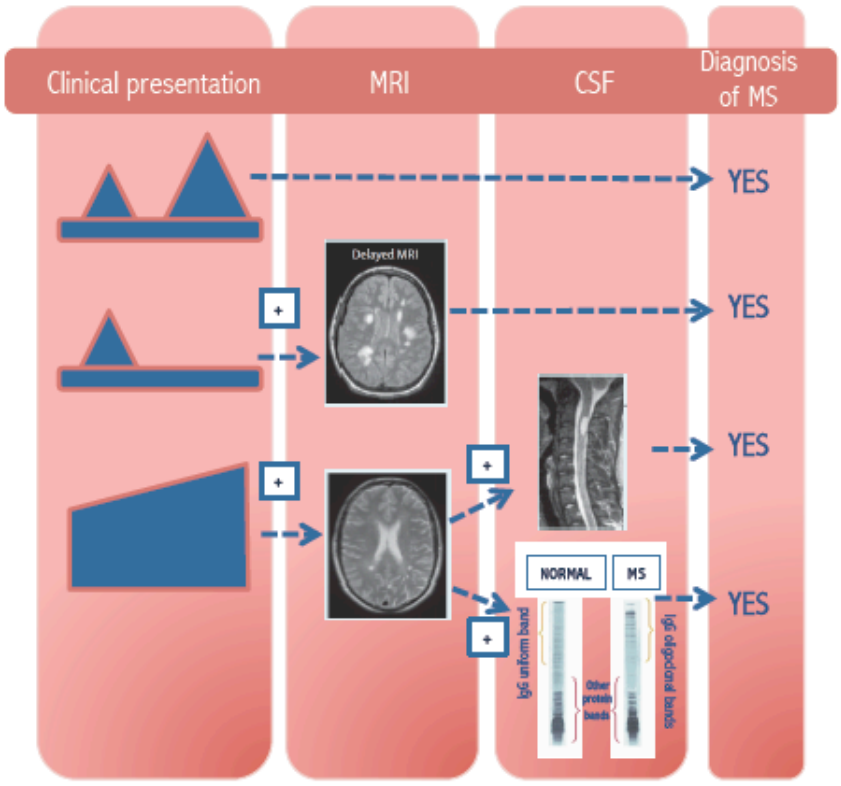

Figure 3. Algorithm for the diagnose of MS
Dissemination in time of magnetic resonance lesions requires: a) detection of gadolinium enhancing lesions at least 90 days after the onset of the initial clinical event, if not at the site corresponding to the initial event, and b) detection of a new T2 lesion if it appears at any time compared with a reference scan done at least one month after the onset of the first clinical event. In the case of recurrent stereotyped clinical episodes at the same neurological site, the criteria for MRI definition to determine dissemination in space are three features from: (1) one gadolinium-enhancing lesion or nine T2 MRI lesions, (2) one or more infratentorial lesion, (3) at least one juxtacortical lesions, or (4) three or more periventricular lesions (a spinal cord lesion can replace some of these brain lesions). PP-MS can be diagnosed after 1 year of a progressive deficit and two of: (1) a positive brain MRI, (2) a positive spinal cord MRI, and (3) positive oligoclonal bands. Those patients who do not meet all the diagnostic criteria but who have a specific clinical presentation can be considered as possibly MS. Modified from the McDonald criteria (Polman et al. 2005)

1.3 Treatment

There is no cure for MS, but the current therapeutic strategies aim to slow down the course of the disease, treat relapses and improve symptoms. In the case of severe exacerbations of the disease, high-dose corticosteroids are aimed to reduce inflammation. The Food and Drug Administration (FDA) has approved the use of beta interferon-1α and -1β, glatiramer acetate, fingolimod, mitoxantrone and natalizumab as disease-modifying drugs in the treatment of MS. These agents are effective in the treatment of RRMS, delaying the time of progression to disabling stages. Drugs approved as first- and second-line treatments for MS are described in table 5.

Among the most widely used drugs are the beta interferons (IFNβ), namely IFNβ-1a (Avonex and Rebif) and IFNβ-1b (Betaseron). The exact mechanisms for IFNβ to achieve immunomodulatory and anti inflammatory effects remain uncertain. However, several modes of action have been proposed, including the blockage of T-cell activation and proliferation, apoptosis of autoreactive T cells, induction of Treg cells, inhibition of leukocyte migration across the BBB, cytokine modulation, and potential antiviral activity (Ann Marrie and Rudick, 2006). The effects of IFNβ can be manifested clinically as decreased MRI lesion activity, decreased brain atrophy, delayed definite diagnose of MS after neurological symptoms onset, reduced relapse rate and decreased risk of maintained disability progression (Rudick et al. 1999).

Glatiramer acetate (Copaxone) is a random polymer of four amino acids found in the myelin basic protein (MBP), mimicking the conformational regions of this protein. The proposed effects of this treatment include differentiation of CD4+ T cells into Th2 cells, increment of frequency and activity of Treg cells, and modulation of CD8+ T cells (Karandikar et al. 2002).

Fingolimod (Gylena) is the first oral treatment approved by the FDA for MS. It is a structural analogue of sphingosine that is phosphorylated by sphingosine kinases into FTY720-P, a high affinity agonist for sphingosine 1-phosphate (S1P) receptors (Billich et al. 2003). The function of fingolimod is to sequester lymphocytes in the secondary lymph organs (SLO), preventing them from migrating to the central nervous system.

Mitoxantrone (Novantrone) has been approved as a second-line treatment for RRMS; it is a topoisomerase II inhibitor used as an antineoplastic agent in the treatment of certain types of cancer. Its immunosuppresive properties lie on the reduction in the number of B cells, inhibiting T helper cell function and enhancing T cell suppression (Rio et al. 2011).

Table 1. First- and second-line drugs approved for MS

Approved first line drugs	
IFNβ1b (Betaseron, Extavia)	CIS considered at high risk of developing MS. RRMS. SPMS with relapses
IFNβ1a (Avonex)	CIS, RRMS
IFNβ1a (Rebif)	RRMS SPMS with relapses
Glatiramer acetate (Copaxone)	CIS. RRMS
Fingolimod (Gilenya)	Relapsing forms of MS

Approved second line drugs	
Mitoxantrone hydrochloride (Novatrone)	Highly active RRMS or SPMS with frequent relapses and progression of disability during first-line treatment
Natalizumab (Tysabri)	RRMS patients who have not responded to a full and adequate course of interferon beta
Fingolimod (Gilenya)	Rapidly evolving aggressive RRMS and relapsing forms of MS

Drugs approved by the US Food and Drug Administration (FDA) for the treatment of MS. Reference: RIO, J., COMABELLA, M. & MONTALBAN, X. 2011. Multiple sclerosis: Current treatment algorithms. Curr Opin Neurol, 24, 230-7.

1.3.1 Therapies under investigation

1.3.1.1 Oral drugs

Four oral drugs for RRMS, apart of fingolimod, have been reported to have positive results: cladribine, teriflunomide, laquinimod, and dimethyl fumarate. Cladribine (Litak) is a purine analog that, such as Teriflunomide (A77 17269), an enzymatic blocker of dihydroorotate dehydrogenase, inhibits rapidly dividing cells, including activated T cells. Laquinimod (Allegro) is a derivative of linomide that causes a TH1 to Th2 shift profile. It also down regulates MHC class II, chemokines and adhesion related molecules important to inflammation. Dimethyl fumarate is a fumaric acid ester that inhibits microglial and astrocytic activation by suppressing the synthesis of nitric oxide, IL-1β, TNF-α and IL-6 (Wilms et al. 2010).

1.3.1.2 Monoclonal antibodies

Monoclonal antibodies (mAbs) represent a specific therapeutic strategy for the treatment of MS. Three of them (alemtuzumab, rituximab, and daclizumab), approved in the treatment of other diseases, are already in clinical trials for MS. Natalizumab is the only mAb approved by the FDA for the treatment of MS.

Natalizumab (Tysabri) is a humanized recombinant monoclonal antibody that inhibits the interaction between VLA-4, a surface molecule found on nearly all leukocytes, and the vascular cell adhesion molecule-1 (VCAM-1) expressed on endothelial cells to block leukocyte migration into the CNS. This treatment decreases by 50% the risk of sustained disability progression in comparison to healthy donors, and by 25% when compared to control group treated with IFNβ. Although this drug is in general well tolerated, of 96,582 patients treated with Tysabri as of January 4, 2012, 201 patients developed progressive multifocal leukoencepalopathy (PML) (www.fda.org).

Alemtuzumab (Campath) is a humanized monoclonal antibody that targets the CD52 antigen present on the surface of B and T lymphocytes, natural killer (NK) cells, monocytes, macrophages and some dendritic cells (Hale et al. 1990). Alemtuzumab induces a rapid depletion of lymphocytes followed by a differential recovery of lymphocyte subsets with prolonged suppression of CD4+ T cells. Evidence from phase II trials suggests that alemtuzumab reduces relapse rates in MS by over 70% compared to interferon β−1a, improves MRI parameters, and may also reduce disability. However, at least 20% of patients with MS treated with alemtuzumab develop de novo antibody-mediated autoimmune diseases (AID) during follow-up such as Grave´s disease and immune thrombocytopenic purpura (Coles et al. 1999 and Cossburn et al. 2011).

Daclizumab (Zenapax) is a humanized monoclonal antibody that targets the alpha chain of the IL-2 receptor complex (IL-2Rα or CD25) inhibiting the binding of IL-2 to its receptor on activated T-cells without causing T-cell depletion. In the CHOICE study, a phase II clinical trial to test daclizumab in MS, daclizumab given as monotherapy or combined with IFNβ produced a reduction in the number of brain lesions detected by MRI. Unexpectedly, a consistent increase in the frequency of $CD56^{bright}$ NK cells in peripheral blood related to the clinical effect was observed (Bielekova et al. 2006). The exact mechanism by which daclizumab expands this NK cell population is not understood.

The receptor for IL-2 (IL-2R) consists of three subunits, IL-2Rα (CD25), IL-2Rβ (CD122), and the common gamma chain (CD132). All three subunits are required for the formation of the high affinity IL-2R (Kd~ 10 pM). CD122 and CD132 have intracellular signaling motifs and together form the intermediate affinity receptor (Kd~ 0.1-1 nM), whereas CD25 does not induce a signal transduction by itself and binds with low affinity to IL-2 (Kd ~10 nM). IL-2 bound to the high-affinity IL-2R is short-lived on the cell surface because this complex is rapidly internalized (t1/2 10–20 min) (Malek 2008). CD25 is expressed constitutively at low levels only in resting human T cells and on $CD56^{bright}$ NK cells, but it is highly up regulated upon activation. A possible explanation would be that the blockage of IL-2 signaling in T cells by daclizumab induces an increase in the availability of IL-2 for the $CD56^{bright}$ NK cells, since these cells express high levels of CD122, the intermediate affinity chain of the IL-2 receptor, and therefore these cells expand.

1.4 Pathophysiology

MS is characterized by an inflammatory process in the CNS (neuroinflammation) that leads to a disruption of the myelin sheath (demyelination). Events such as remyelination, oligodendrocyte depletion, astrocytosis, and neuronal and axon degeneration are also involved in the development of the disease.

The disease process starts in the periphery with the activation of autoreactive CD4+ T cells, for example after recognition of a viral peptide, inducing the expression of adhesion molecules such as LFA-1 and VLA-4 that promote the transmigration to the brain parenchyma after adhesion to the BBB endothelium. These cells become reactivated by CNS-resident antigen presenting cells (APC) and release cytokines that, subsequently activate macrophages and microglia to finally recruit CD8+ T cells, B cells and mast cells.

1.4.1 Role of the immune system in MS

1.4.1.1 Effector cells

1.4.1.1.1 CD4/Th1 cells

MS is considered an autoimmune disease initiated by MHC class II-restricted CD4+ Th1 lymphocytes, which polarize to the production of IFNγ, tumor necrosis factor-α (TNFα) and lymphotoxin (LT) (Lassmann and Ransohoff 2009). Myelin-specific CD4+ rather than CD8+ T cells are the primary mediators in most models of EAE, probably because the induction method favors the activation of MHC class II-restricted T cells. Other facts supporting the role of CD4+ T cells in the pathology of MS are, 1) the possibility to passively or adoptively transfer EAE by *in vitro* reactivated myelin-specific CD4+ T cells (Zamvil and Steinman 1990), but not by antibodies; 2) the susceptibility to EAE of humanized transgenic mice expressing HLA-DR4 (DRB1*0401) (Forsthuber et al. 2001); 3) the development of spontaneous or induced EAE in mice expressing both MS-associated HLA-DR molecules and specific TCR for MS-patient derived MBP (Quandt et al 2004); and 4) the induction of CD4+T cells cross reactivity by an altered peptide ligand of MBP (83-99) leading to disease exacerbation (Bielekova et al. 2000).

However, many MS features are not reflected in Th1-mediated brain inflammation suggesting that besides Th1 cells, other immune mechanisms and neurodegenerative components within the target tissue might contribute to the initiation, propagation and modification of this disease.

1.4.1.1.2 Th17 cells

It was largely accepted that Th1 cells, driven by IL-12, were the pathogenic T cells in MS and EAE. Recently, the role of IL-17-producing CD4+ T cells, driven by IL-23 and referred to as Th17 cells, has been associated to the active disease in MS and to the pathogenesis of EAE. In RRMS patients the frequency of Th17 cells increases in CSF (Brucklacher-Waldert et al. 2009) and in peripheral blood (Durelli et al. 2009).
Concentrations of IL-17 in CSF are significantly higher in MS patients than in healthy donors. Moreover, memory T cells producing IL-17 and IL-22 infiltrate into the MS lesion (Kebir et al. 2007), where transcripts of IL-17 have been found highly upregulated (Lock et al. 2002).
Th17 cells in MS show higher basal levels of activation markers, costimulatory molecules and adhesion molecules than Th1 cells. In comparison to Th1, the Th17 cell population binds better to human endothelial cells due to surface expression of CD146, possesses a higher proliferative capacity and is less susceptible to regulatory T cell-mediated suppression (Brucklacher-Waldert et al. 2009 and Kebir et al. 2007). These characteristics give Th17 cells a high pathogenic potential for MS.
In the EAE model, mice lacking components of the IL-12/Th1 axis (Zhang et al. 2003) are prone to a more severe development of the disease than those mice lacking components of the IL-23/Th17 axis (Langrish et al. 2005). Interestingly, NK cell enrichment in the CNS improves disease. The activity of CNS resident NK cells has been associated with suppression of myelin-reactive Th17 cells (Hao et al. 2010). This and other studies show that the regulatory effect of various ameliorative mechanisms in EAE and possibly MS is exerted in part through Th17 modulation.
Apart of the described Th1 and Th17 cells, another effector T cell subset, Th9 cells, has recently been described. Driven by the combined effects of TGF-β and IL-4, Th9 cells produce large amounts of IL-9 and IL-10 (Veldhoen et al. 2008). Th9 cells are capable of inducing EAE upon adoptive transfer (Jager et al. 2009).

1.4.1.1.3 CD8+ T cells

A recent interest for CD8+ T cells in the pathology of MS has revived due to their greater abundance than CD4+ T cells in acute and chronic multiple sclerosis lesions (Hauser et al. 1986), their preferential clonal expansion (Babbe et al. 2000), and their pathogenicity in some animal models of MS (Sun et al. 2001 and Huseby et al. 2001). Other data supporting a role of CD8+ T cells are 1) enhanced production of LT in

SPMS patients (Buckle et al. 2003), 2) increased adhesion to brain venules (Battistini et al. 2003), 3) augmented frequency of CD8+ T cells against EBV epitopes in MS patients, and 4) a correlation between proliferating CD8 T cells in MS secreting both IL-10 and IFN-γ, and MRI- documented tissue destruction (reviewed by Killestein et al. 2003). However, the exact contribution of CD8 T cells in the pathogenesis of MS remains ambivalent as the HLA-A*0301 allele is reported to increase susceptibility (Fogdell-Hahn et al. 2000), whereas the HLA-A*0201 allele confers protection from the disease (Brynedal et al. 2007). New humanized mouse models of MS have revealed key contributions of MHC class I-restricted CD8+ T cell responses in initiating autoimmunity and additional contributions from MHC class II-restricted CD4+ T cells for disease progression (Friese et al. 2008). However, in other models of MS, the role of CD8+ T cells may be protective (Koh et al. 1992 and Najafian et al. 2003), remaining the possibility of a mixture of both pathologic and regulatory CD8+ T cells in MS.

1.4.1.2 Regulatory cells

1.4.1.2.1 Tregs

The function of Tregs is to suppress harmful immune responses against foreign and self antigens. In autoimmunity, Treg cells control the number and function of autoreactive T cells (Mills 2004). An imbalance between effector and regulatory cells is suggested as a possible cause of MS. In comparison to neurological diseases of non-inflammatory and non-autoimmune component, the frequency of CD4+ $CD25^{high}$ FoxP3+ Treg cells in CSF is higher in MS patients. However, frequency of Treg cells in periphery does not differ in MS patients and healthy donors (Feger et al. 2007). In MS, Treg cells are functionally impaired or have deficits in their maturation as their potential to inhibit myelin-specific and antigen nonspecific T-cell proliferation is reduced (Viglietta et al. 2004, Haas et al. 2005 and Kumar et al. 2006).

CD39, an ectonucleotidase that hydrolizes ATP to AMP, is expressed primarily by immune-suppressive FoxP3+ Treg cells. RRMS patients show reduced frequency of $CD39^{pos}$ Tregs in periphery (Borsellino et al. 2007). Treg cells suppress proliferation and IFNγ production by responder T cells, but only the CD4+ $CD25^{high}$ $CD39^{pos}$ population, suppress IL-17 production, whereas the $CD39^{neg}$ Treg subset produces IL-17. In MS patients, a reduced suppressive function of $CD39^{pos}$ Treg cells against Th17 cells is observed (Fletcher et al. 2009).

In the EAE model, CD4+ CD25+ cells have a clear beneficial role in the disease, suppressing cytokine production by myelin-specific pathogenic TH1 cells. Their transfer into normal mice prior to immunization results in decreased disease severity (Kohm et al. 2002).

1.4.1.2.2 NK cells

As effector cells, they play key roles in host immune responses against tumors, viruses, intracellular bacteria and parasites. Human NK cells are classified into two major subsets. Regulatory NK cells expressing high levels of CD56 and no CD16 are known as $CD56^{bright}$, whereas the naturally cytolytic subset CD16+ $CD56^{low}$ is known as $CD56^{dim}$. $CD56^{dim}$ cells are the most abundant subset in blood (≈90%). These cells produce large amounts of granzymes and perforin and mediate robust cytotoxicity toward MHC class-I deficient targets. $CD56^{bright}$ represent a small fraction (≈10%) of NK cells in blood, but in lymph nodes and in inflammatory compartments, they are found in higher proportions (up to 90% of NK cells). Because of their low production of perforin and Granzyme B, $CD56^{bright}$ NK cells were considered non cytotoxic; however, recent data has shown that these cells release high amounts of Granzyme A and K (Jiang et al. 2011). In addition to killing MHC class I-deficient targets, $CD56^{bright}$ NK cells can also kill different targets than the ones killed by the $CD56^{dim}$ subset, specifically the MHC class I-expressing immature dendritic cells (iDC) (Della Chiesa et al. 2003) and activated T cells (Bielekova et al. 2006).
NK cells have been classified into different subsets based on their cytokine profile. NK1 and cells produce IFNγ and IL-10, whereas NK2 produce IL-5 and IL-13 (Peritt et al. 1998 and Takahashi, 2001 #9824). NK17/NK1 cells secrete IL-17 and IFNγ, are generated upon activation via IL-2, and are more abundant in CSF of MS patients, but their precise role in autoimmune diseases is not yet clear (Pandya et al. 2011). The NK-22 subset produces the Th17 cytokine IL-22, probably playing an important role in mucosal immunity (Cella et al. 2009).

1.4.1.2.2.1 NK cell development

NK cell differentiation in humans occurs in the secondary lymphoid tissue (SLT), where 95% of all CD34+ cells have a phenotypic pattern of NK progenitor cell (NKP) expressing CD34+ CD45RA+ and integrin $b7^{bright}$, whereas only 6% have this phenotype in the periphery. The differentiation process has been divided into maturational stages, starting with a precursor NK or multipotential stage 1 (CD34+

CD117- CD94-), immature NK cells or stage 2 (CD34+CD117+CD94-), going through NK-committed stage 3 cells (CD34-CD117+CD94-) and finally becoming mature stage 4 (CD34-CD117+/-CD94+) NK cells (Freud and Caligiuri 2006) (Fig. 4).

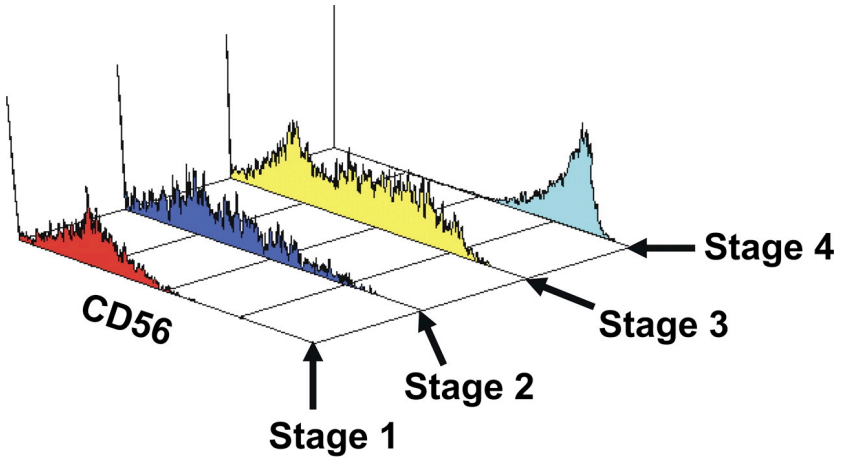

Figure 4. Progressive expression of CD56 during NK cell differentiation
The progressive CD56 expression by in vivo stages of human NK cell differentiation is shown. Stage 1 corresponds to CD34+ CD117neg CD94neg cells. Stage 2 is characterized by expression of CD34+ CD117+ CD94 neg. Stage 3 cells have a CD34neg CD117(+/-) CD94neg phenotype and stage 4 are those CD34neg CD117(+/-) CD94+ cells. Figure taken from Freud A G et al. J Exp Med 2006;203:1033-1043.

In this multistep process, the expression of other specific NK cell surface molecules allows the identification of precise stages of maturation. The first surface molecules acquired during stage 1 and 2 are CD161, 2B4 and CD56. The acquisition of LFA-1, NKp46, NKp30, NKG2D, and DNAM-1 activating receptors is correlated with the gradual acquisition of cytotoxicity. Expression of CD16 and Killer-cell immunoglobulin-like receptors (KIR) occurs at later stages (Freud and Caligiuri 2006). The acquisition of other surface markers occurs gradually from a more immature CD94+ NKG2A+ CD62L+ CD57- KIR-CD56^{bright} NK cell profile to a CD94^{high} NKG2A+/- CD62L+/- CD57-/+ KIR-/+ CD56^{dim} intermediate phenotype that progress further towards the terminally differentiated CD94^{low} (Yu et al. 2010) NKG2A- CD62L- (Juelke et al. 2010) CD57+ (Bjorkstrom et al. 2010) KIR+ CD56^{dim} NK cells. Blood CD56^{bright} CD16^{neg} NK cells are equivalent to stage 4 and, since CD56^{bright} cells can differentiate to CD56^{dim} cells *in vitro*, blood CD56^{dim} CD16+ NK cells may represent stage 5 (Yu et al. 2010). A scheme of differential expression of NK markers on different stages is shown in figure 5.

1.4.1.2.2.2 NK cells in central nervous system

In non pathogenic conditions, only a small number of immune cells cross the BBB to enter the CNS (reviewed by Cayrol et al. 2008). Although NK cell-related genes are

detected in the CNS (Bryceson et al. 2005), direct evidence on the presence of NK cells in the healthy human brain and spinal cord are scarce. Disruption of BBB leads to recruitment of large amounts of leukocytes into the CNS, among them NK cells (Hammarberg et al. 2000) during the course of infections, as well as in autoimmune CNS inflammation (Hansen et al. 2007 and Huang et al. 2006). However, the functional significance of NK cell recruitment and their mechanisms of action during brain inflammation are unknown.

The chemokines CX3CL1 (fraktaline), produced by neurons, and CXCL10 and CCL2, produced by macrophage/microglia and astrocytes, recruit NK cells into the CNS (Rollins 1997, Sorensen et al. 2002 and Dogan et al. 2008). Expression of CX3CR1, the chemokine receptor for CX3CL1, is decreased in MS patients, specifically on NK cells. This expression is dependent on disease activity and is particularly reduced in patients with a stable disease course. CXC3R1 is an additional differentiation marker in humans that may link NK cell maturation with the ability to migrate to different organs including the central nervous system (Hamann et al. 2011).

1.4.1.2.2.3 NK cells in EAE

In the EAE model, NK cells in the CNS can control the magnitude of the inflammatory response. Severity of EAE dramatically increases after depletion of NK cells (Zhang et al. 1997 and Xu et al 2005). Mice lacking CXCR3, a chemokine that recruits NK cells to CNS, exhibit a selective deficit in NK cell homing to the CNS that results in increased EAE-related mortality (Huang et al. 2006). The use of IL-2/Anti-IL-2 mAb complexes in the EAE model induces a specific expansion of NK cells in peripheral blood and CNS and an attenuation of disease activity and CNS pathology. In contrast, treatment with IL-2 complexes in animals depleted of CNS-resident NK cells does not alter the course of EAE (Hao et al. 2011), underlining the importance of NK cells in disease development.

1.4.1.2.2.4 NK cells in MS

In both RRMS and CIS patients, the frequency of $CD3^{neg}$ $CD56^{pos}$ $CD8^{dim}$ $CD4^{neg}$ lymphocytes, consistent with the NK cell profile is low (De Jager et al. 2008).

Interestingly, an increase in the frequency of the $CD56^{bright}$ NK cell subset has been reported during the last trimester of pregnancy, a time of reduced MS relapses in women with MS (Airas et al. 2008). Similarly, an elevated proportion of circulating $CD56^{bright}$ NK cells has been observed in patients with RRMS after treatment with IFN-

β and daclizumab, which correlates with a good response to treatment (Saraste et al. 2007, Vandenbark et al. 2009 and Bielekova et al. 2006).

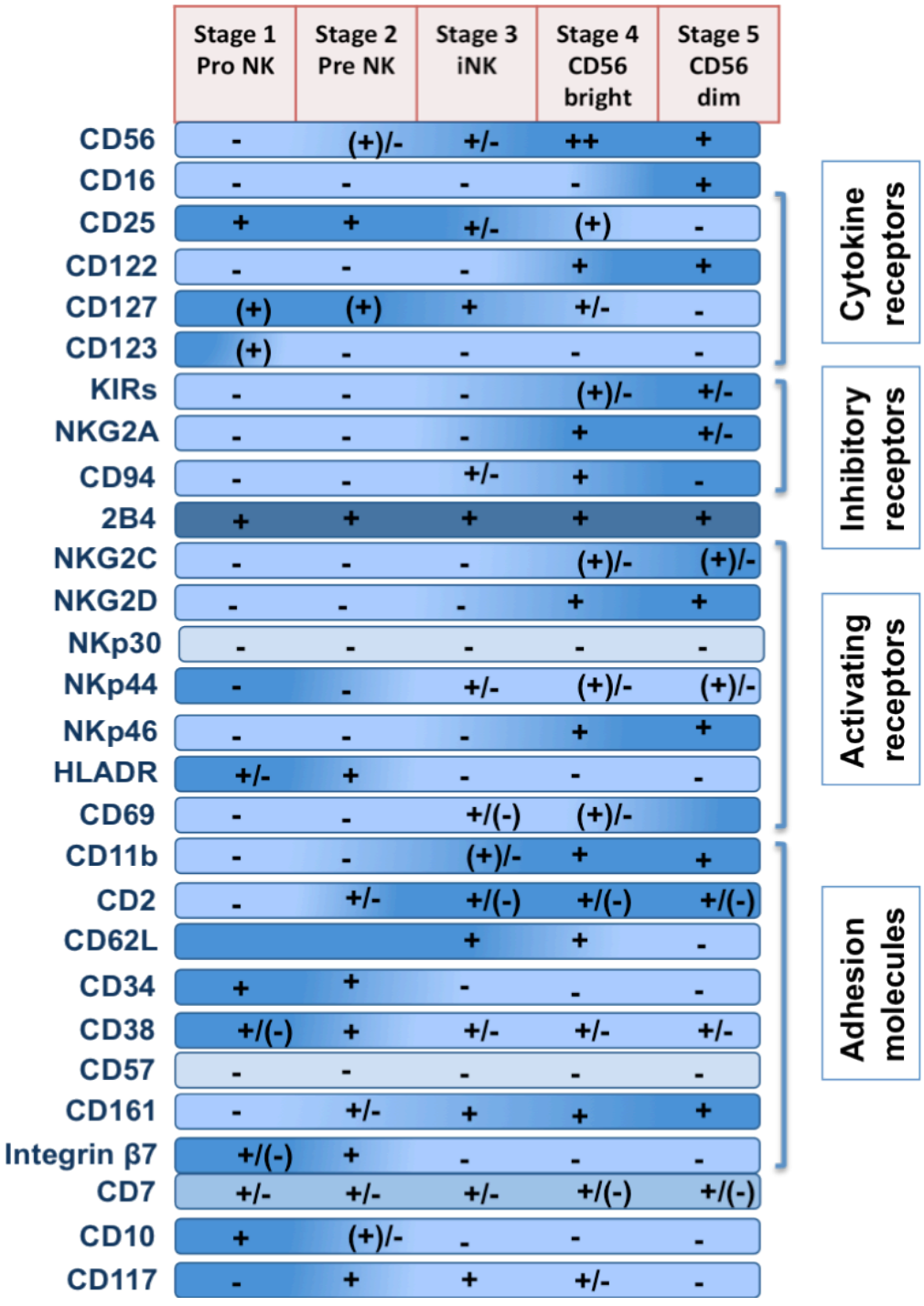

Figure 5. Surface antigen expression during NK development in SLT
+_All cells are positive, -_all cells are negative, +/- donor-dependent/ variable expression, (+)/- most of cells are negative, +/(-) most of the cells are positive, (+) almost not detectable on cells.

In vitro, NK cells isolated from daclizumab-treated MS patients, as well as activated NK cells from healthy donors, are capable of killing activated T cells, suggesting that NK cell cytotoxicity against activated T cells may represent one physiological mechanism for the termination of adaptive immune responses that is enhanced *in vivo* by daclizumab administration.

The emerging role of $CD56^{bright}$ NK cells as critical suppressor cells in target organs of autoimmunity gives us the chance to explore their phenotypical and functional characteristics in health and autoimmune disease such as MS, and the better understanding might wide the possibilities for new therapeutic targets.

2. Materials and methods

2.1 Materials

Media and supplements	Company	Catalog No.
Bovine serum albumin (BSA)	PAA	K45-001
Dimethyl sulfoxid (DMSO) for cell culture	AppliChem	A3672.0100
Dubelcco´s Phosphate Buffered Saline (PBS) 1x	PAA	H15-002
Dubelcco´s Phosphate Buffered Saline (DPBS) 10x	PAA	H15-011
Isocove´s Modified Dubelcco´s Medium (IMDM)	Gibco	31980-022
L-Glutamine, 200mM	Invitrogen	25030-024
Lymphocyte Separation Medium	PAA	J15-004
Penicillin/Streptomycin, 100x	PAA	P11-010
RPMI 1640	Gibco	61870
Serum, fetal bovine	Biochrom AG	S0115
Serum, human type AB	PAA	C21-021
X-VIVO 15, serum free medium	Lonza	BE 04-418F

Cell stimulation	Company	Catalog No.
Anti-CD3/Clone OKT3	Bioxell	BE00001-2
Ionomycin	Sigma Aldrich	I0634-1mg
Phorbol myristate acetate (PMA)	Calbiochem	524400
hr IL-2 (tecin)	Hoffmann-Roche	RO 23-6019
hr IL-15	Peprotech	200-15
rh IL-18	R&D (MBL)	B001-5
rh IL-21	Peprotech	200-21
Live/dead fixable dead cell kit	Invitrogen	L34960
Basiliximab (Simulect)	Novartis	
Daclizumab (Zenapax)	Hoffmann-La Roche	

Reagents for cell culture	Company	Catalog No.
2-beta Mercaptoethanol, 50 mM	Invitrogen	31350010
Brefeldin A	eBioscience	00-4506
eFluor670 cell proliferation dye	eBioscience	650840-90
MEM Non Essential Amino acids 100x	Gibco	11140-035
Mycoalert mycoplasma detection kit	Lonza	LT07-118
Sodium pyruvate 100 mM	Gibco	11360-039
Trypan blue solution, 0.4%	Sigma Aldrich	T8154
Vybrant CFDA SE cell tracer kit	Invitrogen	V12883

Magnetic cell separation/activation	Company	Catalog No.
NK isolation kit, human	Miltenyi Biotech	130-092-657
NK cell activation/expansion kit human	Miltenyi Biotech	130-094-483

Antibodies for flow cytometry

Specificity	Fluorochrome	Clone	Company	Catalog No.	Isotype
anti-CD14	V450	MϕP9	BD Biosciences	560349	IgG2b,k
anti-CD14	PB	M5E2	BD Biosciences	558121	mIgG2a, κ
anti-CD16	FITC	3G8	BioLegend	302006	IgG1,k
anti-CD16	APCCy7	3G8	BioLegend	302018	IgG1,k
anti-CD16	APCCy7	3G8	BioLegend	302018	IgG1,k
anti-CD16	FITC	3G8	BD Biosciences	555406	m IgG1, κ
anti-CD3	PeCy5.5	UCHT1	Biozol	9515-16	IgG1
anti-CD3	PE-Cy5.5	UCHT1	Beckman Coulter		rIgG2a,k
anti-CD4	V450	RPA-T4	BD Biosciences	560345	IgG1,k
anti-CD4	PECy7	RPA-T4	BD Biosciences	560649	IgG1,k
anti-CD45	V500	HI30	BD Biosciences	560777	IgG1,k
anti-CD56	PE	Hanti-CD56	BioLegend	318306	IgG1,k
anti-CD56	APC	N901 (NKH-1)	Beckman Coulter	IM2474	IgG1
anti-CD8	V500	RPA-T8	BD Biosciences	560775	IgG1,k
anti-CD8a	Brilliant violet 570	RPA-T8	BioLegend	M15168	
anti-CD8a	PacBlue	RPA-T8	BioLegend	301033	IgG1, κ
anti-CD56	Qdot-605	MEM-188	Invitrogen	Q10307	
anti-CD3	Q-dot 655	S4.1	Invitrogen	Q10012	
Cytokine receptors					
anti-CD122	PE	Mik-β3	BD Biosciences	554525	IgG1,k
anti-CD127	FITC	HIL-7R-M21	BD Biosciences	560549	IgG1,k
anti-CD127	PE	eBioRDR5	eBioscience	12-1278-73	mIgG1
anti-CD132*	PE	TUGh4	BioLegend	338606	IgG2b, k
anti-CD132	PE	AG184	BD Biosciences	555900	mIgG1
anti-CD25	PE	2A3	BD Biosciences	341011	IgG1
anti-CD25	PE	M-A251	BD Biosciences	555432	IgG1,k
anti-CD25	PE	B1.49.9	Beckman Coulter	A07774	mIgG2a
anti-IL18Rα	PE	H44	eBioscience	12-7183-71	mIgG1
Adhesion molecules					
anti-CD11a	FITC	G-25.2	BD Biosciences	347983	mIgG2a
anti-CD11b	PE	ICRF44	BioLegend	301306	IgG1,k
anti-CD11b	PE	VIM12	Caltag	CD11b04	IgG1
anti-CD11c	PE	B-ly6	BD Biosciences	555392	IgG1, κ
anti-CD11c	AF488	BU15	ABDSerotec	MCA2087A488	IgG1
anti-CD2	FITC	RPA-2.10	BD Biosciences	555326	IgG1,k
anti-CD2	FITC	S5.2	BD Biosciences	347593	IgG2a
Activating receptors					
anti-HLA-DR	FITC	G46-6	eBioscience	555811	IgG2a,k
anti-HLA-DR	FITC	G-46-6	BD Biosciences	555811	IgG2a κ
anti-NKG2D	PE	1D11	eBioscience	12-5878-42	mIgG1

anti-NKG2D	PE	1D11	BD Biosciences	557940	IgG1,k
anti-NKG2D	PE	1D11	BioLegend	320806	IgG1,k
Chemokine receptors					
anti-CXCR3	AF488	1C6/CXCR3	BD Biosciences	558047	IgG1,k
anti-CD197 (CCR7)	PE	3D12	BD Biosciences	552176	IgG2a,k
anti-CD197 (CCR7)	PE-Cy7	3D12	BD Biosciences	557648	IgG1
Markers of NK cell development					
anti-CD161	AF647	HP-3G10	BioLegend	339910	IgG1,k
anti-CD57	FITC	Hanti-CD57	BioLegend	322306	IgM, k
anti-CD62L	PE	DREG-56	BD Biosciences	555544	mIgG1
anti-CD94	FITC	HP-3D9	BD Biosciences	555888	IgG1,k
Activation markers					
anti-CD26	PE	M-A261	BD Biosciences	555437	IgG1,k
anti-CD27	APCCy7	M-T271	BD Biosciences	560222	IgG1,k
anti-CD69	APCCy7	FN50	BioLegend	310914	IgG1, k
Ectonucleotidases					
anti-CD39	FITC	eBioA1 (A1)	eBioscience	11-0399	IgG1
anti-CD39	PECy7	A1	BioLegend	328212	IgG1
anti-CD73	PE	AD2	BioLegend	344004	IgG1,k
Intracellular cytokines/transcription factors					
anti-IFN-γ	PacB	4S.B3	eBio	57-7319	IgG1,k
anti-IFN-γ	PE	4S.B3	eBio	12-7319-	IgG1,k
anti-IFN-γ	FITC	4S.B3	eBio	11-7319-71	IgG1,k
anti-IL10	PE	JES3-9D7	eBio	12-7108-	IgG1,k
anti-IL22	AF647	22URTI	eBio	51-7229-42	IgG1
anti-TNFα	V450	PCH101	eBioscience	77-5776	IgG1,k
anti-FoxP3	FITC	PCH101	eBioscience	11-4776-71	IgG1,k
anti-FoxP3	Alexa647	206D	Biolegend	320114	
anti-FoxP3	APC	PCH101	eBioscience	17-4776	IgG2a,k

Reagents for flow cytometry	**Company**	**Catalog No.**
FACS Flow, 20L	BD Biosciences	342003
Fixation buffer	eBioscience	00-8222-49
Lysing solution	BD Biosciences	349202

Permeabilisation buffer, 10x	eBioscience	00-8333-56
FoxP3 Fix/Perm buffer 4x	Biolegend	421401

Buffers and solutions	Content
FASC buffer	0.1% BSA, 00.2% NaN3. In 1x PBS
MACS/Sorting buffer	0.5% human Serum, 2mM EDTA. In 1x PBS
Standard Medium	FCS 10%, Pen/Strep 1%, L-Glu 2mM. In RPMI
	FCS 10%, Pen/Strep 1%, L-Glu 2mM, MEM NEAA, sodium pyruvate, 2-βMercaptoethanol. In
NK medium	RPMI
Freezing medium	RPMI 40%, FCS 40%, DMSO 20%
Fixation buffer	2% PFA in 1xPBS
HEK-Medium	D-MEM/Glutamax, 10% FCS, 1% Pen/Strep

Kits	Fluorochrome	Company	Catalog No.
Anti-human FoxP3 staining set			
Clone PCH101	APC	eBioscience	775776-40
Human IL-2 Biotinylated Fluorokine Kit	Avidin-FITC	R&D Systems	NF200

Consumables	Company
Cryotubes	Grenier
Eppendorf tubes	Eppendorf
FACS tubes	Sarstedt
LS/MS columns for magnetic isolation	Miltenyi Biotech
Parafilm "M"	Pechiney
Pipette tips	Sarstedt
Serological pipettes	Falcon
Sterile surgical blades	Braun
Syringe filters	VWR
Tissue culture flasks	Sarstedt
Tissue culture plates	Sarstedt and Greiner

Equipment	Company
Benchtop refrigerated centrifuges	Hereaus, Eppendorf, BC
FACS Aria cell sorter	BD Bioscience
FACSCanto	BD Bioscience
Freezers	Liebherr
Freezing container, Nalgene Cryo 1°C	Roth
Fridges	Liebherr / Thermo
Incubator, IncuSafe	Scientific, Sanyo
MACS Multistand	Miltenyi Biotech
Magnet, MACS Mini and Midi	Miltenyi Biotech
Microscope	Zeiss
Neubauer improved chamber	Marienfeld
Nitrogen tank	MVE

Pipettes	Eppendorf/Gilson
Racks	Roth
Sterile bank, class II standard	Thermo Scientific
Vortex-Genie 2	Scientific Industries
Waterbath	Eppendorf, GFL

Software	**Company**
FACS DIVA Software v6.1.3	BD
GraphPad Prism	GraphPad Software, Inc.
Adobe Illustrator CS3	Adobe Systems Incorporated

2.2 Methods

2.2.1 Donors

Healthy volunteers were recruited either from the blood bank at the UKE, or from our colleagues in the Institute of Immunology. All MS patients were recruited by the MS outpatient clinic of the neurology department at the UKE. This study was approved by the local ethics committee (Ethik-Kommission der Ärztekammer Hamburg, Nr. 2758, title 'Heterogenität der Multiple Sklerose – Studie zu Phänotypisierung, biologischen Markern un zur Risikokommunikation bei Multiple Sklerose') and written informed consent was obtained from all study subjects.

The study population consisted of 66 patients with RRMS diagnosed by McDonald criteria (Polman et al. 2005). Age range was 20-61 years. Score for the expanded disability status scale (EDSS) ranged 0-6 (Kurtzke 1983). All patients were in remission. The group included untreated (n=16) and treated (n=50) patients. Among the latter, we counted with Avonex- (n=12), Rebif- (n=18), Tysabri- (n=15), daclizumab- (n=4) and basiliximab-treated patients (n=1).

We obtained clinical data such as EDSS at the time of diagnose and at the time of sampling, duration of the disease and rate of relapse per year. Treated patients were treated for at least three months and a maximum of three years, and did not receive any steroidal or other immunomodulatory therapy within the last four weeks prior to sampling.

Patients were classified as responders when there were no relapses during the follow-up period, no new lesions detected by MRI and no increase in the EDSS score. A partial responder was indicated by the presence of at least one new T2-hyperintense or enhancing lesion detected by MRI without clinical impairment. Patients labeled as non-responders included those who experienced one or more relapses, or increased at least one point in the EDSS score that persisted for a minimum of two consecutive

visits separated by a 6-month interval, or presented one or more new T2-hyperintense or enhancing lesions detected by MRI. We included only good responders to treatment. As control group, we included 26 healthy donors (HD) matched in age and sex. The characteristics of MS and control donors are listed in Tables 1 and 2, respectively.

We could follow longitudinally two MS patients treated with basiliximab. One was followed for two years and was considered a good responder, whereas the other was retired from treatment after 6 months with basiliximab due to a bad response.

Table 2. Cohort of MS patients analyzed

Code	Age	Sex	Initial EDSS	EDSS at time of sampling	Disease duration	Rate of relapse/year
UNTREATED						
UN001	27	M	1	1	1 month	low
UN002	21	F	2	1	5 months	low
UN003	37	F	3	3	baseline	middle
UN004	46	M	2	2	6 years	low
UN005	26	F	2	3	6 years	middle
UN006	51	F	0	0	14 years	low
UN007	20	M	4,5	4,5	baseline	middle
UN008*	41	M	2	2	baseline	
UN009	33	M	2	0	6 months	middle
UN010	32	F	0	0	15 months	middle
UN011	25	F	0	0	3 months	low
UN012	29	M	2	2	4 months	low
UN013	48	F	0	2	18 years	middle
UN014	57	F	1	1	1 year	low
UN015	54	F	0	0	7 years	low
UN016	31	F	0	0	2 years	low
AVONEX						
AV001	25	F		3	5 years	middle
AV002	25	F	2	3	8 years	low
AV003	20	F	0	2	4 months	middle
AV004	26	M	2	2	4 months	middle
AV005	41	M		3,5	13 years	low
AV006	25	F	2	4	3 years	high
AV007	26	F	2	2	1 year	middle
AV008	42	F	2	2	4 years	low
AV009	61	M	2	3,5	9 years	low
AV010	34	F	0	2	1 year	low
AV011	47	F	3	6	5 years	none
AV012	39	F	1	2	2 years	low

REBIF						
RE001	34	F	0	0	9 months	low
RE002	34	M	3	2	37 months	low
RE003	24	F	0	0	1 month	low
RE004	46	F		3	36 months	low
RE005	40	F		6	18 years	low
RE006	44	F	2	2,5	5 years	low
RE007	27	M	1	1	4 months	low
RE008	21	F	2	1	8 months	low
RE009	38	F	0	1	20 months	middle
RE010	24	F	0	0	14 months	middle
RE012	49	F	3	4	1 year	high
RE013	53	F	3	3	1 year	middle
RE014	48	F	0	3,5	10 years	low
RE015	36	F		2,5	7 years	low
RE016	38	F	2	2,5	11 years	low
RE017	29	M	2	2	7 months	low
RE018	35	M	2	2	9 months	low
TYSABRI						
TYS001	49	M	2	4,5	11 years	low
TYS002	27	F	1	4	3 years	middle
TYS003	46	F	2	4	11 years	low
TYS004	36	F	5	3,5	2 years	low
TYS005	29	F		2,5	9 years	low
TYS006	37	M	0	2	4 years	high
TYS007	42	F		3	9 years	low
TYS008	38	F		4	15 years	low
TYS009	41	F	0	6	15 years	low
TYS010	40	F		3,5	16 years	low
TYS011	51	F	0	4	11 years	low
TYS012	44	F		3	13 years	low
TYS013	32	M	0	3,5	13 years	low
TYS014	51	M	2	3,5	12 years	low
TYS015	36	F	0	2,5	9 years	low
Anti-CD25						
A25001	36	F	2	5,5	9 years	middle
A25002	28	F	2,5	6	1 year	high
A25003	32	F	0	2,5	3 years	low
A25004	43	M	2	4	12 years	high
A25005	38	F		6	10 years	low

EDSS: Expanded Disability Status Score, scale 0-10. **Initial EDSS** is the score obtained at the moment of MS diagnose. **Rate of relapse/year** indicates the number of relapses suffered within 12 months; low <1/year, medium 1-2/year and high >2/year. All Tysabri-treated patients had high annual relapse rate before treatment. *Patient diagnosed with CIS, not confirmed as MS at the moment of sample. **F**: female, **M**: male

Table 3. Healthy controls

Code	Sex	Age
HD001	F	25
HD002		
HD003	F	29
HD004	M	28
HD005	M	32
HD006	F	28
HD007	M	29
HD008	F	30
HD009	M	23
HD010	F	28
HD011	M	57
HD012	F	23
HD013	F	31

Code	Sex	Age
HD014	F	28
HD015	F	31
HD016	M	32
HD017	M	20
HD018	F	23
HD019	F	46
HD020	M	24
HD021	F	45
HD022	F	43
HD023	F	56
HD024	F	20
HD025	F	28
HD026	F	29

2.2.2 Isolation of Peripheral Blood Mononuclear Cells

PBMC were obtained from leukocyte concentrates or from peripheral venous blood. Samples were diluted 1:3 in PBS. PBMC were isolated after density gradient centrifugation over lymphocyte separation medium (room temperature, 30 min, 2000 rpm), and washed twice in phosphate buffered saline (PBS). Cells were resuspended in PBS containing 2% FCS and counted in the microscope in an improved Neubauer counter chamber. Cells were either used immediately or cryopreserved. When using frozen samples, PBMC were thawed immediately before use.

2.2.3 Cell surface staining of NK cell markers

To determine the expression of specific markers in the NK cells subsets, we elaborated a panel of 23 surface molecules according to their function as cytokine receptors, adhesion molecules, chemokine receptors, lectin like receptors, ectonucleotidases, activation and maturation markers (table 3).

Table 4. Panel of cell surface markers analyzed

Cytokine receptors	Adhesion molecules	Chemokine receptors	Markers of NK cell development	Ectonucleo-tidases	Activation markers
CD25 (IL-2Rα)	CD11c	CCR7	CD62L	CD39	CD69
CD122 (IL-2Rβ)		CXCR3	CD94	CD73	CD26
CD132 (IL-2Rγ)			CD161		CD6
CD127 (IL-7Rα)			CD57		CD2
IL-18Rα			CD27		CD11b
					NKG2D
					HLADR

Immunophenotypical analysis of cells was performed using monoclonal antibodies conjugated either to fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin-chlorophyll proteins (PerCP), peridinin-chlorophyll proteins-Cy5.5 (PECy5.5), PECy7, allophycocyanin (APC), allophycocyanin-Cy7 (APCCy7), alexa fluor 488 (AF488), alexa fluor 670 (AF670), alexa fluor 647 (AF647), pacific blue (PacBlue), or V500.
Nine ml peripheral whole blood were collected by venipuncture into anticoagulated (EDTA) tubes and processed within 2 hours of collection. We transferred 75µl of blood into each of 17 FACS tubes per donor (5 ml polypropylene round-bottom tubes) and 10µl of each antibody were added according to the combination of surface markers shown in table 4. After 30 min incubation at room temperature in darkness (RTD), erythrocytes were lysed in 1 ml of lysis buffer (BD Biosciences) for 15 minutes at RTD, followed by two washing steps with PBS.
When staining freshly isolated or thawed PBMC, 1 million cells per tube were used. Cells were washed in PBS, blocked with 5µl of human IgG (hIgG) for 10 minutes, and then stained on the surface in FACS buffer containing the respective antibodies for 30 min at 4°C. Finally cells were washed and resuspended in FACS buffer.

2.2.4 Gating strategy and FACS analysis

To analyze surface markers in the different lymphocytic cell subsets (CD4, CD8, $CD56^{dim}$ and $CD56^{bright}$), the initial category in our hierarchical gating strategy was the lymphocytic gate in a FSC/SSC plot, followed by an exclusion of doublets in an SSC-A/SSC-H plot. A quadrant gate was set in the CD3/CD56 dot plot. NK cells were defined as $CD3^{neg}$ $CD56^{pos}$, the double positive cells represented the NKT cell population, and T cells were defined as $CD3^{pos}$ $CD56^{neg}$. Cells in the double negative quadrant were considered as B cells and debris. Three further gates were applied in a CD56/CD16 plot; the $CD56^{high}$ $CD16^{neg/low}$ represented the $CD56^{bright}$ cells, whereas the $CD56^{low}$ $CD16^{high}$ defined the $CD56^{dim}$ population; a third gate was set for $CD56^{low}$ $CD16^{low/neg}$ as intermediate NK cells. CD4+ and CD8+ T cells were gated in a CD4/CD8 plot, derived from the T cell gate (figure 6). The surface markers of interest were subsequently analyzed on the $CD56^{bright}$, $CD56^{dim}$, CD4+ and CD8+ populations, therefore each tube contained the basic markers (CD3, CD4, CD8, CD56, CD16) to define cell subpopulations. Because our immunophenotypical analysis of cells was performed with 8-color flow cytometry, in each tube were added 1, 2 or maximum 3 other surface antibodies according to our combination of antibodies described in table 4. The expression of the different markers was determined as the percentage of cells positive for a specific surface molecule. In cases of bimodal or trimodal expression, we

also evaluated the median fluorescence intensity (MFI). An example is shown in detail in figure 7. All samples were measured on a FACS Canto flow cytometer (BD Biosciences), using the Diva software v6.1.3 for further analysis.

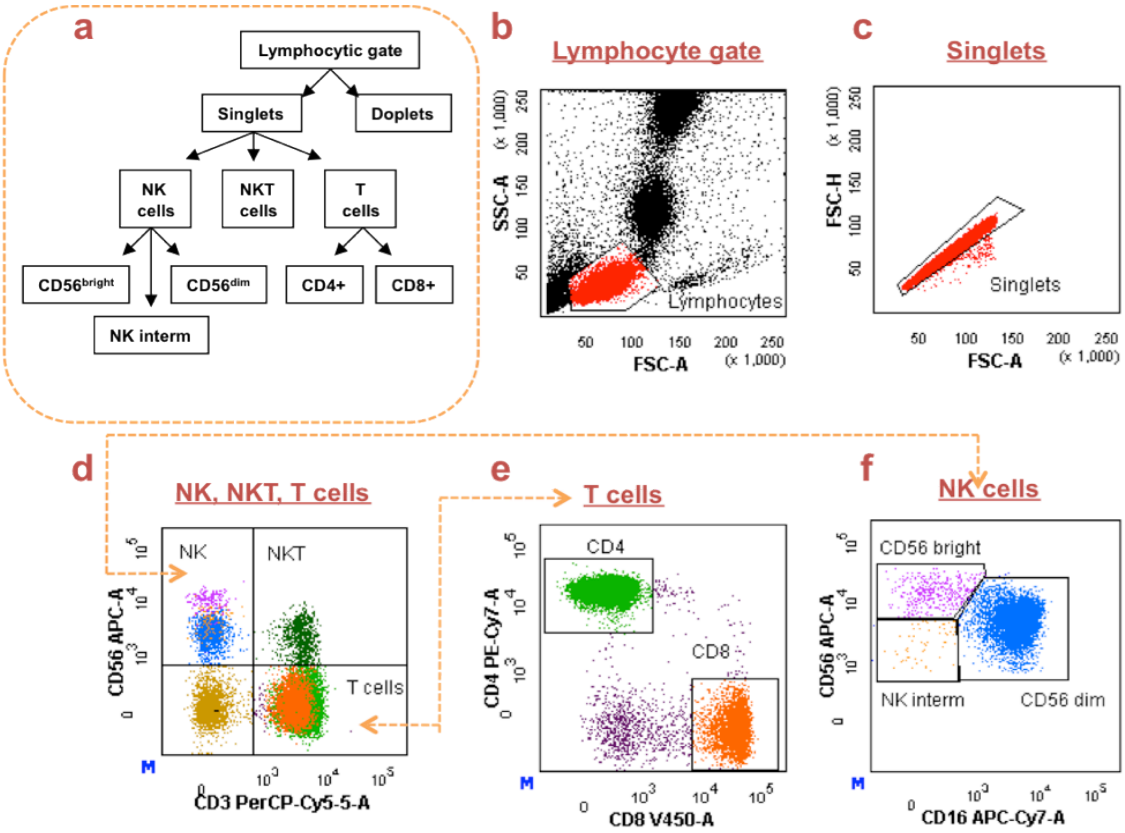

Figure 6. Gating strategy for the analysis of NK and T cell subsets
Representative dot plots of eight color flow cytometric evaluations from a healthy donor sample. All plots depict in the X and Y axes a log10 fluorescent intensity; a minimum of 100,000 events was collected. The gating strategy is depicted in a logarithm (a). The classic lymphocytic gate (b) is followed by the exclusion of doublets (c). A quadrant gate in the CD3/CD56 plot is set in order to identify the NK, NKT and T cell populations (d). The latter are subdivided into CD4+ and CD8+(e). From the NK cell gate derive the $CD56^{bright}$ and $CD56^{dim}$ subsets according to the low or high expression of CD16, respectively (f).

2.2.5 Treg cells staining

Expression of FoxP3 on Treg cells was evaluated using the Anti-human FoxP3 staining set (eBioscience), according to manufacturer's instructions. Briefly, after cell surface staining with anti-CD3, -CD4, -CD8, -CD25 and –CD127 as described above, cells were resuspended in Fix/Perm buffer for 30 minutes at 4°C, followed by two washing steps with permeabilisation buffer. Fc regions were blocked with rat serum for 10

minutes, followed by FoxP3 staining for 30 minutes at 4°C. FoxP3 expression was determined as the percentage of FoxP3 positive out of the CD4 positive cell population.

2.2.6 Intracellular cytokine staining

To release intracellular cytokines, PBMC were activated with PMA (50 ng/ml) and ionomycin (1µg/ml) for at least 5 hours in X-Vivo 15 medium at conditions of 37°C and 5% CO2. Protein transport from the endoplasmic reticulum to the Golgi apparatus was inhibited with 10 µg/ml of Brefeldin A after one hour of incubation with PMA/Iono.

Table 5. Combination of markers used for flow cytometry

→ Fluorochrome / ↓ Tube #	V500	V450 PacBlue	FITC AF488	PE	PECy5.5	PECy7	APC AF647	APCCy7
1	-	-	-	-	-	-	-	-
2	-	CD8	-	-	CD3	CD4	CD56	CD16
3	CD45	CD14	CD4	-	CD3	CD19	CD56	CD16
4	-	CD8	CD16	CD56	CD3	CD4	CD161	-
5	CD8	CD4	CD127	CD25 Tac	CD3	CD39	CD56	CD16
6	-	CD8	CD16	CD122	CD3	CD4	CD56	CD69
7	-	CD8	CD57	CD25 non tac	CD3	CD4	CD56	CD16
8	-	CD8	CD11c	IL-18Ra	CD3	CD4	CD56	CD16
9	-	CD8	CD94	CD132	CD3	CD4	CD56	CD16
10	-	CD8	HLA-DR	CD62L	CD3	CD4	CD56	CD16
11	-	CD8	CD2	NKG2D	CD3	CD4	CD56	CD16
12	-	CD8	CXCR3	-	CD3	CD4	CD56	CD16
13	-	CD8	-	CD26	CD3	CD4	CD56	CD16
14	-	CD8	CD11a	CD6	CD3	CD4	CD56	CD16
15	-	CD8	-	CCR7	CD3	CD4	CD56	CD16
16	-	CD8	-	CD11b	CD3	CD4	CD56	CD16
17	-	CD8	CD39	CD73	CD3	CD4	CD56	CD16

Tube #1 was used as negative control. Tube #2 was used to determine the 'fluorescence minus one' (FMO) control for setting the threshold values for the expression of specific markers.

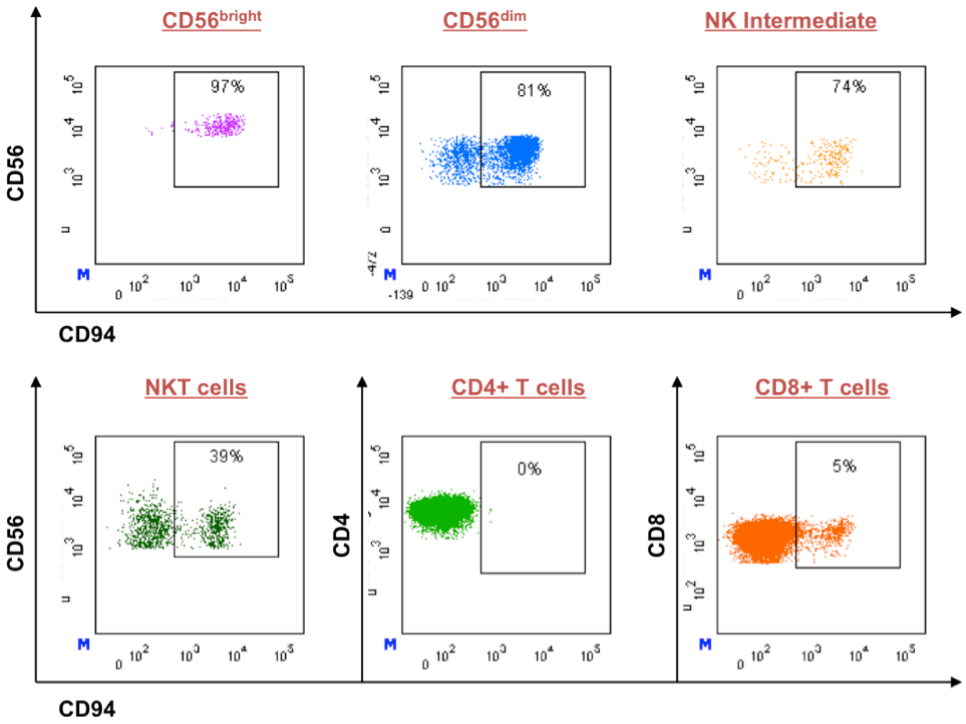

Figure 7. Expression of CD94 in the different lymphocytic cell populations
Representative staining to identify the expression of CD94 cells in PBMC from one healthy donor. In each plot is shown the expression of the marker in percentage of CD94 on $CD56^{bright}$, $CD56^{dim}$, CD4+, CD8+ and NKT, respectively.

After 6 hours of incubation, cells were harvested, washed and resuspended in 100 µl of live/dead cell dye for 20 minutes at RTD. A further wash step was followed by cell surface staining by resuspension of the cell pellet in 50µl of FACS buffer plus 10µl of each cell surface antibody (CD3-PerCPCy5.5, CD4-PECy7, CD56-APC, CD57-FITC and CD94-PE). Cells were incubated for 30 minutes RTD, then washed in permeabilisation buffer and stained for intracellular IFNγ and TNFα with APCCy7 anti-IFNγ and V450 anti-TNFα for 30 minutes at RTD. Finally cells were fixed, washed and resuspended in FACS buffer for flow cytometry analysis. To analyze the intracellular production of IFNγ and TNFα, a first gate was set up on live cells in the FSC/SSC plot, followed by an exclusion of doublets in an SSC-H against SSC-A dot plot. NK cells were gated on $CD3^{neg}$ $CD56^{pos}$, and T cells on $CD3^{pos}$ $CD56^{neg}$. Two further gates on $CD56^{high}$ $CD57^{pos}$ and $CD56^{dim}$ $CD94^{high}$ cells defined respectively the $CD56^{dim}$ and $CD56^{bright}$ populations. Their respective markers defined CD4 and CD8 T cells.

2.2.7 IL-2 binding assay

To investigate the effect of treatment on the binding capability of IL-2 for its receptor on different cell populations, PBMC were washed twice with PBS, followed by incubation for 1 hour at 4°C with either 10 µl of biotinylated IL-2, or 10 µl of biotinylated negative control reagent, or 10 µl of biotinylated IL-2 complexed with a blocking antibody diluted in wash buffer. For cell surface staining, 10µl of avidin-fluorescein in addition of CD3, CD4, CD8, CD56 and CD16 antibodies were added incubated for 30 minutes at 4°C. Cells were then washed three times with wash buffer and analyzed by flow cytometry.

2.2.8 Cell proliferation assay

To determine the proliferation effect of anti-CD25 antibodies and IFNβ on the different cell populations, PBMC were labeled either with 2-µM CFSE or 2µM eFluor dye. A scheme of the proliferation assay principle is described in figure 8. Cell stimulation was performed with anti-CD3 antibody (clone OKT3, 500 ng/ml, Bioxcell) in presence or absence of daclizumab (20 ng/ml, Zenapax, Hoffmann-La Roche), basiliximab (20 ng/ml, Simulect, Novartis) or IFNβ1a (100 or 1000 IU/ml, Avonex, Biogen**)** for 3 to 7 days. As positive controls, cells were activated with IL-2 (100 U/ml, Tecin, Hoffmann Roche) or IL-15 (20 ng/ml, PreProtech, London, UK). Proliferating cells were determined for each cell population.

2.2.8.1 CFSE

The CFDA-SE (Carboxyfluorescein diacetat succinimidyl ester) stock (10 µM) was resuspended in prewarmed (37°C) PBS for sequential dilutions (25, 10, 5, 2 and 0.5 µM). PBMC were previously washed in PBS and resuspended in 2µM CFSE dilution ($1x10^7$ cells/1ml). After 15 min of incubation at 37°C, cells were washed and incubated in prewarmed standard medium for 30 minutes at RTD. Two further washing steps with RT standard medium and one with PBS were done. For further cell culture, cells were resuspended in medium and transferred into 48 or 96-well plates with the respective stimuli. For subsequent NK enrichment and/or sorting, cells were resuspended in MACS buffer.

2.2.8.2 eFluor 670

In contrast to CFDA-SE, the eFluor670 stock reconstitution and dilutions were done with cold (4°C) PBS. Incubation with the dye was performed at RTD for 10 minutes at a

concentration of 2 µM. To stop labeling, RPMI + 10% FCS was added 4 times the volume used for staining, followed by incubation on ice for 5 minutes. Two washing steps followed, one with complete medium and one with PBS. Cells were resuspended either in standard medium for subsequent stimulation, or in MACS buffer for NK enrichment or sorting.

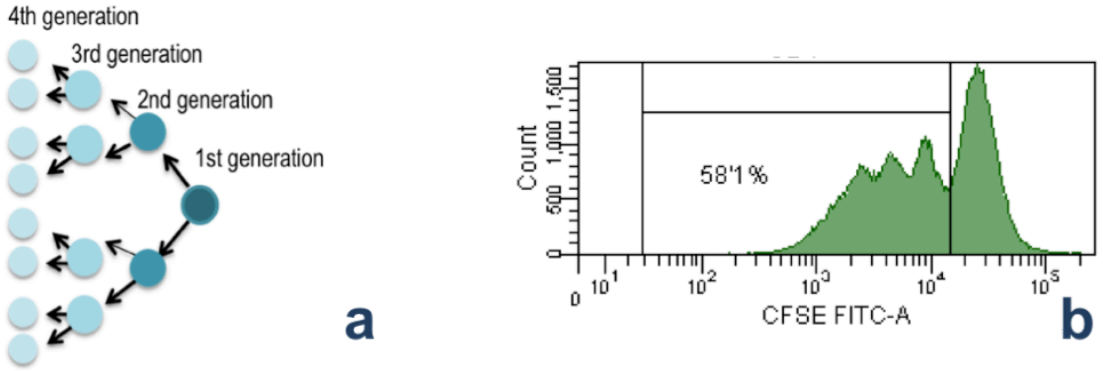

Figure 8. CFSE-based proliferation assay
The dye is inherited by daughter cells after cell division, and is not transferred to adjacent cells in a population. a) Cell division produces sequential halving of the dye's fluorescence, and up to 8 divisions can be monitored before a decrease to the background fluorescence of unstained cells, resulting in a histogram in which the peaks represent successive generations. B) CFSE profile for PBMC labeled at 2 µM CFSE and cultured with 200ng/ml of anti-CD3 for 3 days. The peak on the right represents the undivided cells, which correspond with the control unstimulated CFSE-labeled cells.

2.2.8.3 Isolation of NK cells

Natural killer cells were enriched from freshly isolated PBMC by negative selection using the NK cell isolation kit (Miltenyi) according to manufacturer's instructions. The principle of this technique is the depletion of T cells, B cells, stem cells, dendritic cells, monocytes, granulocytes, and erythroid cells. All these cells are magnetically labeled with a cocktail of biotin-conjugated antibodies that are bound to a cell microbead-cocktail. Susequently, CFDA-SE- or eFluor 670-labelled PBMCs were incubated in NK cell biotin antibody cocktail and then conjugated with NK cell Micro bead cocktail. Cell suspension was rinsed in LS or MS columns (Miltenyi) through a magnetic field using a midi or mini magnet (Miltenyi). Enriched NK cells constituted the total cell effluent collected.

2.2.8.4 Sorting of CD56^{bright} and CD56^{dim} NK subsets

Activation of NK cells results in changes of CD56 and CD16 expression, therefore, we initially sorted CD56^{bright} and CD56^{dim} cells in order to longitudinally trace each cell subset. For this purpose, CFSE- or eFluor670-labelled MACS-isolated NK cells were further stained with anti-CD56 and -CD16 antibodies for 30 minutes at 4°C. Stained cells were washed in MACS buffer and resuspended in RPMI. Cells were sorted in a FACS Aria (BD) from the FACS sorting core unit, according to the expression of CD16: CD56^{bright} expressing low or null CD16, and CD56^{dim} showing a high expression of CD16. The collected subpopulations were washed in PBS + 5% FCS and then plated in 96 wells-plates in standard medium as following: unstained PBMC were mixed with CFSE-labeled CD56^{bright} cells and eFluor-labeled CD56^{dim} cells (or vice versa) at ratios of 1:10 NK/T cells and 1:10 CD56^{bright}/CD56^{dim} NK cells. Cell proliferation within the cell PBMC mix was analyzed at day 3, 5 and 7 as the percentage of diluted CFSE or eFluor labeled cells in reference to the initial peak of the original parent generation.

2.2.9 Statistical analysis

GraphPad Prism (version 5.0) software was used for statistical evaluation of data. The unpaired t test was performed to compare healthy donor and untreated MS patients; all t tests were two tailed. Comparison of untreated patients and treated with different therapies, was performed using one-way analysis of variance (ANOVA) followed by a Dunnett´s multiple comparison test, using the untreated group as reference. Normal distribution was determined with the D'Agostino-Pearson omnibus test. P values less than 0.05 were considered significant.

3. Results

3.1 Effect of treatment on the relative frequency of T and NK cells in MS patients

The frequency of CD4, CD8, CD56^{dim} and CD56^{bright} cells was assessed in 26 healthy donors and 66 MS patients. From the patients, 16 were untreated, 29 treated with IFNβ-1a (12 Avonex, 17 Rebif), 15 with natalizumab (Tysabri) and five with anti-CD25 mAb. The frequencies of CD56^{dim} and CD56^{bright} were determined from the NK cell gate, whereas CD4+ and CD8+ were determined from T cells.

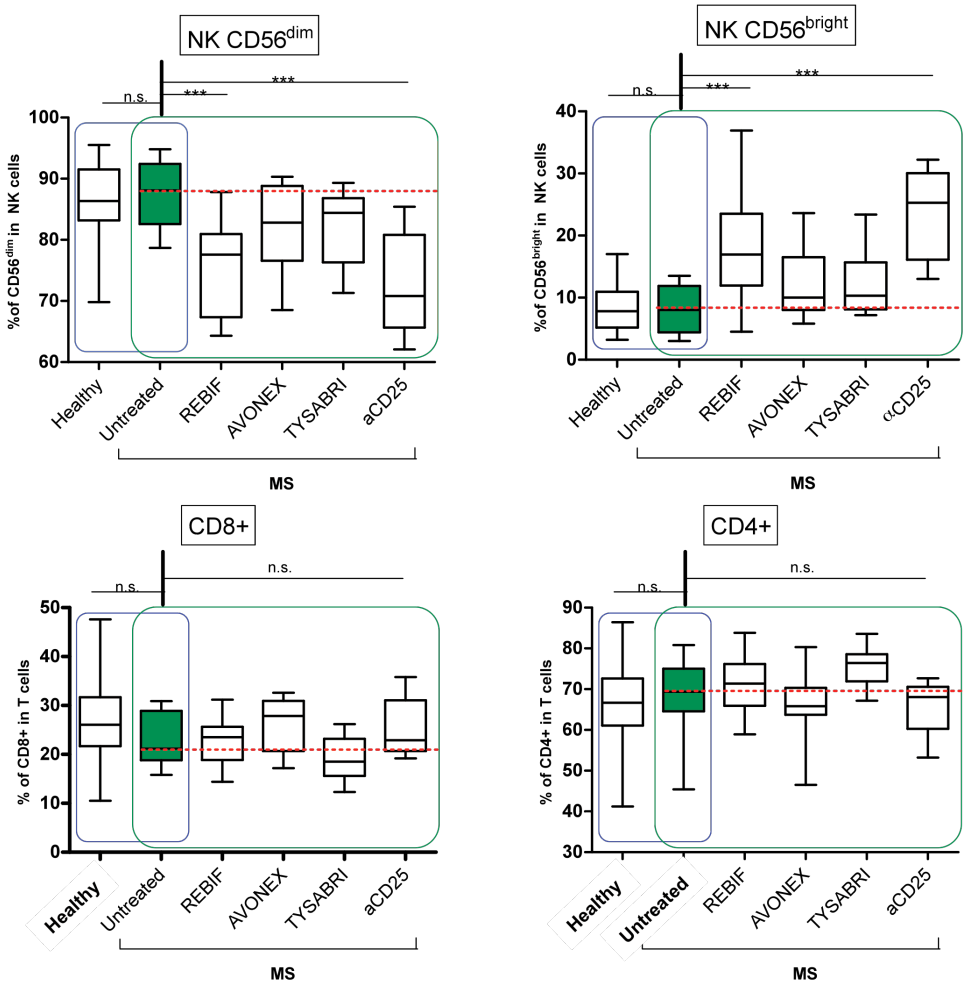

Figure 9. Frequency of lymphocytic cells in healthy donors and MS patients
Depicted are the percentages of CD56^{dim}, CD56^{bright}, CD4+ and CD8+ cells analyzed by flow cytometry. Cell populations were gated according to the strategy described above in figure 6. Frequencies correspond to healthy donors (n=26), untreated (n=16) and treated MS patients (total n=66). Among the latter group, we dissected the effect of different treatments, i.e. Rebif (n=17), Avonex (n=12), natalizumab (n=15) and anti-CD25 mAb (n=5). Unpaired t test was performed to compare healthy donor and untreated MS patients (blue rectangle). Comparison of untreated patients and treated with different therapies, was performed using one-way analysis of variance (ANOVA) followed by a Dunnett´s multiple comparison test, using the untreated group as reference (green rectangle). The broken line represents the median of untreated MS patients as reference to compare the treated groups. *** p<0.001, **p<0.01, p*<0.05

We first compared the percentage of cell subpopulations between untreated MS patients and healthy donors. In a subsequent step, we compared untreated MS patients against each treated group. In our cohort, frequencies of NK and T cell subsets did not differ between healthy donors and untreated MS patients (fig. 9). An increase in the frequency of CD56^{bright} NK cells in MS patients has been reported as an effect of anti-CD25 (Bielekova et al. 2006) and IFNβ-1a treatment (Saraste et al. 2007 and Vandenbark et al. 2009). Consistent with these reports, we observed a higher percentage of CD56^{bright} NK cells in patients treated with IFNβ-1a (15.9±8%, p<0.01) and, to a higher extent, with anti-CD25 antibodies (23.5±7.5%, p<0.01) in comparison to untreated MS patients (7.9±3.6)(fig. 9).

The percentage of CD56^{bright} cells in RRMS patients treated with IFN-β1a was further dissected between the two commercial IFNβ-1a used: Avonex and Rebif. Rebif induced a higher increase in the frequency of CD56^{bright} NK cells than Avonex, but the difference did not reach statistical significance (p=0.072) (figure 10).

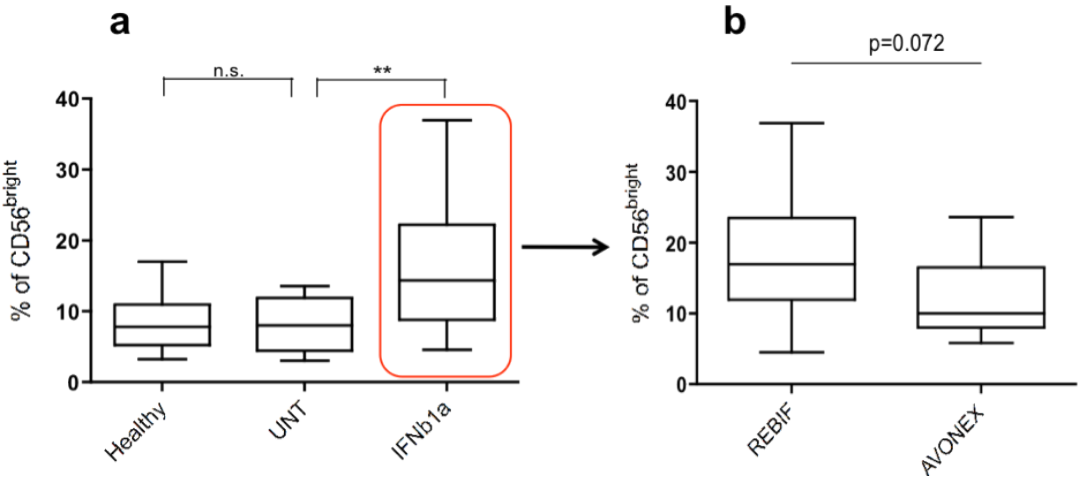

Figure 10. Effect of IFN-β-1a on the frequency of CD56^{bright} NK cells
Percentage of CD56^{bright} NK cells (out of total NK cells) in PBMC from 26 healthy donors, 15 untreated RRMS subjects and 29 IFN-β-1a-treated patients (18 Rebif, 11 Avonex). Horizontal bars indicate median values. Unpaired t test was used for statistical analysis (**p<0.01, n.s. non significant).

Treatment with natalizumab results in an increase of total NK cells (Skarica et al. 2011 and Putzki et al. 2010); however, its effect on NK cell subsets has not been analyzed. Interestingly, we observed that treatment with natalizumab, similar to IFNβ-1a and anti-CD25 mAb, also resulted in an increased frequency of CD56^{bright} NK cells (12.2±4.8%). CD4+ T cells were also found elevated in peripheral blood in these patients (p<0.05) (fig. 9).

To determine the effect of different treatments on the frequency of Treg cells in MS, we established our gating strategy for flow cytometry analysis based on the expression of CD25pos and CD127neg on the CD4+ T cells (fig. 11a). Treg from MS patients treated with anti-CD25 mAb were analyzed separately with a different staining and gating strategy due to the lack of CD25 expression after treatment; details are described later. As reported by other groups (revised by Costantino et al. 2008 and Venken et al. 2008), we first confirmed that the frequency of Tregs in MS patients was similar to healthy donors. In contrast, natalizumab induced a significant decrease of CD4+ CD25+ CD127neg regulatory T cells (P<0.01) (fig. 11b).

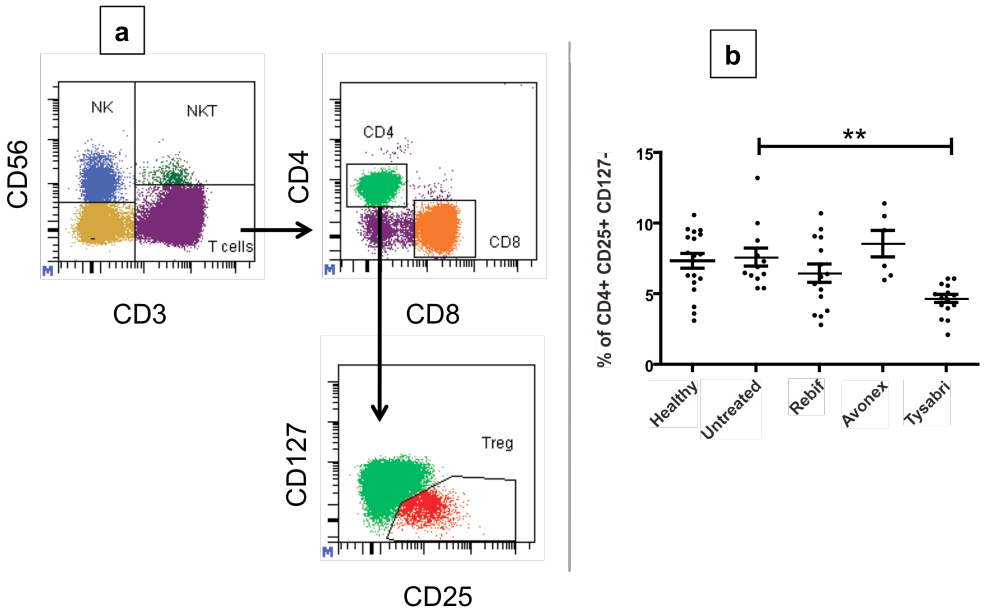

Figure 11. Natalizumab induces a decrease in the frequency of Treg cells
PBMC from 18 healthy donors, 12 untreated MS patients, 15 Rebif-treated, 6 Avonex-treated and 15 Rebif-treated MS patients were analyzed. Samples were stained for CD3, CD56, CD4, CD8 CD25 and CD127 and analyzed by flow cytometry. a) Gating strategy for Treg cells. Cells were first gated on an FSC/SSC plot, followed by an exclusion of doublets in an SSC-A/SSC-H plot (not shown). A quadrant gate was set in the CD3/CD56 dot plot. T cells were defined as CD3pos CD56neg. CD4+ and CD8+ T cells were gated in a CD4/CD8 plot, derived from the T cell gate. Treg cells were gated from the CD4 subset according to negative expression of CD127 and CD25 positive/high. b) Percentages of CD4+ CD25+ CD127neg cells in healthy donors and MS patients. Each dot represents one subject. Mean values and SEM are shown. ** p<0.01

3.1.1 Effect of treatment with anti-CD25 monoclonal antibodies

3.1.1.1 A good response to treatment with anti-CD25 induces a gradual increase in CD56^{bright} NK cells over time

We had the chance to longitudinally follow two patients treated with anti-CD25 mAb. One of them developed a good response to treatment, whereas the other was a bad responder.

In the good responder, we observed a sustained increase in the frequency of CD56^{bright} cells over the first year of treatment with basiliximab followed by a plateau between the first and second year of treatment (baseline: 7.7%, 12 months: 20%, 24 months: 26.7%). Consequently, a decrease of CD56^{dim} cells occurred (baseline 78.9%, 12 months 60%)(fig.12a).

The second patient was initially treated with daclizumab for two years. In 2009, daclizumab was withdrawn from the market, resulting in a change of the therapeutic scheme from daclizumab to basiliximab. He developed a bad response to treatment with basiliximab, including an evident reduction of CD56^{bright} cells with a subsequent increase in CD56^{dim} NK cells (fig. 12b). After six months of non-response to treatment, basiliximab was eliminated from his medication.

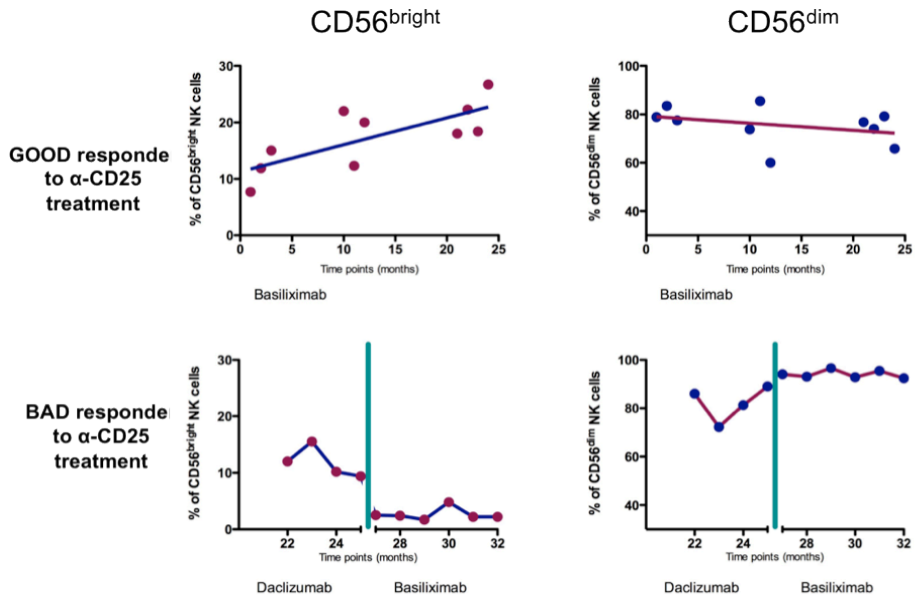

Figure 12. Response to anti-CD25 mAb treatment in two MS patients
The time course of CD56^{bright} and CD56^{dim} NK cells in the peripheral blood from two MS patients responding differently to basiliximab was assessed. Patients were sampled monthly; PBMC or fresh blood were obtained right before the i.v. administration of basiliximab. NK and T cell subpopulation frequencies were determined by flow cytometry using CD3, CD4, CD8, CD56 and CD16 antibodies. a) Basiliximab-treated MS patient followed for two years; a good response to treatment was observed. b) MS patient initially treated with daclizumab, responding favourably to treatment. After two years, his treatment was changed to basiliximab, resulting in a bad response including a drastic decrease of CD56^{bright} NK cells with a consecutive increase of CD56^{dim} NK cells.

Daclizumab and basiliximab bind the same epitope as IL-2 in the alpha chain of the IL-2 receptor (Binder et al. 2007). This epitope is referred to as “tac” epitope, in contrast to “non tac” epitopes, which are recognition sites within the CD25 molecule other than

the IL-2 binding site. To assess the expression of IL-2Rα, we used two different antibodies: the anti-CD25 clone 2A3 recognizing the tac epitope, and anti-CD25 clone M-A251 recognizing the non tac epitope. PBMC from the patient responding well to basiliximab were obtained at trough levels, i.e. right before the administration of the next dose.

As expected, the CD25 tac epitope remained blocked throughout the duration of basiliximab therapy, whereas the CD25 non tac epitope persisted detectable on the cell surface. Total CD25-non tac expression on CD4+ T cells declined from 8.2 to 5.1% between the first and second year of treatment with basiliximab (fig. 13).

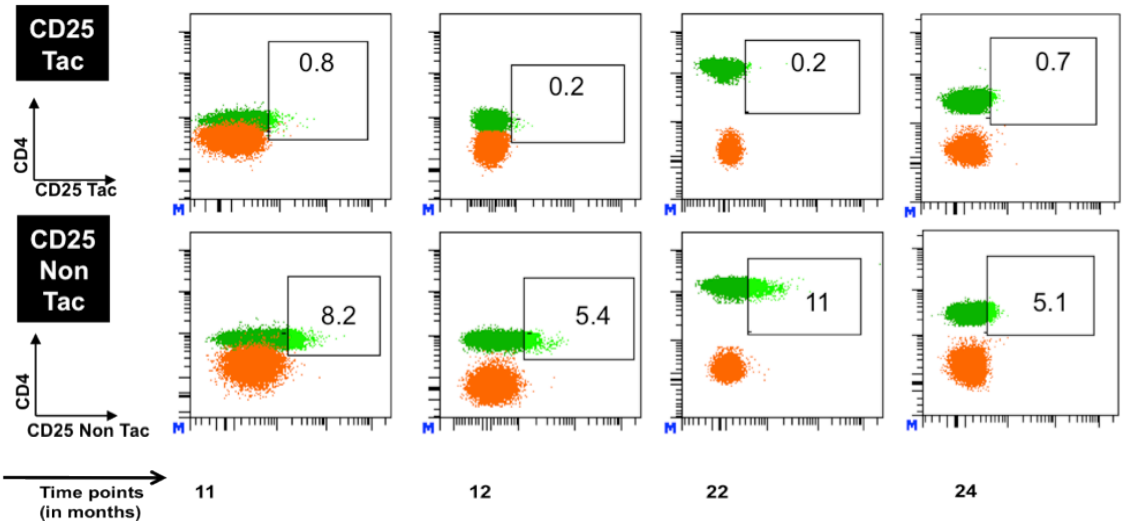

Figure 13. Effect of basiliximab on CD25 expression
CD25 expression was evaluated by FACS analysis in PBMC from a basiliximab-treated MS patient at different time points. 2 fluorochrome-labeled antibodies directed at a competing (tac, clone 2A3) and noncompeting epitope on CD25 (anti tac, clone M-A251) were used.

3.1.1.2 Treatment with anti-CD25 mAb does not deplete CD4+ FoxP3+ cells

Basiliximab and daclizumab are not depleting antibodies; still, the effect of depriving Treg cells from IL-2 has not been analyzed in MS patients. Since treatment with anti-CD25 mAb masks the tac epitope and decreases the total expression of the non tac epitopes in the CD25 molecule, we used FoxP3 to identify Treg cells. We observed that the frequency of Treg cells was not affected by treatment with daclizumab of basiliximab in comparison to healthy donors (fig. 14).

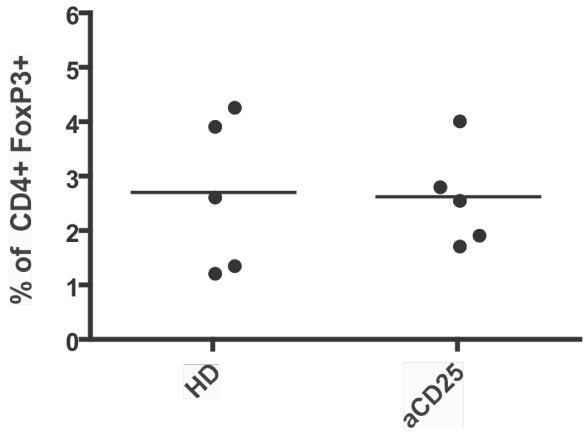

Figure 14. Effect of anti-CD25 treatment on regulatory T cells the frequency in MS
PBMC from healthy donors and anti-CD25 mAb-treated MS patients were stained for CD3, CD4 and CD8 with a subsequent intracellular staining for FoxP3. Shown are the percentages of FoxP3 positive cells within the CD4+ cells.

3.2 Phenotypical characterization of NK cells in MS patients

To phenotypically characterize the expanded CD56^{bright} NK cells in MS patients after treatment with anti-CD25 mAb, IFNβ-1a and natalizumab, we elaborated a panel of cell surface markers based on molecules expressed normally on CD56^{bright} NK cells. These markers are not exclusive of this cell population; therefore their expression on other cell subsets such as CD56^{dim}, CD4 and CD8 cells was also examined.
We first analyzed the expression of these molecules on PBMC from healthy donors in comparison to untreated MS patients to determine an expression pattern in MS. We then compared the expanded cells after different treatments to the untreated group.

3.2.1 Cytokine receptors

3.2.1.1 IL-2 receptor complex

CD25, CD122 and CD132 are the α-, β- and γ-chains (γc) of the IL-2 receptor, respectively. Daclizumab targets the α-chain of the high-affinity receptor. Blocking IL-2 signaling in T cells with daclizumab could thus increase the availability of IL-2 for NK cells, leading to their expansion (Martin et al. 2010). We determined the expression of the IL-2R components in T and NK cells after treatment with anti-CD25 mAb, and also with treatments that do not target the IL-2 receptor such as IFNβ-1a and natalizumab. In healthy donors, surface expression of **tac and non tac** epitopes of CD25 was observed mainly on CD4+ T cells and to a minor extent, CD56^{bright} NK cells, whereas CD8+T cells and CD56^{dim} NK cells expressed this marker scarcely. In comparison to healthy donors, expression of CD25 tac and non tac was slightly higher on CD4+ T

cells of untreated MS patients. CD25 tac epitope expression was decreased significantly on CD4+ T cells after treatment with IFNβ-1a and natalizumab. Anti-CD25 mAb treatment masked the expression of CD25 tac epitope on all cell types analyzed. Differences in the expression of CD25 non tac epitope were only marginal in all treatment groups (fig 15).

In healthy donors, **IL-2 receptor beta chain (CD122)** expression was exclusively expressed on NK cells. Among the NK cell subsets, CD122 expression predominated on $CD56^{bright}$ cells. In MS, CD122 expression was not altered in comparison to healthy controls. Interestingly, Tysabri significantly reduced CD122 expression on both NK cell subsets. Anti-CD25 mAb treatment resulted in a slight increase of CD122 expression on $CD56^{bright}$ NK cells (fig 15).

Expression of **IL-2 gamma chain receptor (IL-2Rγ or CD132)** was expressed on T and NK cells, showing no differences between healthy donors and MS. Tysabri and anti-CD25 treatment induced a significantly lower expression of CD132 on $CD56^{bright}$ NK cells. CD8+ T cells also showed a reduction in CD132 expression after anti-CD25 mAb treatment (fig.15).

3.2.1.2 IL-7 receptor α or CD127

IL-7 enhances the survival of the $CD56^{bright}$ NK subset (Michaud et al 2010). We observed that in untreated MS patients, NK cells showed a decreased expression of **CD127** in comparison to healthy donors, whereas no significant changes were observed on T cells. Treatment with IFNβ-1a brought the expression of CD127 on NK cells to healthy donor levels predominantly on $CD56^{dim}$ NK cells (fig. 16).

3.2.1.3 IL-18 receptor α

IL-18 has been found increased in serum and CSF of MS patients (Losy and Niezgoda 2001). We observed the expression of its receptor, **IL-18Ra**, predominantly on $CD56^{bright}$ NK cells in all groups. A remarkable increase of IL-18Ra expression on $CD56^{bright}$ NK cells of patients treated with anti-CD25 was observed (fig. 16).

3.2.2 Activation markers

3.2.2.1 CD69, CD26 and CD6

Next, we assessed the expression of the activation markers CD69, CD26 and CD6 (fig. 17). The expression of these molecules has been documented in diseases characterized by T cell activation, such as systemic lupus erythematosus (Wong et al.

2009), rheumatoid arthritis (RA) and multiple sclerosis (Sellebjerg et al. 2005, Constantinescu et al. 1995 and Tak et al. 1996). However, the expression of these markers on NK cells in the context of MS has not been addressed.

In healthy donors, we confirmed that CD4+ T cells were the predominant cell type bearing **CD26** on their surface, followed by CD8+ T cells, $CD56^{bright}$ and $CD56^{dim}$. In untreated MS individuals, the expression of CD26 was significantly decreased only on NK cells. Remarkably, Avonex and anti-CD25 mAb induced an increase of CD26 expression on $CD56^{bright}$ NK cells (fig. 17).

CD6 molecule, a surface glycoprotein primarily expressed on lymphocytes, plays a crucial role in cell adhesion, activation, differentiation, and survival processes. CD6 expression was observed mainly on T cells, whereas on NK cells, CD6 was limited to a subset of the $CD56^{dim}$ cells. CD4 T cells expressed CD6 at a higher extent than CD8 T cells. We confirmed that in healthy donors, $CD56^{bright}$ NK cells do not express CD6; however, treatment with natalizumab and anti-CD25 mAb induced a minor but significant overexpression of this molecule on $CD56^{bright}$ cells. Avonex exerted a clear reduction of CD6 expression on $CD56^{dim}$ cells (fig. 17).

CD69, an early T cell activation antigen, is involved in lymphocyte proliferation, functioning as signal-transmitting receptor in lymphocytes, NK cells, and platelets. In our cohort, $CD56^{bright}$ NK cells represented the population with higher frequency of CD69-bearing cells. Treatment with anti-CD25 mAb exerted a reduction in the expression of CD69 on this cell population (fig. 17).

3.2.2.2 CD2, CD11b, NKG2D and HLADR

NK cell activation *in vivo* most likely represents a coordinated effort of various receptors with activating potential (Orange et al. 2003). Among others, CD2, 2B4, CD11a, CD11b, HLADR, NKp30, NKp46, and NKG2D have been described as NK cell activating receptors. We analyzed the expression of CD2, CD11b, NKG2D and HLADR in our cohort.

CD2 regulates NK cell lytic activity and inflammatory cytokine production upon engagement of ligands on neighboring NK cells (McNerney and Kumar 2006). We observed that anti-CD25 mAb therapy produced a prominent increase of CD2 expression on $CD56^{bright}$ and, to a lesser extent, on $CD56^{dim}$ cells. Rebif decreased CD2 expression on $CD56^{dim}$ and CD8+ T cells, whereas natalizumab only on CD8+ T cells (fig. 18).

CD11b is an integrin expressed on monocytes, neutrophils, NK cells, granulocytes and macrophages. CD11b participates both in adhesion between the NK cell and its target

cell and in stimulation. Regarding NK cells cell activation signaling, ligation of CD11b induces phosphorylation-dependent NK cell activation (Orange et al. 2003). CD11b is of particular interest, because NK cells are the only lymphocytes that uniformly express this subunit in high density. We confirmed in healthy donors that the expression of CD11b is restricted to the NK cell subpopulations. $CD56^{dim}$ showed a higher expression of this marker than the $CD56^{bright}$ counterpart. Untreated MS patients showed a marked decrease of CD11b on the $CD56^{bright}$ cells. Treatment with IFN-β1a and, predominantly anti-CD25 mAb, produced a recovery of CD11b expression on $CD56^{bright}$ cells to higher levels than in healthy donors (fig. 18).

NKG2D is an activating receptor expressed on human NK cells, CD8+ T cells, and gamma/delta T cells. In the inflamed CNS, NKG2D-NKG2D ligand interactions may contribute to cytotoxic responses mediated by activated immune effector cells. All treatments induced a reduction of NKG2D expression on $CD56^{dim}$ and CD8 cells, whereas Rebif and natalizumab exerted an increased expression of this marker on CD4+ and $CD56^{bright}$ cells (fig. 18).

HLA-DR expression marks a distinct subset of NK cells, present at low frequency in circulating blood but readily expanded by IL-2, which can play an important role during immune responses (Evans et al. 2011). Untreated MS patients were characterized by a significant decrease in the expression of this molecule on NK and T cell populations. All treatments induced normalization to healthy baseline levels of HLA-DR expression in all cell subsets (fig.18).

3.2.3 Markers of NK cell differentiation

3.2.3.1 CD57, CD161, CD94, CD27 and CD62L

CD94, CD57, CD62L, CD27 and CD161 are surface molecules expressed at different stages of NK cell maturation. We examined the expression of these molecules to determine a possible pattern indicative of a developmental stage of the expanded $CD56^{bright}$ NK cells after treatment either with anti-CD25, IFNβ1a or natalizumab.

In NK cells, **CD57** defines a functionally distinct population of mature cells in the human $CD56^{dim}$ NK cell subset (Lopez-Verges et al. 2010). On chronically stimulated T cells, CD57 is upregulated. In our healthy donor cohort, among all cell subsets studied, the expression of CD57 was confined to the $CD56^{dim}$ NK cells, and to a lesser extent, to CD8+ T cells. Consistent with prior observations in healthy donors (Bjorkstrom et al. 2010), CD57 was expressed on 10 to 75% of $CD56^{dim}$ NK cells, which represent mature NK cells, whereas less than 1% of $CD56^{bright}$ NK cells expressed CD57. We did not find

differences in the frequency of CD57-bearing cell subsets between healthy controls, untreated and treated MS patients (fig. 19).

CD161 (human NKR-P1A) is a molecule among the first NK cell receptors to be detected during NK cell development. Analysis of peripheral blood of patients with MS and RA have shown an upregulation of CD161 at the protein level, as well as a significant excess of $CD161^{high}CD8+$ T cells. The expression of CD161 on CD8+ T cells marks a subset of non-cytotoxic, proinflammatory T lymphocytes that may play a role in multiple sclerosis immunopathogenesis by acting as effectors and targeting the CNS (Annibali et al. 2011). We were interested in the expression of this molecule on NK cells as a marker of early NK development, as well as a possible player contributing to the immunopathogenesis of MS. In healthy donors, CD161 was expressed on a minority of CD4 and CD8+ T cells in comparison to its expression on the majority of NK cells. CD161 showed a bimodal pattern expression on NK cells and CD4+ cells, while on CD8+ T cells, the pattern was trimodal. Consistent with other studies in MS and in RA, we observed an increased frequency of CD161+ $CD56^{bright}$ and CD161+$CD56^{dim}$ cells in untreated MS patients in comparison to healthy donors. Rebif reduced the expression of CD161 on $CD56^{bright}$ $CD56^{dim}$ and CD4+ T cells, whereas Tysabri reduced it on CD8+ T cells. We also observed a non-significant, but evident reduction in the frequency of $CD56^{bright}$ CD161+ cells in MS patients treated with anti-CD25 mAb. Remarkably, from all the markers studied within our panel, CD161 was the only molecule expressed at higher levels in untreated MS patients in comparison to healthy donors (fig. 19).

CD94 expression on NK cells identifies a functional and likely developmental intermediary between $CD56^{bright}$ and $CD56^{dim}$ NK cells (Yu et al. 2010). In our cohort, $CD56^{bright}$ NK cells were distinguished as the cell population with the highest density of CD94 on their surface, whereas two thirds of $CD56^{dim}$ cells were positive for CD94. On CD8 cells, the expression was low, while on CD4+ cells, it was nearly null. CD94 expression was diminished significantly on $CD56^{dim}$, CD4+ and CD8+ cells in untreated MS patients. All treatments induced a recovery of the CD94 expression on CD4+, CD8+ and $CD56^{bright}$ cells, reaching healthy baseline levels. CD94 expression on $CD56^{bright}$ NK cells of Rebif- and Avonex-treated MS patients increased to higher levels than in healthy donors. Remarkably, anti-CD25 mAb treatment caused a two-fold increase of CD94 expression on $CD56^{bright}$ cells in comparison to untreated patients (fig. 19).

Expression of **CD27** dissects mature NK cells into two subsets with distinct responsiveness and migratory capacity: the majority of $CD27^{neg}$ NK cells are $CD56^{dim}$, whereas a large proportion of $CD27^{pos}$ NK cells are $CD56^{bright}$ (Vossen et al. 2008).

We observed that the main expression of CD27 was on T cell subsets; however, we confirmed the higher expression of this marker on the $CD56^{bright}$ NK cell population rather than in the $CD56^{dim}$. Notably, natalizumab caused a significant decrease in the expression of CD27 on all cell subpopulations in comparison to untreated MS patients (fig. 20).

The expression of **CD62L** on NK cells has been recently reported to identify a unique subset of polyfunctional $CD56^{dim}$ NK cells, representing an intermediate stage of NK-cell maturation, which after restimulation can develop into terminally differentiated effectors (Juelke et al. 2010). Our healthy donor samples showed a CD62L expression pattern on NK cells similar to that of CD94, in which nearly all $CD56^{bright}$ cells were positive, whereas $CD56^{dim}$ displayed a bimodal expression, confirming the presence of the $CD56^{dim}$ CD62L+ subset. Interestingly, anti-CD25 treatment exerted a significant decrease in the expression of CD62L on CD4+ and CD8+ cells, and an increase on $CD56^{bright}$ cells. Natalizumab also induced an increase of CD62L expression on $CD56^{bright}$ NK cells. Additionally, natalizumab produced a differential expression of CD62L on $CD56^{bright}$ cells; i.e., some patients expressed $CD56^{high}$ $CD62^{low}$ and some $CD56^{high}$ $CD62L^{high}$ (fig. 20).

3.2.4. Adhesion molecules

3.2.4.1 CD11c

CD11c, an integrin involved in the binding of iC3b, adhesion to stimulated endothelium, and phagocytosis of apoptotic cells is expressed highly during relapses in MS. This expression reflects the temporal activity of MS, suggesting that the $CD11c^{high}$ group of patients may be in more unstable condition than $CD11c^{low}$ (Aranami et al. 2006).

We observed that, similar to CD11b, the expression of CD11c was limited to NK cells, with predominance on $CD56^{bright}$ NK cells. In untreated MS patients, CD11c expression on $CD56^{bright}$ NK cells was significantly reduced. Avonex, Rebif, and predominantly anti-CD25 mAb induced an increase of CD11c expression on $CD56^{bright}$ NK cells (fig. 21).

3.2.5 Chemokine receptors

3.2.5.1 CCR7 and CXCR3

The frequency of $CD56^{bright}$ NK cells is ten times higher in lymph nodes than in blood. This difference has been attributed to the expression of CCR7, since entry of lymphocytes to lymph nodes can be directed via expression of CCR7, in response to

the CCR7 ligands CCL19 and CCL21 expressed in the lymph node. The $CD56^{bright}$ NK cell subset differentially expresses CCR7, allowing its accumulation in lymph nodes (Johnson et al. 2011). In MS patients after treatment with IFNβ, CCR7 expression on peripheral blood lymphocytes is up-regulated (Vallittu et al. 2007).

In untreated MS patients, **CCR7** expression was significantly diminished on $CD56^{dim}$ and $CD56^{bright}$ NK cells. We confirmed that treatment with Rebif in MS patients caused an increased expression of CCR7 on both NK cell subsets, as well as after treatment with natalizumab and anti-CD25 mAb. Of interest, anti-CD25 mAb produced an outstanding increase of CCR7 expression on the CD4+ T cell subset in comparison to untreated MS patients (fig. 21).

CXCR3 mediates the migration of effector T cells and NK cells to inflamed tissues, where the cognate ligands CXCL9, CXCL10 and CXCL11 are expressed. CXCL9 mediates the recruitment of circulating CXCR3+ monocytes from blood to inflamed lymph nodes and it is likely to be the basis of the observed recruitment of plasmacytoid DC precursors and NK cells. In addition, CXCL10 produced by DCs leads to retention of TH1 lymphocytes in lymph nodes, a trapping mechanism that may also contribute to the increase in NK cell numbers. In our cohort, we observed the frequency of CXCR3 positive cells mainly on the $CD56^{bright}$ and CD8+ cell subsets. All treatments caused a decrease of CXCR3 expression on $CD56^{bright}$ and CD8 cells, more notoriously with anti-CD25 mAb (fig. 21).

In addition to the described markers, we also evaluated the expression of cell surface ectonucleotidases such as CD39 and CD73 and no changes in the expression of these two molecules were observed between healthy donors and untreated or treated MS patients (data not shown).

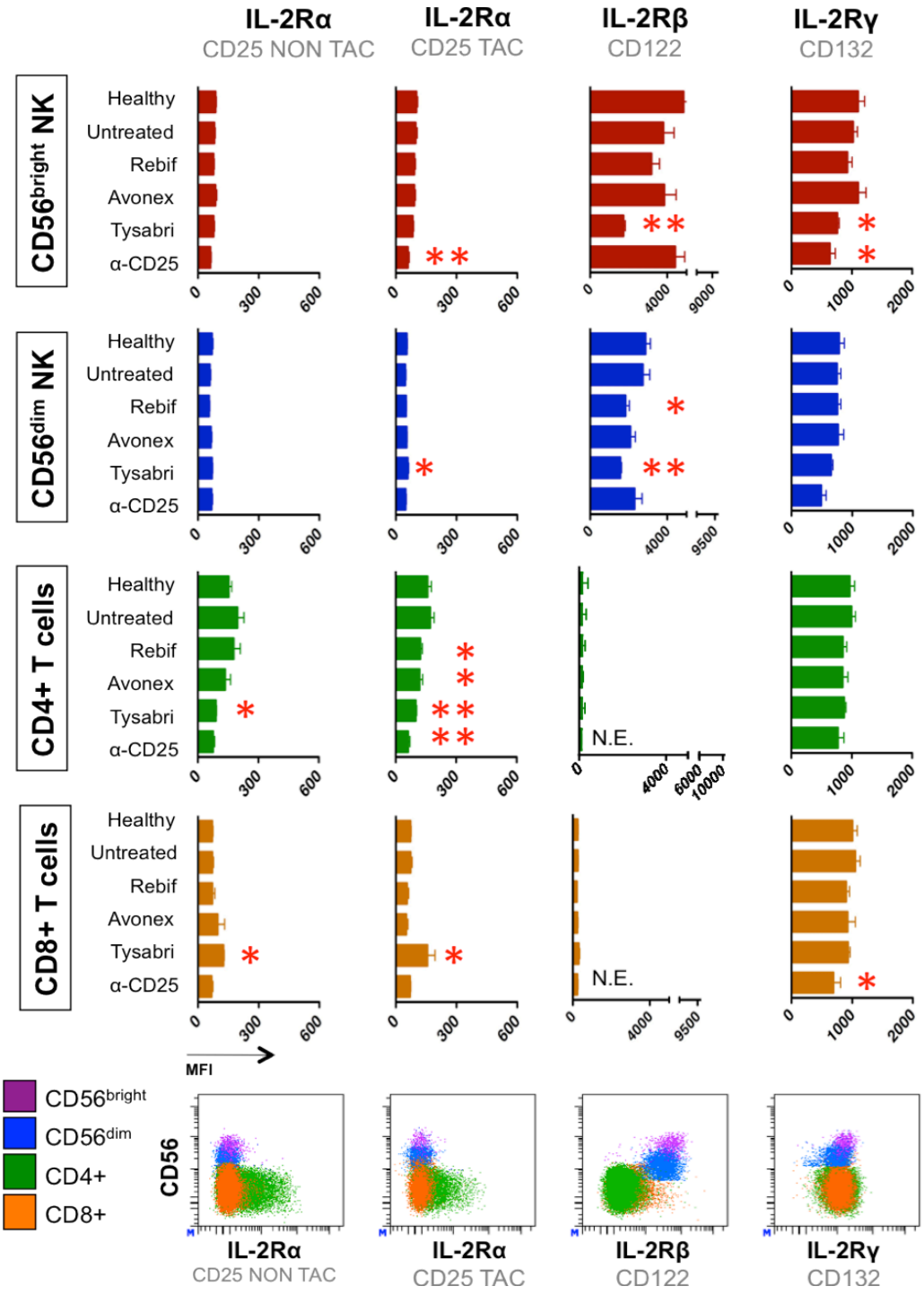

Figure 15. Expression of IL-2R subunits on T and NK cells of healthy donors and MS patients

Cell surface marker expression was analyzed in PBMC from healthy controls (n=26), untreated (n=14) and treated MS patients with Rebif (n=14), Avonex (n=8), Tysabri (n=15) or anti-CD25 mAb (n=5). Horizontal column bars indicate mean and error bars represent SEM. The mean fluorescence intensity (MFI) of CD25 tac, CD25 non tac, CD122 and CD132 for each cell subpopulation was analyzed by flow cytometry. T test was applied to determine statistical differences between healthy donors and untreated MS patients; whereas an ANOVA followed by Dunnett´s multiple comparison test were used to compare all columns (treated patients) to a control column (untreated patients). *p ≤ 0.05, **p<0.01, ***p<0.001.

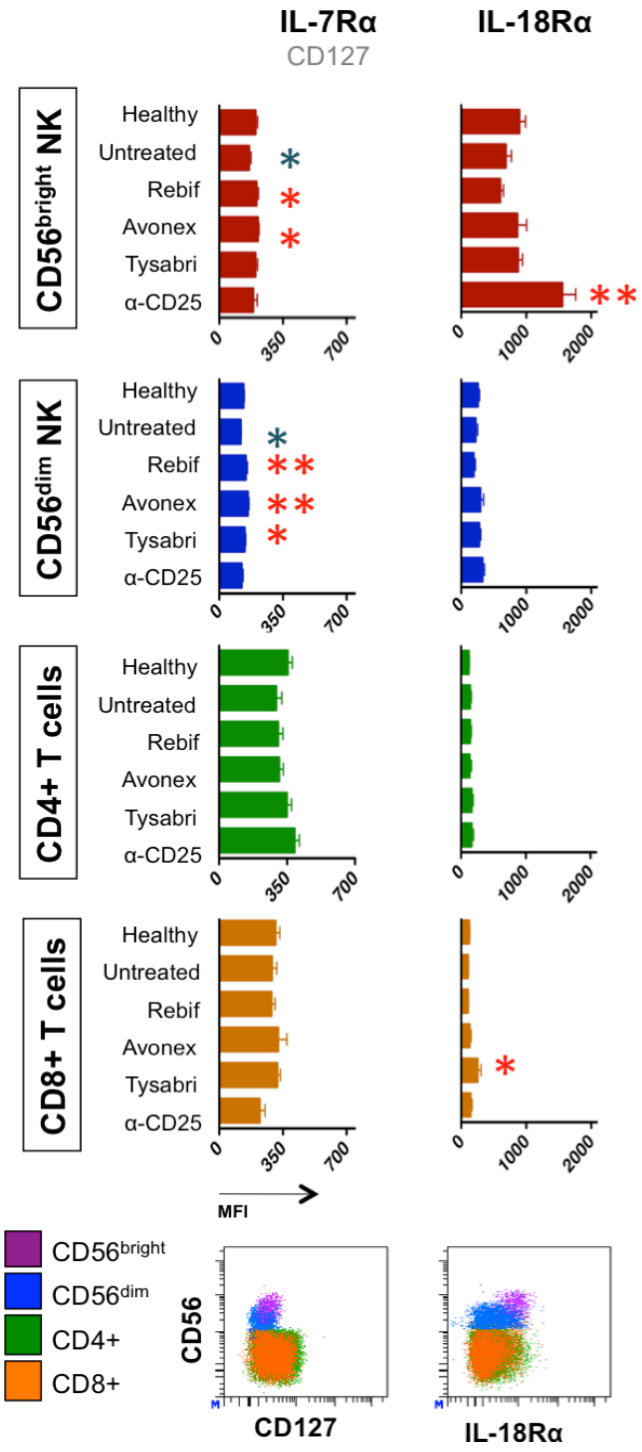

Figure 16. Expression of IL-7Rα (CD127) and IL-18Rα on T and NK cells of healthy donors and MS patients

Cell surface marker expression was analyzed in PBMC from healthy controls (n=26), untreated (n=14) and treated MS patients with Rebif (n=14), Avonex (n=8), Tysabri (n=15) or anti-CD25 mAb (n=5). Horizontal column bars indicate mean and error bars represent SEM. The mean fluorescence intensity (MFI) of CD127 and IL-18Rα for each cell subpopulation was analyzed by flow cytometry. T test was applied to determine statistical differences between healthy donors and untreated MS patients; whereas an ANOVA followed by Dunnett´s multiple comparison test were used to compare all columns (treated patients) to a control column (untreated patients). *p ≤ 0.05, **p<0.01, ***p<0.001.

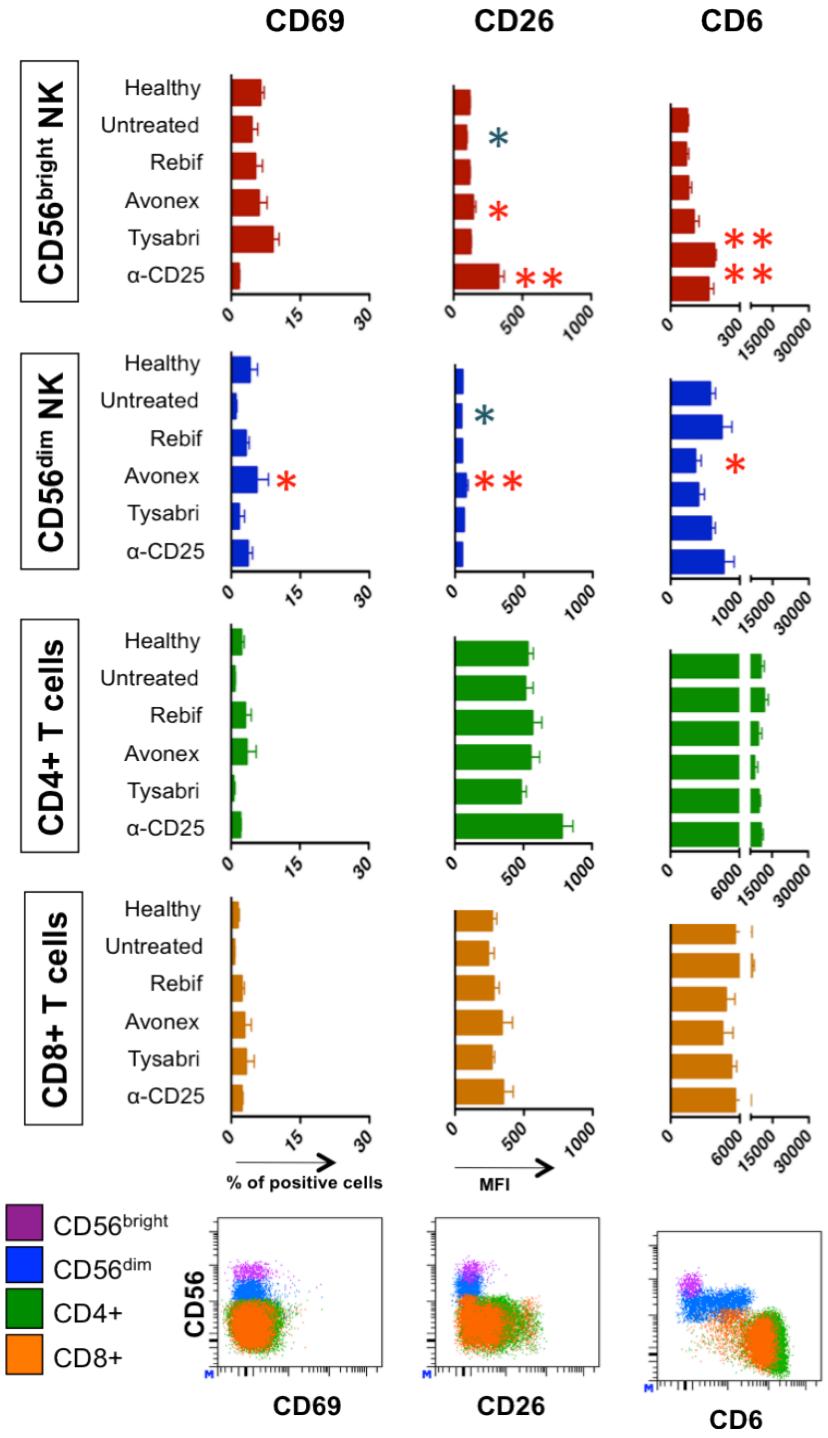

Figure 17. Expression of activation markers on T and NK cells of healthy donors and MS patients

Cell surface marker expression was analyzed in PBMC from healthy controls (n=26), untreated (n=14) and treated MS patients with Rebif (n=14), Avonex (n=8), Tysabri (n=15) or anti-CD25 mAb (n=5). Horizontal column bars indicate mean and error bars represent SEM. The percentage of positive cells to CD69, and the mean fluorescence intensity (MFI) of CD26 and CD6 for each cell subpopulation were analyzed by flow cytometry. T test was applied to determine statistical differences between healthy donors and untreated MS patients; whereas an ANOVA followed by Dunnett´s multiple comparison test were used to compare all columns (treated patients) to a control column (untreated patients). *p ≤ 0.05, **p<0.01, ***p<0.001.

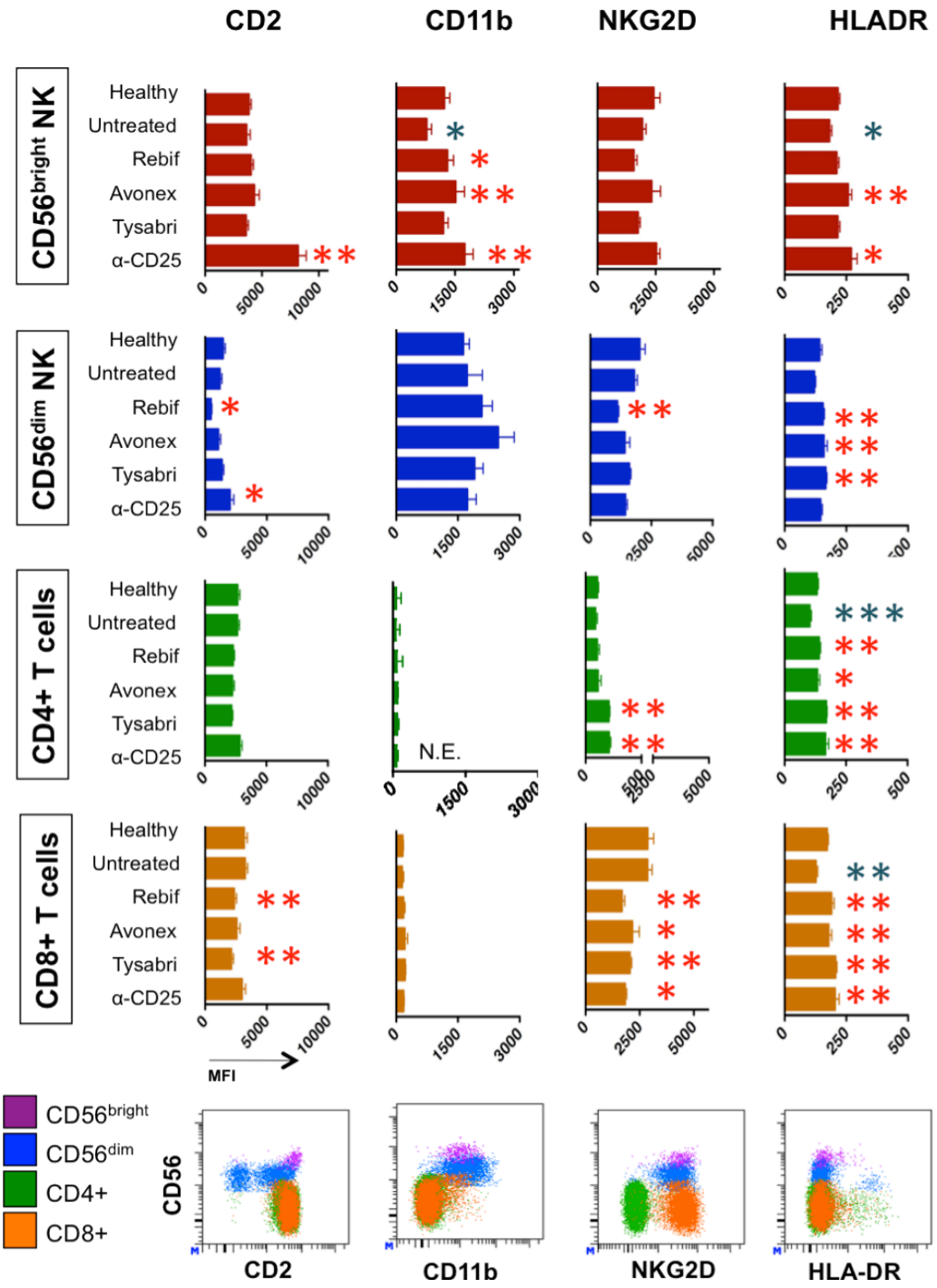

Figure 18. Expression of activation markers on T and NK cells of healthy donors and MS patients

Cell surface marker expression was analyzed in PBMC from healthy controls (n=26), untreated (n=14) and treated MS patients with Rebif (n=14), Avonex (n=8), Tysabri (n=15) or anti-CD25 mAb (n=5). Horizontal column bars indicate mean and error bars represent SEM. The mean fluorescence intensity (MFI) of CD2, CD11b, NKG2D and HLA-DR for each cell subpopulation was analyzed by flow cytometry. T test was applied to determine statistical differences between healthy donors and untreated MS patients; whereas an ANOVA followed by Dunnett´s multiple comparison test were used to compare all columns (treated patients) to a control column (untreated patients). *p ≤ 0.05, **p<0.01, ***p<0.001.

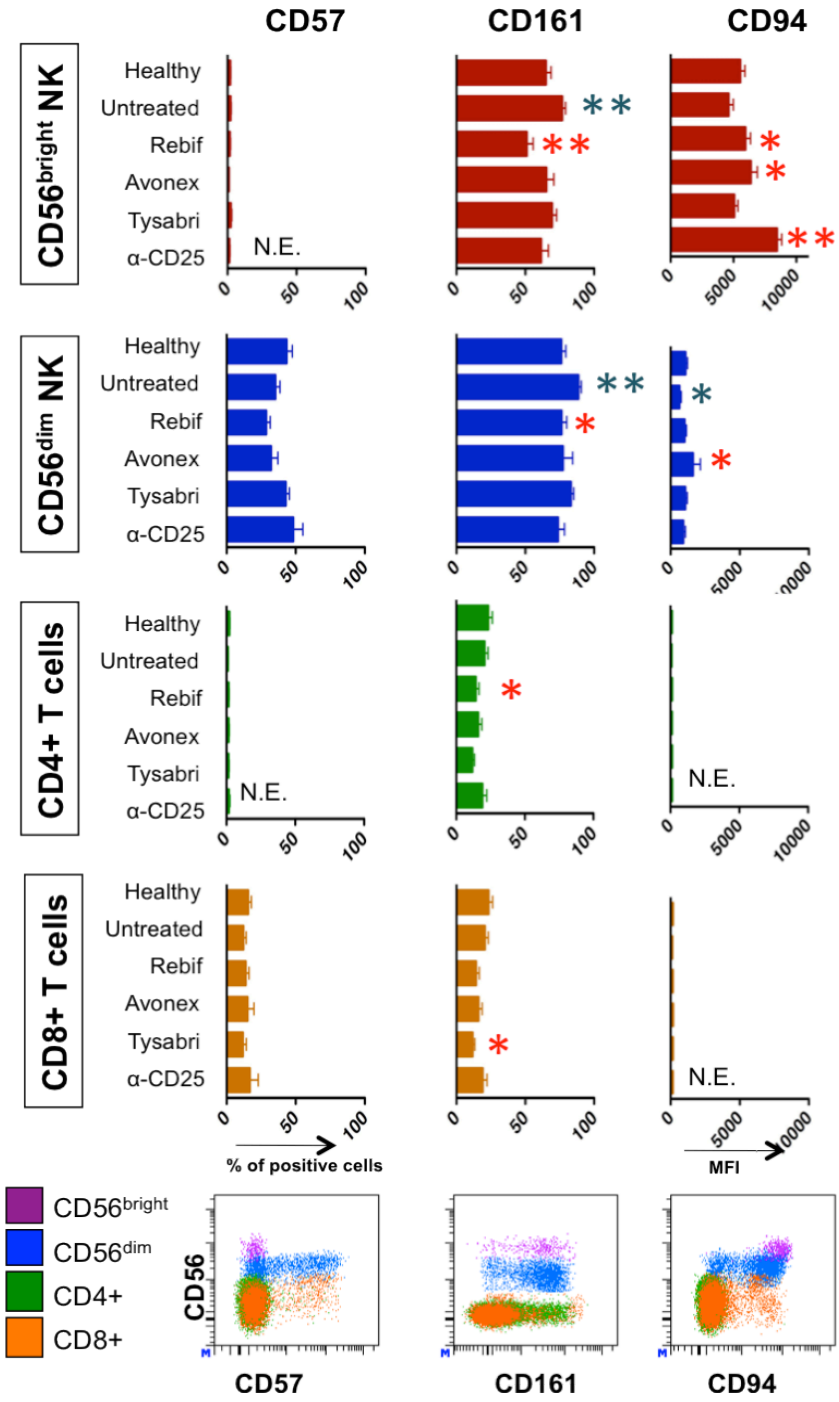

Figure 19. Expression of NK cell differentiation markers in healthy donors and MS patients (I)

Cell surface marker expression was analyzed in PBMC from healthy controls (n=26), untreated (n=14) and treated MS patients with Rebif (n=14), Avonex (n=8), Tysabri (n=15) or anti-CD25 mAb (n=5). Horizontal column bars indicate mean and error bars represent SEM. Percentage of positive cells to CD57 and CD161, and the mean fluorescence intensity (MFI) of CD94 for each cell subpopulation were analyzed by flow cytometry. T test was applied to determine statistical differences between healthy donors and untreated MS patients; whereas an ANOVA followed by Dunnett´s multiple comparison test were used to compare all columns (treated patients) to a control column (untreated patients). *p ≤ 0.05, **p<0.01, ***p<0.001.

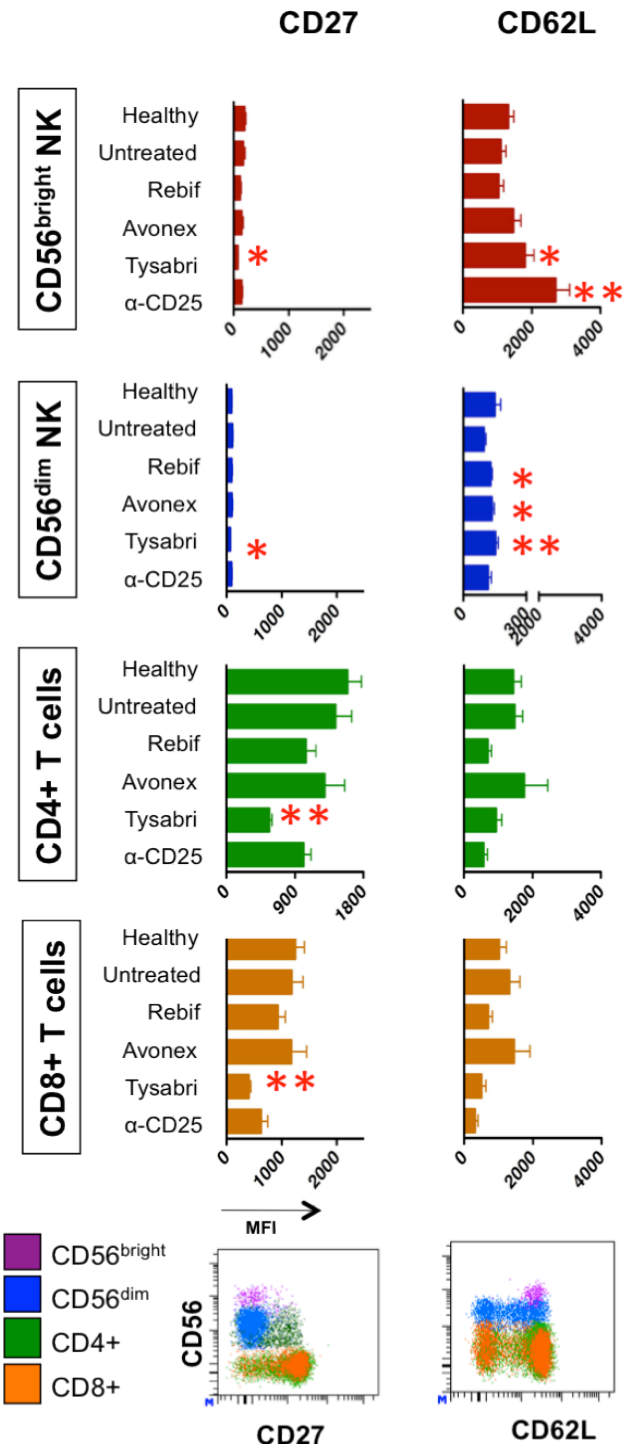

Figure 20. Expression of NK cell differentiation markers in healthy donors and MS patients (II)

Cell surface marker expression was analyzed in PBMC from healthy controls (n=26), untreated (n=14) and treated MS patients with Rebif (n=14), Avonex (n=8), Tysabri (n=15) or anti-CD25 mAb (n=5). Horizontal column bars indicate mean and error bars represent SEM. Mean fluorescence intensity (MFI) of CD27 and CD94 for each cell subpopulation was analyzed by flow cytometry. T test was applied to determine statistical differences between healthy donors and untreated MS patients; whereas an ANOVA followed by Dunnett´s multiple comparison test were used to compare all columns (treated patients) to a control column (untreated patients). *p ≤ 0.05, **p<0.01, ***p<0.001.

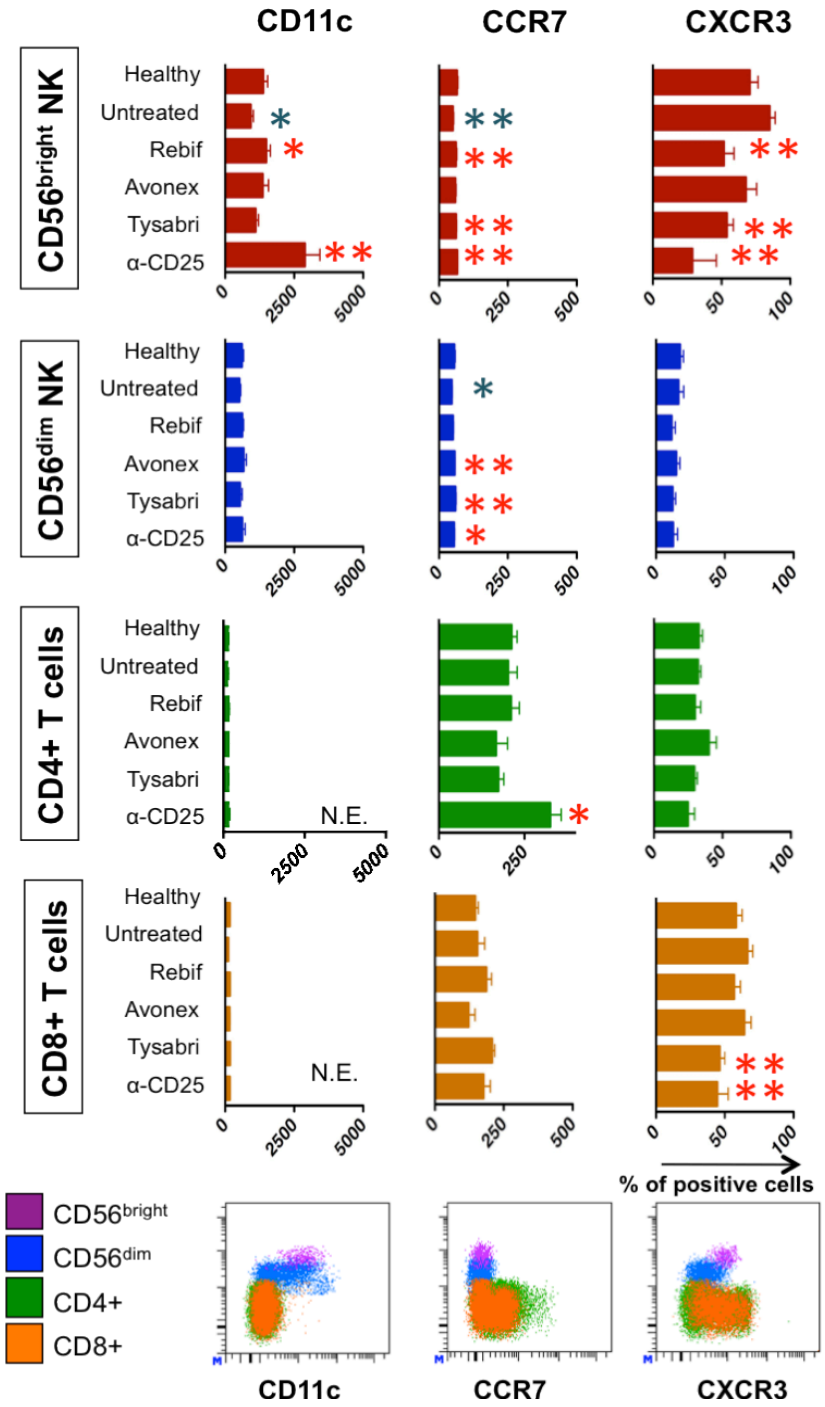

Figure 21. Expression of cell adhesion molecules and chemokine receptors on T and NK cells of healthy donors and MS patients

Cell surface marker expression was analyzed in PBMC from healthy controls (n=26), untreated (n=14) and treated MS patients with Rebif (n=14), Avonex (n=8), Tysabri (n=15) or anti-CD25 mAb (n=5). Horizontal column bars indicate mean and error bars represent SEM. Mean fluorescence intensity (MFI) of CD11b and CCR7, and percentage of positive cells for CXCR3 in for each cell subpopulation were analyzed by flow cytometry. T test was applied to determine statistical differences between healthy donors and untreated MS patients; whereas an ANOVA followed by Dunnett´s multiple comparison test was used to compare all columns (treated patients) to a control column (untreated patients). *p ≤ 0.05, **p<0.01, ***p<0.001.

3.3 Functional characterization of NK cells

3.3.1 Cytokine production

3.3.1.1 CD94 and CD57 identify CD56^{bright} and CD56^{dim} NK cells after PMA/Iono activation

CD56^{dim} and CD56^{bright} NK cells display functional differences. CD56^{dim} cells are the subset with a superior cytotoxic capacity, whereas CD56^{bright} NK cells represent the cell subset with greater ability to produce proinflammatory cytokines such as IFNγ and TNFα. This cytokine release response has been exclusively studied after 1-3 days exposure to cytokines such as IL-2, IL-12, IL-15, IL-18 and IL-1β.

Our interest was the analysis of cytokine production of NK cells ex vivo after PMA/Iono, stimulation. Our first observation was that CD16 was not detectable on NK cell surface after stimulation, a fact that hampered the identification of NK cell subsets. We then searched for markers that could unmistakably identify CD56^{bright} and CD56^{dim} cells without the need of CD16. We found that expression of CD94 and CD57 was not affected after PMA/Iono stimulation and the combination of these two markers could clearly identify the CD56^{bright} and CD56^{dim} NK cells, respectively (fig. 22).

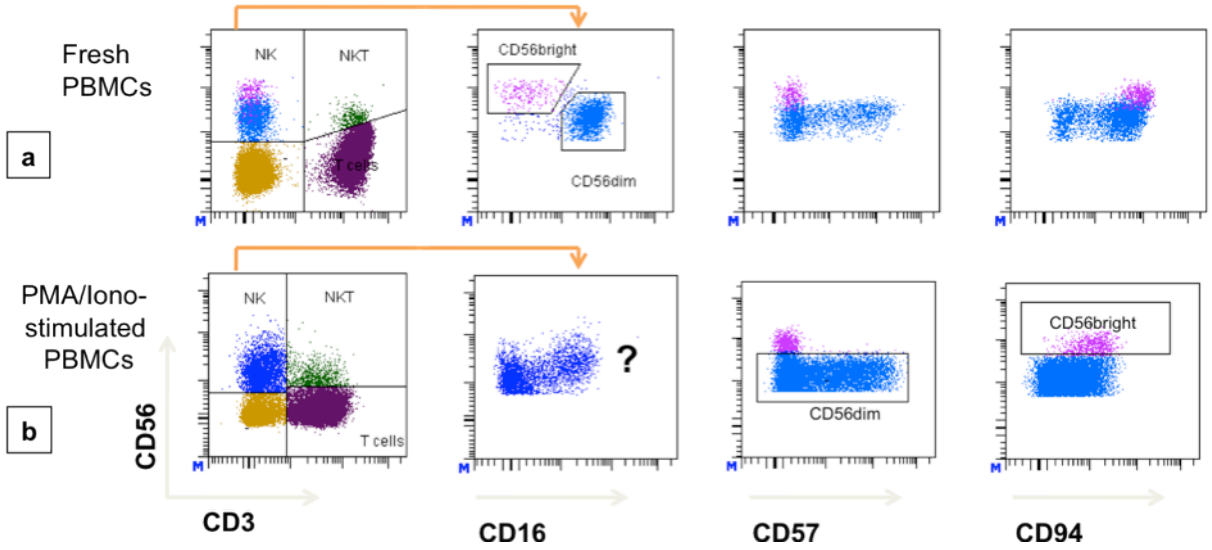

Figure 22. Intracellular cytokine staining for CD56^{bright} and CD56^{dim} NK cells
Unstimulated **(a)** and PMA/Iono-stimulated **(b)** PBMC were stained with CD3, CD56, CD16, CD57 and CD94. NK, NKT and T cells were identified in a CD3/CD56 dot plot. CD56^{dim} and CD56^{bright} were gated according to expression of CD16 and CD56. The question mark points out the decreased expression of CD16 after stimulation. CD56^{dim} cells, shown in light blue, correspond to the low expression of CD56 and either positive/negative expression of CD57. CD56^{bright} cells, shown in magenta, correspond to the cells with the highest level of CD56 and CD94 positive expression.

Next, we found that while NK cells produced more IFNγ than T cells, TNFα was produced mainly by CD4+ and CD8+ T cells. In line with previous findings that $CD56^{bright}$ cells are the principal cytokine-producing NK cell subset, we found a tendency of $CD56^{bright}$ cells to produce IFNγ and TNFα at a major extent than $CD56^{dim}$ NK cells, although the difference was minor (Fig. 23).

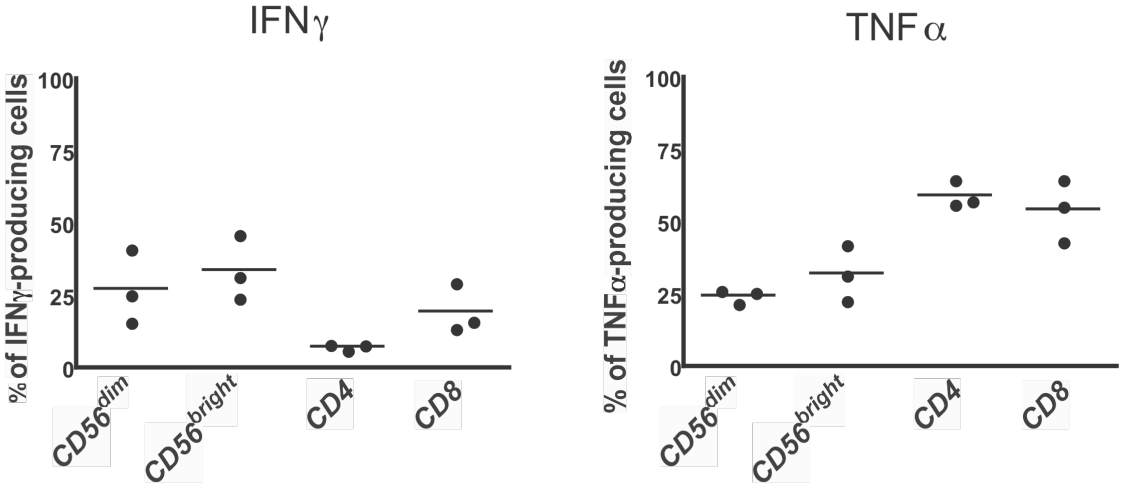

Figure 23. NK cells are the major source of IFNγ, while T cells produce more TNFα
PBMC from 3 healthy donors were stimulated with PMA/Iono for 5 hours and subsequently stained with CD3, CD4, CD56, CD57 and CD94. Intracellular staining was done for IFNγ and TNFα. To identify cytokine-producing $CD56^{bright}$ and $CD56^{dim}$ NK cells, the method described above was used. The graphs show the percentage of cytokine positive cells after gating on each cell population.

3.3.1.2 Production of IFNγ and TNFα by T and NK cell subsets is increased in patients with MS

The exact roles of the cytokines IFN-γ and TNF-α in MS remain controversial, with evidence suggesting both detrimental and protective effects of these cytokines in MS and EAE. Excessive TNF production is associated with MS (Hofman et al 1989), rheumatoid arthritis, septic shock (Tracey et al 1986), and many animal models of disease; however, there is a lack of studies regarding the production of these cytokines by NK cell subsets in MS. Therefore we aimed to identify the ex-vivo production of these two cytokines from $CD56^{brigh}$ and $CD56^{dim}$ NK cells according to the method above described. Interestingly, we found that in untreated MS patients, IFNγ and TNFα production by $CD56^{bright}$ and $CD56^{dim}$ NK cells was significantly higher in comparison to healthy donors. CD4+ and CD8+ T cells from untreated MS patients also showed a highly significant increased production of TNFα and a clear tendency to

produce more IFNγ than healthy donors. (Fig. 24). In contrast to the methods of Lünemann and colleagues (Lünemann et al 2011), where they exposed PBMCs to the NK cell-activating cytokines IL-2 and IL-12 for 72 h in *in vitro* culture, we stimulated PBMC with PMA/Iono for 5 hours in order to induce the release of cytokines without compromising signaling pathways that might exert artificial results by prolonged times of activation. Our results show a clear differential cytokine production by the two groups, indicating the likely role of these two cytokines in the pathophisiology of the disease.

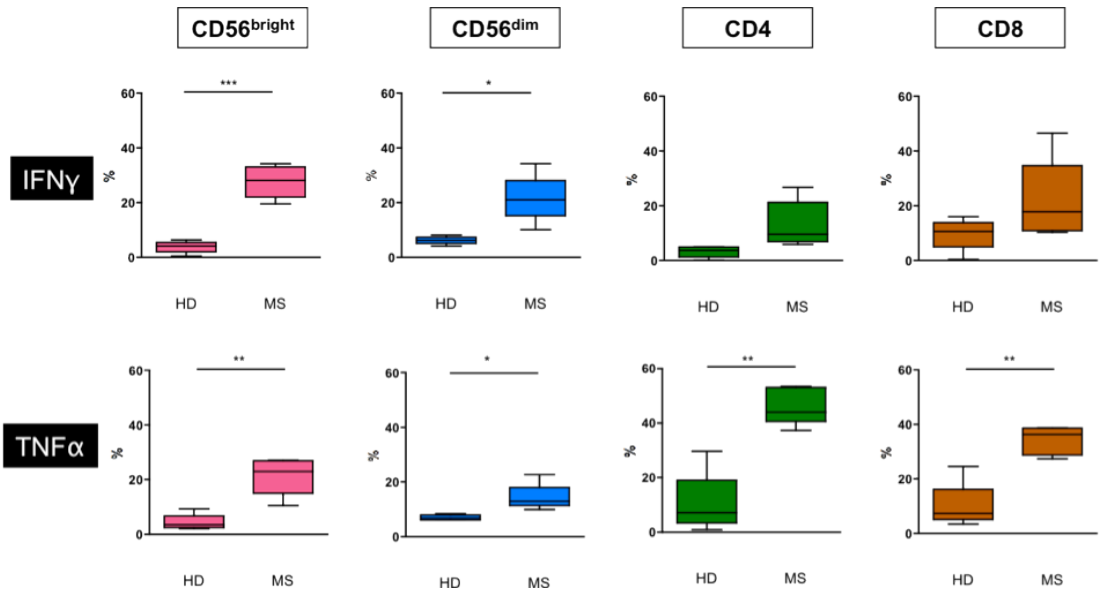

Figure 24. IFNγ- and TNFα- producing cells are increased in MS patients
Intracellular cytokine staining for IFNγ and TNFα was assessed for CD4, CD8, CD56^{dim} and CD56^{bright} cells in healthy donors (n=4) and untreated MS patients (n=4). Each box-and-whisker plot shows the median, quartiles, and extreme values within a category. *** p<0.001, **p<0.01, p*<0.05

3.3.1.3 Treatment with IFNβ, anti-CD25 mAb or natalizumab results in reduced IFNγ and TNFα production by NK cells

To further investigate the effect of treatment with IFNβ-1a, natalizumab and anti-CD25 on NK cell cytokine release in MS, particularly on the expanded CD56^{bright} NK cells, we determined IFNγ and TNFα production by intracellular cytokine staining. Remarkably, in all cases, treatment resulted in a reduced production of these two cytokines. This reduction was more pronounced in the case of TNFα by CD56^{bright} NK cells, reducing the cytokine release to levels seen in healthy donors. Our results support the possible role of these cytokines in the development of the disease, as the effect of treatment brings the elevated levels of TNFα and IFNγ to healthy baseline levels (fig. 25).

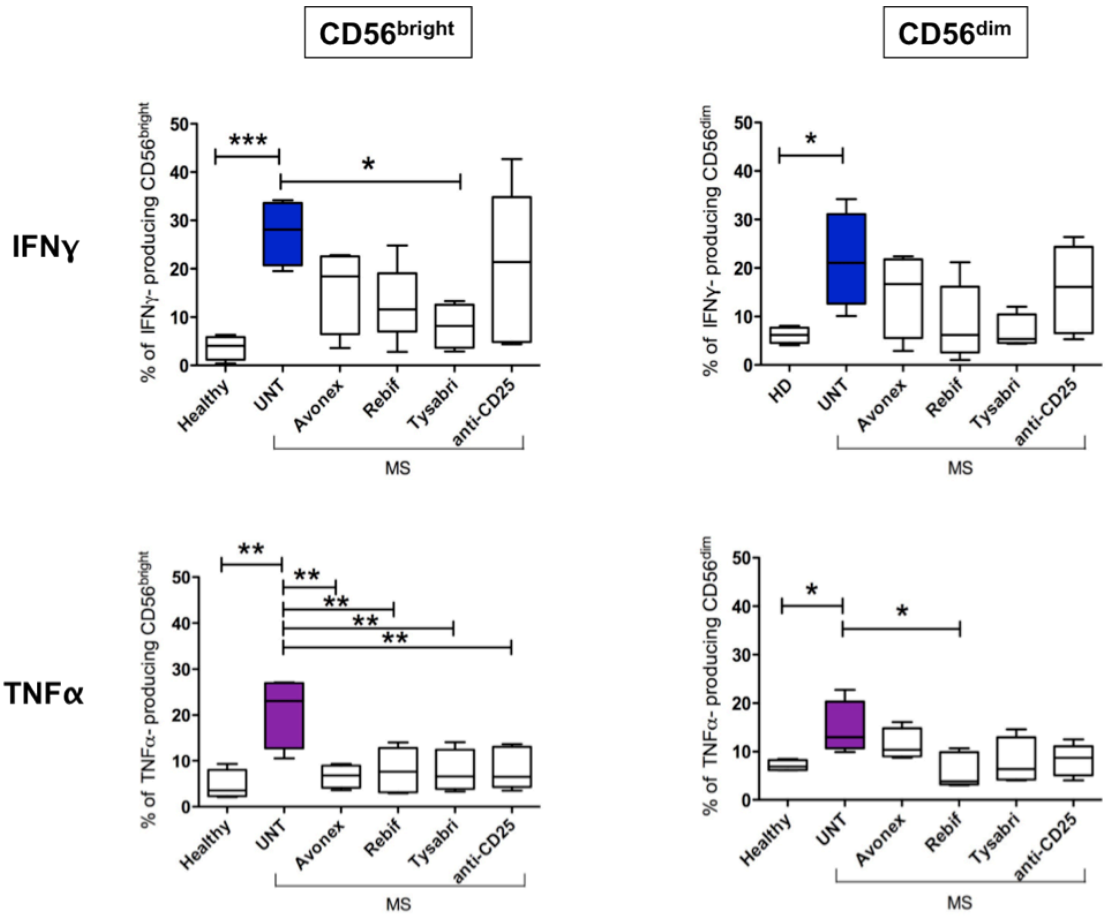

Figure 25. IFNγ and TNFα profiles differ between untreated and treated MS patients
Percentages of IFNγ- and TNFα-producing CD56^{bright} and CD56^{dim} NK cells from PBMC of healthy donors (n=4), untreated MS patients (n=4), and MS patients treated either with Rebif (n=5), Avonex (n=4), natalizumab (n=4) and anti-CD25 mAb (n=5) are shown. Staining and gating strategy were done as described before. Plots show the median, quartiles, and extreme values within a group. *** p<0.001, **p<0.01, p*<0.05

3.4 Mechanistic basis of CD56^{bright} NK cell expansion

3.4.1 Daclizumab and basiliximab bind the CD25 tac epitope

To assess the *in vitro* effect of basiliximab and daclizumab on CD25 expression, we used two different antibodies that recognize the tac and non tac epitopes, respectively. The expression of CD25 tac and non tac on untreated PBMC was invariably and minimally observed on CD4+ T cells and CD56^{bright} NK cells, whereas anti-CD3-activated PBMC up regulated the expression of CD25, both the tac or non tac epitope, up to 80% on those cell populations. Treatment with basiliximab and daclizumab induced a complete blocking of CD25 tac epitope on activated cells, whereas the CD25

non tac epitope remained detectable up to 60-70% on CD8, CD4 and CD56^{bright} cells (Fig. 26).

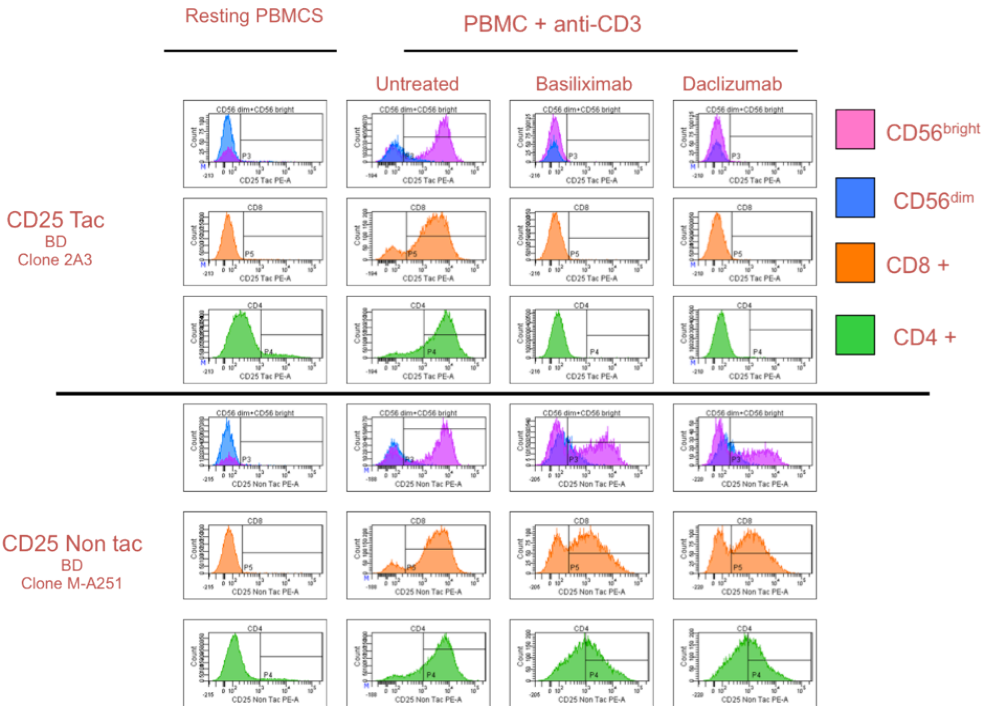

Figure 26. Daclizumab and basiliximab bind the tac epitope of the IL-2 receptor
CD25 expression on resting and anti-CD3-activated PBMC was assessed on cells treated with daclizumab or basiliximab. We used two different antibodies recognizing the tac (2A3) or the non tac epitope (M-A251) on the CD25 molecule. Expression of CD25 was analyzed on CD4+, CD8+, CD56^{bright} and CD56^{dim} cells.

3.4.2 IL-2 binding to its receptor decreases after treatment with anti-CD25 mAb

To investigate the effect of anti-CD25 antibodies on the binding capability of IL-2 to its receptor we directly assessed IL-2 binding on resting or anti-CD3- activated PBMC. IL-2 binding capability of activated PBMC was high in CD4+, CD8+ and CD56^{bright} cells. On CD56^{dim} cells, IL-2 binding was notably low, confirming that this cell population possesses low amounts of IL-2R. Among NK and T cell subsets, CD4+ showed the highest IL-2 binding capability, followed by CD8 and CD56^{bright}. Treatment with basiliximab or daclizumab induced a 75% reduction on the binding capability of all cell subsets to the cytokine (Fig. 27).

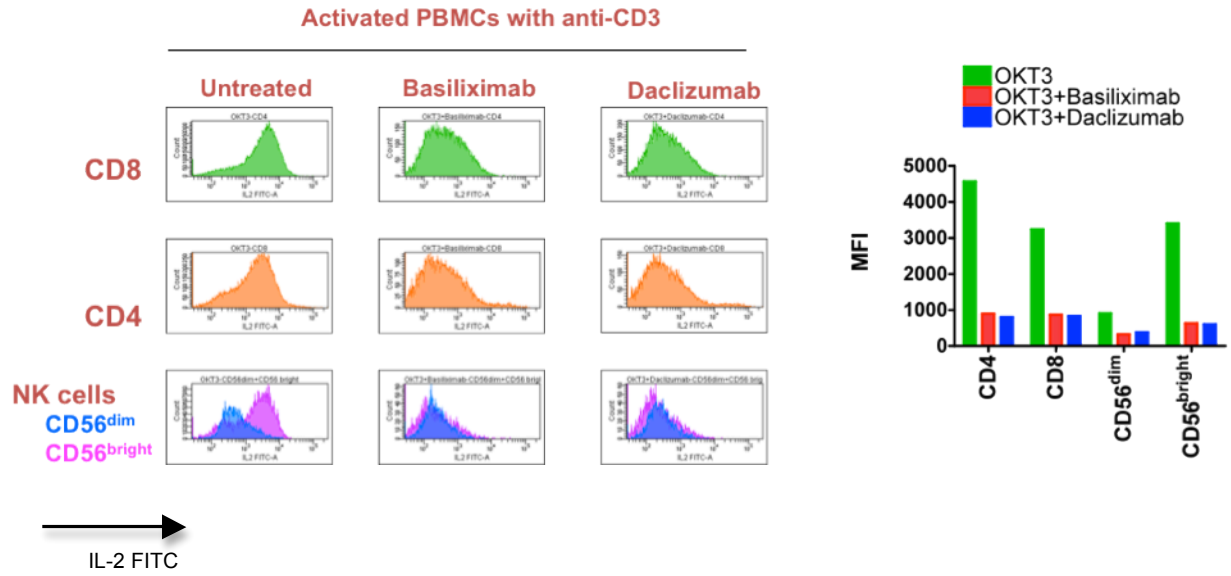

Figure 27. Binding of IL-2 to CD4+, CD8+ T cells and CD56^{bright} cells after CD25 blockade
Binding of IL-2 to T and NK cells was analyzed in anti-CD3-activated PBMC, either untreated or treated with basiliximab or daclizumab mAb. IL-2 binding was determined after cell incubation with biotinylated IL-2 as percentage of CD4+, CD8+ CD56^{dim} or CD56^{bright} cells positive for anti-IL-2 detected by a FITC-streptavidin conjugate, as shown in histograms. The corresponding bar graph shows the MFI values.

3.4.3 *In vitro* effect of treatment with anti-CD25 or IFNβ1a on CD56^{bright} NK cells expansion

To assess the effect of anti-CD25 and IFNβ-1a treatment on CD56^{bright} NK cells *in vitro*, we designed an experiment to mimic the conditions of lymphocytic activation that we presume happen in vivo in MS. PBMC from healthy donors were stimulated with anti-CD3, assuming that the IL-2 produced by the T cells after polyclonal activation would suffice to induce the proliferation of NK cells. Basiliximab was added to the cell cultures to assess the effect of anti-CD25 blockade under such culture conditions. Because activation of NK cells results in changes in the expression of CD56 and CD16, in order to trace CD56^{bright} and CD56^{dim} cells longitudinally we labeled them previously with CFSE and eFluor, respectively, as explained in methods section. NK cells proliferation within the PBMC mix was evaluated at different time points as the percentage of CFSE or eFluor dilution in reference to the initial peak of the original parent generation (fig. 28).

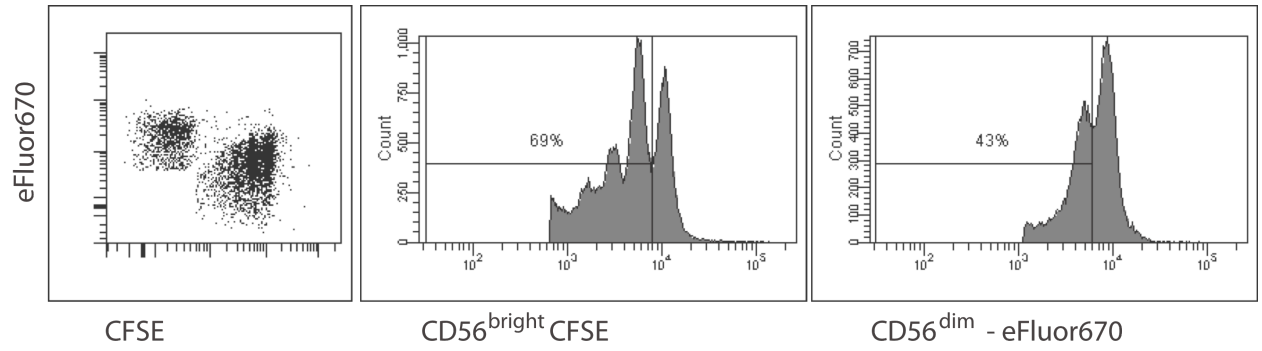

Figure 28. CFSE and eFluor labeled NK cell proliferation
Representative dot plot and histogram of IL-2 activated NK cells after 5 days of culture. Unstained PBMC were mixed with 2µM CFSE-labeled $CD56^{bright}$ cells and 2µM eFluor-labeled $CD56^{dim}$ cells at ratios of 1:10 NK/T cells and 1:10 $CD56^{bright}$/$CD56^{dim}$ NK cells. With this method it is possible to track specific cell subpopulations without additional staining.

Activation of T cells with anti-CD3 resulted in an 'indirect' proliferation of $CD56^{bright}$ NK cells (50% of dividing cells), while only a minimal effect was observed on $CD56^{dim}$ NK cells, probably due to the low expression of IL-2 receptors on this cell subset. Addition of basiliximab did not result in enhancement of $CD56^{bright}$ proliferation as we had expected given the expansion observed in patients treated with this antibody (fig. 29, upper panels). One possibility for the lack of response to basiliximab would be that the levels of IL2 produced by the T cells are not enough to induce NK cell proliferation. Indeed, when exogenous IL-2 was added to the cell culture as the sole source of this cytokine (in the absence of anti-CD3), both NK cell subsets proliferated readily, and the $CD56^{bright}$ again at higher levels (middle panels). In the presence of excess IL-2, basiliximab had no further effect on NK cell proliferation (Fig. 29 middle panels).

However, when we added exogenous IL-2 in addition to anti-CD3, again both NK subsets proliferated, and, very interestingly, addition of basiliximab to the cultures resulted in the expected effect of increased proliferation of the $CD56^{bright}$ NK cell subset exclusively, while the $CD56^{dim}$ NK cells did not show differences (fig. 29 lower panels). In this case we have a condition with exogenous cytokine and activated T cells, which consume IL-2, but still there is enough IL-2 to bind both high affinity and intermediate affinity receptors, and consequently to support proliferation of the NK cells. When basiliximab is added to the system, the high affinity receptor on T cells is blocked, and only cells with the intermediate affinity receptor respond to IL-2. We have previously shown that T cell proliferation is impaired in the presence of basiliximab (fig. 26). Given the higher levels of CD122 expressed by $CD56^{bright}$ NK cells in comparison to $CD56^{dim}$ cells, it is the $CD56^{bright}$ subset the one that 'uses' the IL-2 and proliferates more

readily. Remarkably, even in the absence of the T cell stimulus, a certain level of proliferation was detected in the CD56^{bright} subset exclusively.

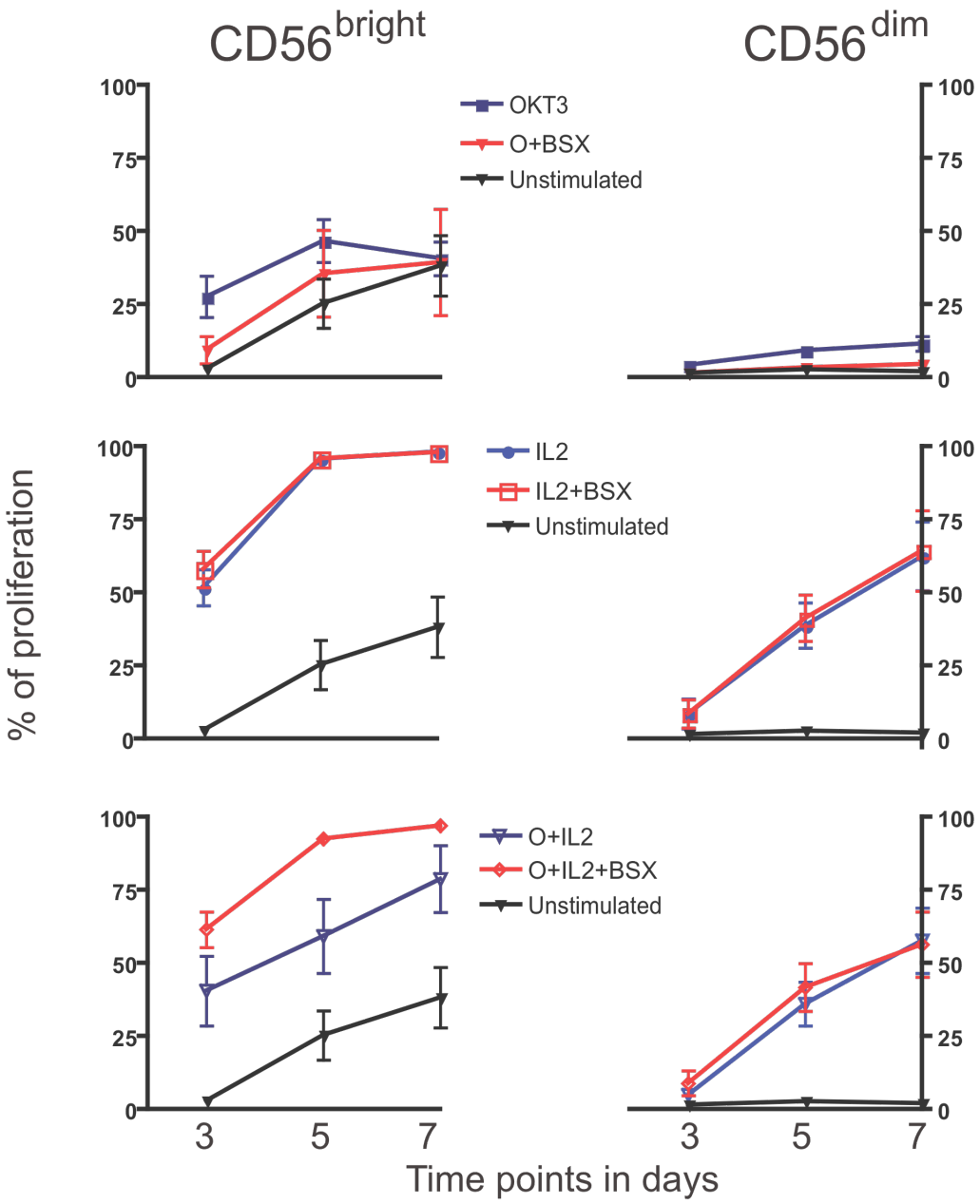

Figure 29. Effect of basiliximab in anti-CD3-, IL-2- or anti-CD3/IL-2- activated cells
The in vitro effect of basiliximab (20 ng/ml) in the proliferation of CFSE-labeled CD56^{bright} and e-Fluor labeled CD56^{dim} cells, was assessed in untreated, anti-CD3 (OKT3 - 500 ng/ml), IL-2 (100 U/ml) and anti-CD3/IL-2 activated cells after 3, 5 and 7 days of incubation.

We took a similar approach to analyze the in vitro effect of IFNb-1a treatment on NK cells. However, in all conditions tested we observed a decreased proliferation of NK

cells when IFNb-1a was added to the system (fig. 30), in contrast to *in vivo* observations in MS patients after treatment with IFNb-1a, namely increased frequency of CD56^{bright} NK cells. This effect might indicate that IFNb-1a does not act directly on NK cells to induce their proliferation, but has an indirect effect that we have not been able to reproduce in our in vitro conditions.

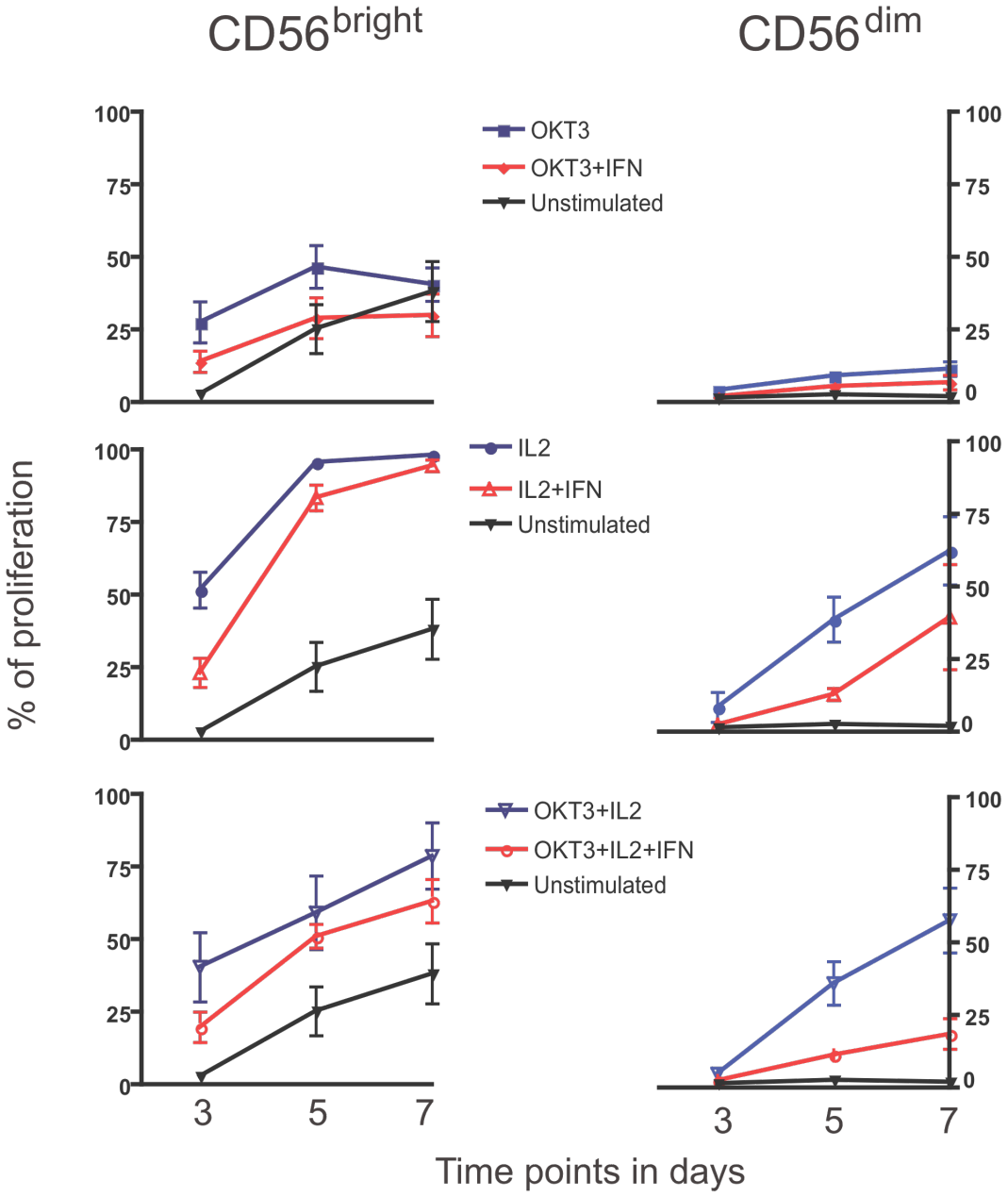

Figure 30. Effect of IFNβ1a in anti-CD3-, IL-2- or anti-CD3/IL-2- activated cells
The in vitro effect of IFNb-1a (100UI/ml) on proliferation of CFSE-labeled CD56^{bright} and e-Fluor labeled CD56^{dim} cells, was assessed in untreated, anti-CD3 (OKT3 - 500 ng/ml), IL-2 (100 U/ml) and anti-CD3/IL-2 activated cells after 3, 5 and 7 days of incubation.

4. Discussion

4.1 Effect of treatment on the relative frequency of T and NK cells in MS patients

Because the beneficial effect of daclizumab is attributed to the specific expansion of $CD56^{bright}$ NK cells, we focused our study on the phenotypic and functional characterization of this cell population. Recent studies suggest similar effects induced by other treatments for MS: IFNβ-1a (Rebif or Avonex) exerts an expansion of $CD56^{bright}$ NK cells, whereas treatment with natalizumab increases the frequency of NK cells in MS, but its particular effect on a specific NK cell subpopulation has not been described. In this study, we confirmed the daclizumab- and IFNβ-1a-induced increased frequency of $CD56^{bright}$ cells, and additionally, we found that treatment with natalizumab also resulted in higher frequency of $CD56^{bright}$ cells in circulation.

Rebif and Avonex are both IFNβ-1a drugs, but we have observed that Rebif induced a higher increase in the frequency of $CD56^{bright}$ NK cells than Avonex. Clinically, Rebif is administered subcutaneously three times per week at a higher dose than Avonex, which is given intramuscularly once per week. Clinical trials report a better therapeutic response to Rebif in comparison to other IFNβ1a, probably due to higher doses and higher frequency of administration (Manfredonia et al. 2008), and this may also explain the higher frequency of $CD56^{bright}$ NK cells in these patients.

Natalizumab controls leukocyte adhesion, attachment and migration across the BBB into the CNS by blocking the interaction of VLA-4 with its ligand VCAM on the brain endothelial cells. In MS patients treated with natalizumab, we observed an increased frequency of CD4+ T cells in the periphery and a complementary decreased percentage of CD8+ T cells. These results support the documented analyses of CSF in natalizumab-treated MS patients showing that this drug blocks CD4+ more efficiently than CD8+ T cells from migration into the CNS (Putzki et al. 2010). In MS, no differences in the frequency of Treg cells have been observed in comparison to healthy controls. However, treatment with natalizumab resulted in a decreased percentage of Treg cells in periphery. This finding indicates that a) a stronger impact of the mAb on T effector cells inhibits their influx into the CNS and b) Treg cells are less prone to the effect of natalizumab probably due to the fact that Treg cells express on their surface low levels of VLA-4, the target molecule of natalizumab (Skarica et al 2011).
In our study, we could follow longitudinally two patients treated with anti-CD25 mAb. One of them was initially treated with daclizumab for 2 years and showed a good

response to treatment. In 2009, daclizumab was withdrawn from the market and in the therapeutic scheme of this patient daclizumab was replaced by basiliximab. These two antibodies recognize the same epitope of the high-affinity IL-2 receptor, but differ in their structure. Daclizumab is a humanized monoclonal antibody, whereas basiliximab is a chimeric mouse-human monoclonal antibody. In previous decades, murine anti-CD25 monoclonal antibodies significantly reduced acute rejection episodes, but the high immunogenicity and short half-life of these antibodies limited their clinical use (Sageshima et al. 2009). To overcome this problem, humanized and chimeric monoclonal antibodies were developed. However, in several clinical studies, murine mAbs and chimeric mAbs could induce human anti-mouse and anti-chimeric antibody responses, respectively, and the resulting neutralization would lead to limitation in efficacy. In the CHOICE study (daclizumab as an add-on therapy to IFNβ-1a) 8% of patients were positive for human anti-human neutralizing antibodies (Wynn et al. 2010). Therefore we cannot discard the appearance of neutralizing antibodies as an explanation to the bad response in the referred patient.

Long-term treatment with anti-CD25 mAb in the good responder led to a reduction of circulating Treg cells. This reduction has been associated with a differential effect on inflammatory activity depending on the tissue. Whereas CNS inflammation is clearly reduced with daclizumab, an increased susceptibility to newly emerging inflammatory activity in skin has been observed (Oh et al. 2009). The possibility of compartmental differences in the capacity to maintain tolerance in the setting of reduced numbers of Treg cells should not be discarded.

An allele in 17q12 has been associated with NK cell frequency by demonstrating that a single nucleotide polymorphism (SNP) in this region is associated with a reduction in the $CD56^{dim}$ NK cells and an expansion of the $CD56^{bright}$ NK cells. Additionally, this SNP correlates robustly with enhanced degranulation (CD107a) by NK cells (Xia et al. 2012). An association of this SNP in MS patients would explain the outcome of treatment.

4.2 Phenotypical characterization of NK cells in MS patients

Human NK cell development is associated with the sequential acquisition of NK cell receptors, with CD161 and NKp46 being among the first NK cell receptors to be detected, followed by CD94 and CD62L, and lastly CD16 and the killer-cell immunoglobulin-like receptors (KIRs).

Our results show that the expression of CD62L and CD94 is increased after treatment in $CD56^{bright}$ cells in MS patients. Expression of these two markers identifies a functional and likely developmental intermediary cell population between $CD56^{bright}$ and $CD56^{dim}$ NK cells which are located predominantly in secondary lyphoid tissues (SLT) (Yu et al. 2010 and Juelke et al. 2010). The high expression of these two markers might indicate a release of $CD56^{bright}$ cells from SLT to the periphery.

CD161 is a marker that identifies an early NK cell developmental stage. However, the decreased number of $CD56^{bright}$ cells bearing CD161 after treatment, did not reflect an increase of cells in early stage of maturation. CD161 is a C-type lectin receptor, but little is known about its function in autoimmune diseases and it can be expressed on pathogenic as well as on immunoregulatory cells. The recently described mucosal-associated invariant T cells (MAIT), are IL-17–secreting T cells characterized by the expression of IL-18Rα $CD8\alpha\beta^{int}$ Vα7.2 and high levels of CD161. We observed increased expression of CD161 on NK cells and CD8+ T cells of untreated MS patients. Annibali and colleagues reported an upregulation of CD161 at the protein level and a significant excess of $CD161^{high}$ CD8+ T cells in MS and RA (Annibali et al. 2011), whereas the group of Miyazaki, documented a significant reduction of MAIT cells in MS patients in remission and even more profoundly reduced in those with relapse (Miyazaki et al. 2011). Interestingly, the frequency of MAIT cells positively correlates with the frequency of CD4+ invariant NKT cells and of $CD56^{bright}$ NK cells in healthy controls but not in MS patients (Miyazaki et al. 2011). This might suggest the existence of an immunoregulatory link between MAIT cells and the other two cell populations with disruption of cross talk in MS.

In NK cells, high levels of CD161 receptor are correlated with impairment of NK cytotoxic activity in RA patients (Richter et al. 2010). Very remarkably, CD161 was the only marker of our panel showing an overexpression on NK cells in untreated MS patients. After treatment either with IFNβ-1a, natalizumab or anti-CD25 mAb, its expression returned to healthy baseline levels. This observation supports the possible immunopathogenic role of CD161 in MS.

IL-18 is involved in the immunopathogenesis of MS. The highly increased levels of IL-18 in serum and CSF of MS patients correlate with an increase in the induction of IFNγ, which in turn augments the production of IL-18 binding protein (IL18BP) (Losy et al. 2001 and Karni et al. 2002). This protein has a high binding affinity for IL-18

(Kd=400 pM) and exerts its action by neutralising the proinflammatory effect of IL-18. IL-18 also binds to the alpha chain of the Il-18 receptor (IL-18Ra) but with less affinity (Kd=18.5 nM), so that it does not induce a signaling response unless the beta chain of the receptor dimerizes with the IL18Ra to form the high affinity complex (Kd=0.4 nM) (Boraschi et al. 2006). IL-18Ra is expressed mainly on $CD56^{bright}$ NK cells. In MS, the abundant levels of IL-18 might lead to a binding availability for both IL18BP and IL18R. This situation could explain our finding of a decreased expression of IL-18Rα in untreated MS patients. Interestingly, treatment with anti-CD25 mAb induced an increased expression of IL18Ra, suggesting an unbound state of the receptor for its ligand. The association between treatment with anti-CD25 mAb and the production of IL-18 in MS patients is not known. IL-18Ra overexpression indicates that concentrations of IL18 might be decreased after treatment and binding capability might be restricted to the high affinity IL-18BP. This model would explain the high expression of IL-18Ra as an unbound state of the receptor. Further studies are necessary to confirm or reject an indirectly proportional relation between IL-18 and IL-18Ra.

From all activation markers studied, we found a prominent increase of CD2, CD11b, CD26 and CD6 on $CD56^{bright}$ NK cells after all treatments but particularly with daclizumab. The expression of these markers suggests an activated status pattern of $CD56^{bright}$ NK cells.

Cell surface receptors participate in the formation of the activating NK cell immunologic synapse (NKIS), the interface between NK cell and susceptible target. A variety of adhesion receptors with activating potential, including β2-integrins, the α-integrins CD11a and CD11b, as well as the Ig superfamily member CD2, all accumulate in the peripheral supramolecular activation cluster (pSMAC) of the activating NKIS. Interactions between these adhesion molecules and their cognate ligands induce conjugate formation between effector cells and target or antigen-presenting cells (Orange et al. 2003). Such cellular interactions can initiate signal transduction and cell activation, resulting in proliferation, cytokine secretion and cytotoxicity. In treated MS patients, particularly with anti-CD25 mAb, overexpression of these two molecules on $CD56^{bright}$ NK cells confers a pattern with the potential to generate strong activating signals.

In the center of the mature immunological synapse on T lymphocytes CD6 is found associated to the TCR/CD3 complex (Ibanez et al. 2006). CD6 interaction with its ligand (ALCAM) results in activation of MAPK cascades, likely influencing the dynamic

balance that determines whether resting or activated lymphocytes survive or undergo apoptosis. On NK cells, CD6 expression marks a subpopulation associated with distinct patterns of cytokine and chemokine secretion (Ibanez et al. 2006); however, its expression on the NKIS has not been studied. Overexpression of CD6 on $CD56^{bright}$ NK cells after treatment with natalizumab and anti-CD25 mAb could indicate an elevated cytokine secretion or a major participation in the immunological synapse.

In NK cells, CD26 expression is restricted to the $CD56^{bright}$ subset. This expression increased significantly after anti-CD25 mAb treatment. *In vitro*, CD26 is inducible in NK cells activated by IL-2, IL-12 or IL-15 (Yamabe et al. 1997). Since CD26 is known to have a binding affinity for collagen and thought to be involved in cellular adhesion to extracellular matrix proteins (Muscat et al. 1994 and Sato et al. 2005), the induction of CD26 on activated NK cells may be related to efficient recruitment and tissue infiltration.

The increased number of $CD56^{bright}$ NK cells after treatment with natalizumab, daclizumab and IFNβ could be either a result of a decreased rate of maturation from $CD56^{bright}$ to $CD56^{dim}$ NK cells, probably due to an accelerated egress of $CD56^{bright}$ NK from the lymph node, or to an increased rate of change from the $CD56^{dim}$ to the $CD56^{bright}$ NK cell phenotype. In line with other studies describing the ability of $CD56^{bright}$ NK cells to develop into $CD56^{dim}$ cells, and according to the phenotypical description of the expanded $CD56^{bright}$ population with an intermediate developmental pattern, we favor the first possibility. Possible mechanisms of $CD56^{bright}$ expansion include differential homing of NK cell populations in disease, specific proliferation of $CD56^{bright}$ NK cells and an increased output of immature $CD56^{bright}$ NK cells from bone marrow or from lymphoid tissue to peripheral blood.

4.3 Functional characterization of NK cells

Most reports defining $CD56^{bright}$ NK cells as the major source of NK cell-derived cytokines rely on *in vitro* data obtained after purification of the NK subsets and subsequent culture of the isolated subset in the presence of stimulating cytokines, such as IL-2, IL-12, IL-15, and IL-18. We were interested in determining the *ex vivo* intracellular production of IFNγ and TNFα by both NK cell subsets without previous cytokine stimulation. A major problem to determine the intracellular cytokine production of PMA/Iono-stimulated NK cells is the downregulation of CD16, a specific marker of $CD56^{dim}$ NK cells that allows unequivocal identification of this subset, with changes in

CD56 expression. Our approach was to use specific markers of NK cells that were unaffected after PMA/Iono stimulation. Among the panel of markers that we had established to study the immune phenotype of NK cells, we found that CD94 and CD57 were reliable markers for $CD56^{bright}$ and $CD56^{dim}$ NK cells even after PMA/Iono stimulation. With this method, we investigated the cytokine profile of $CD56^{bright}$ NK cells and confirmed that, PMA/Iono- activated cells produce large amounts of IFNγ and TNFα. Notably, $CD56^{bright}$ NK cells produce more cytokines in response to PMA/Iono stimulation than $CD56^{dim}$ NK cells (Cooper et al. 2001). This fact has been associated to the relatively low expression of the phosphatase SHIP-1 and the high expression of the phosphatase inhibitor SET in $CD56^{bright}$ NK cells, which facilitates a lower activation threshold for cytokine secretion (Trotta et al. 2005).

$CD56^{bright}$ and $CD56^{dim}$ NK cells are currently seen as developmentally distinct subsets, being $CD56^{bright}$ NK cells the major source of cytokines, whereas $CD56^{dim}$ NK cells are regarded as specialized for cytotoxic function. Recent studies, however, challenge these assumptions. Fauriat and coworkers propose the $CD56^{dim}$ cell subset, rather than $CD56^{bright}$ NK cells, as the important proinflammatory cytokine source during early immune response upon cell target recognition, producing graded responses depending on the multiplicity of activating receptors engaged (Fauriat et al. 2010). This finding points out the capability of both NK subsets to produce cytokines after different activation stimuli. On the other hand, the immunoregulatory role attributed to $CD56^{bright}$ NK cells due to their potent cytokine-producing ability has also been discussed. The observation of $CD56^{bright}$ NK cells expansion after daclizumab has extended the concept of $CD56^{bright}$ function to limit adaptive immune responses by their cytotoxicity toward activated T cells (Bielekova et al. 2006). A supporting fact for this hypothesis is the newly defined mechanism of $CD56^{bright}$ NK cells cytotoxicity by a preferential transfer of Granzyme K (GrK) to target cells (Jiang et al. 2011). More importantly, our results on ex-vivo cells clearly showed that all NK and T cell subsets of untreated MS patients produced higher levels of IFNγ and TNFα than healthy controls. Previous reports on cytokine production by T and NK cells of MS patients have yielded conflicting results: downmodulating effects on IFNγ producing T cells in MS induced by IFNβ have been reported by some groups (Becher et al. 1999 and Furlan et al. 2000), but denied by others Dayal et al. 1995 and Lünemann et al. 2011). Studies on cytokine production of NK cell subsets in MS are limited. Lünemman and coworkers recently showed a diminished IFNγ production by NK cells in MS patients. However, they analyzed the intracellular production of IFNγ in *in vitro* cultures after stimulation with IL-2 or IL-12 (Lünemann et al. 2011). This approach differs very much from our ex-vivo

method and the percentages of IFNγ-producing cells they observed in untreated MS patients and healthy controls differ dramatically with our results. They describe a range of 0-5% of IFNγ producing cells, whereas our range was set between 20-40%. Furthermore, in our hands, TNFα-producing cells were also elevated in untreated MS patients. This finding is widely supported by studies showing an up-regulation of TNFα in PBMC of MS patients (Navikas et al. 1996), presence of TNFα in MS plaques (Merrill and Benveniste 1996) and high production of TNFα in association with the clinical activity of MS. Due to the pathogenic role of TNFα in several autoimmune disorders, a therapeutic success targeting this cytokine could be expected; however, anti-TNFα therapy in MS patients has resulted in a clear failure (Kruglov et al. 2011). The significant reduction of Th1 cytokines in the treated MS patients is such that it could contribute by itself to the therapeutic efficacy of daclizumab, basiliximab, natalizumab and IFNβ1a in our patients. The exact roles of IFNγ and TNFα in MS remain controversial, with evidence suggesting both detrimental and protective effects of the cytokine in MS and EAE (reviewed by Lees and Cross 2007). However, many autoimmune diseases are related to high IFNγ and TNFα serum levels, for instance systemic lupus erythematosus, rheumatoid arthritis, mixed connective tissue disease, uveitis, Type I diabetes, and various dermatological diseases (reviewed by Skurkovich and Skurkovich 2003, Funauchi et al. 1991 and Chatzantoni and Mouzaki 2006), indicating the potential role of these cytokines in the pathophysiology of the disease. Our results are closer to the situation in vivo since cells are stimulated for a short period under a stimuli that only induces the intracellular release of the cytokine content. To our knowledge, this is the first study of the ex-vivo production of TNFα and IFNγ from specific NK cell subsets in MS patients.

4.4 Mechanistic basis of $CD56^{bright}$ NK cells expansion

Our approach to study the mechanism of $CD56^{bright}$ cell expansion after treatment consisted in mimicking the conditions that we presume happen *in vivo*. For this, we activated PBMC from healthy donors with anti-CD3 antibody to mimic the autoreactivity of T cells in MS. Treatment with basiliximab or IFNβ-1a on activated cells did not induce an expansion of $CD56^{bright}$ NK cells as observed *in vivo*, but the opposite, a reduction in the proliferation rate similar to T cells. Remarkably, the *in vitro* effect of adding basiliximab and IFNβ-1a on NK cells induced a reduction in cell proliferation, opposite to what is observed in vivo. The natural cytokine production *in vivo*, i.e. IL-2,

in a coordinated manner and likely in association with the production of other physiological factors such as IL-15 by dendritic cells or activated monocytes/macrophages and IL-1 by stromal cells, are not present in our *in vitro* system. Those cytokines could further enhance the activation of $CD56^{bright}$ NK cells and their successful entry into the proliferation cycle. In this line, the expansion of $CD56^{bright}$ NK cells might occur predominantly in lymph nodes, where IL-2 is produced under physiological conditions in the healthy immune system.

The exact means by which daclizumab expands NK cells is not clear. However, we observed that the IL-2Rβ (CD122), the intermediate affinity receptor for IL-2, was mainly expressed on $CD56^{bright}$ NK cells. Since blocking the high affinity receptor for IL-2 (CD25) decreases the consecutive signaling activation in T cells, we think that an increased availability of IL-2 for NK cells, via CD122, may lead to the specific $CD56^{bright}$ NK cell expansion. In *in vitro* activated cells, the presence of daclizumab or basiliximab, did not produce expansion of $CD56^{bright}$ cells as seen in vivo, indicating that the IL-2 produced by T cells was not sufficient to bind the IL-2Rβ expressed on NK cells. In contrast, when IL-2 was directly added to the culture medium, $CD56^{bright}$ NK cells expanded notoriously in presence or absence of daclizumab or basiliximab, meaning that the IL-2 available in the medium was sufficient to bind the intermediate affinity receptors on NK cells, despite the blocking of the low affinity receptor by anti-CD25 mAbs. This apparent discrepancy between in vitro and in vivo studies can be explained by the redundancy in cytokine systems *in vivo*. However, further studies are necessary to understand the in vivo mechanism of $CD56^{bright}$ NK cells expansion.

The lower expansion observed after treatment with IFNβ1a in both NK subsets, either after anti-CD3 or IL-2 activation, might indicate an increased rate of cell death induced by IFNβ1a as observed in experiments in mice, where activation of NK cells with IFNα or β can induce apoptosis of NK cells themselves through Fas-Fas ligand interactions on the surface of adjacent NK cells (Plett et al 2000). This activation results from the binding of the cytokine to its receptors, which are extensively expressed in all lymphocytic cells (Pfeffer et al 1998). Those findings suggest that NK cell activation via IFNα or β can enhance NK cell cytotoxic function; however in human, further studies are necessary.

Some immune cells have important physiologic functions in the CNS, such as immunosurveillance (reviewed by Wilson et al. 2010), maintenance of adult neurogenesis (Ziv et al. 2006) and promotion of CNS reparative processes (Bieber et

al. 2003). In this regard, Bielekova and colleagues reported the ability of daclizumab to induce effective immune regulation without limiting access of the immune cells to the intrathecal compartment (Bielekova et al. 2011). This is promising, because it implies that $CD56^{bright}$ NK cells might regulate (i.e., kill) activated pathogenic T cells directly in MS tissue. This relevant function has only been studied in the EAE model, where CNS resident natural killer cells suppress Th17 responses and CNS autoimmune pathology (Hao et al. 2010). Our finding in humans of an activated and immature phenotypic profile of $CD56^{bright}$ NK cells after treatment in MS, together with the effect of decreased IFNγ and TNFα production by this cell population after treatment, contributes to shape the immunotherapeutic potential of this regulatory NK cell subset in autoimmune diseases.

5. Summary

Multiple sclerosis is a T cell-mediated autoimmune disease characterized by an interplay between inflammatory and neurodegenerative processes that usually result in neurological disturbance followed by progressive accumulation of disability. In MS, regulatory T cells and $CD56^{bright}$ NK cell exert an immunomodulatory role. We were particularly interested in characterizing the $CD56^{bright}$ NK cell subset in MS because of their significant association with clinical remissions and reduced brain lesions after treatment with daclizumab.

In MS, an increase in the frequency of $CD56^{bright}$ after treatment with daclizumab or IFNβ-1a are known, but a phenotypical and functional characterization of this cell population has not been reported. We therefore carried out a cross-sectional analysis in healthy controls and RRMS patients, either untreated or treated with IFNβ1a, daclizumab or natalizumab. The purposes of our study were to identify patterns of NK cell surface markers expression, cytokine production and the mechanism and dynamics of $CD56^{bright}$ expansion.

We found that in addition to anti-CD25 mAb and IFNβ-1a, natalizumab also expands $CD56^{bright}$ NK cells in MS. Treatment with anti-CD25 mAb exerted the most prominent effect on the expression of NK cell markers on the $CD56^{bright}$ population. The expanded $CD56^{bright}$ cells overexpressed the surface molecules CD26, CD2, CD11b and CD6, which indicate an activated cellular pattern. The high expression of CD94 and CD62L in treated patients, indicates an intermediate state of NK cell maturation and an egress from lymph nodes to the periphery.

Our findings of similar levels of cytokine production by the $CD56^{dim}$ and the $CD56^{bright}$ cells, confront the classical definition of the $CD56^{dim}$ subset as only cytotoxic and the bright subset as exclusively capable to secrete cytokines. The remarkably elevated frequency of IFNγ− and TNFα-producing cells in MS without treatment, which is clearly brought to healthy donor baseline levels after treatment, supports the described pathogenic role of these cytokines in MS.

This study offers a wide phenotypical characterization of NK cells in MS that can potentially offer novel biomarkers useful for the clinical assessment of the disease as well as a potential use of NK cell-directed therapeutic approaches in autoimmune diseases such as MS.

6. List of abbreviations

AF488	Alexa Fluor 488
APC	Allophycocyanin
	Antigen Presenting Cell
AF647	Alexa Fluor 647
APCCy7	Alophycocyanin-Cyanin7
BBB	Blood Brain Barrier
B-CSF-B	Blood-Cerebrospinal fluid barrier
Bref A	Brefeldin A
BSA	Bovine Serum Albumin
CD	Cluster of differentiation
CIS	Clinically Isolated Syndrome
CNS	Central Nervous System
CSF	Cerebrospinal fluid
DMSO	Dimethylsulfoxid
EAE	Experimental Autoimmune Encephalomyelitis
EDSS	Expanded disability status score
EDTA	Ethylenediamine-tetraacetic acid
ER	Endoplasmic reticulum
E: T	Effector: Target ratio
f	Female
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FOXP	Forkhead box protein
HCl	Hydrochloride acid
HD	Healthy donor
HLA	Human leukocyte antigen
hr	Human recombinant
ICS	Intracellular cytokine staining
IFNγ	Interferon gamma
IL	Interleukin
IMDM	Iscove's modified Dulbecco's medium
Iono	Ionomycin
L-Glu	L-Glutamine
m	Male
MAG	Myelin associated protein
mAb	Monoclonal antibody
MBP	Myelin basic protein
MCAM	Melanoma cell adhesion molecule
MFI	Median fluorescence intensity
MHC	Major histocompatibility complex
MOG	Myelin oligodendrocyte glycoprotein
MRI	Magnet resonance imaging
mRNA	Messenger RNA
MS	Multiple sclerosis
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NCAM	Neural cell adhesion molecule
NK	Natural killer cell
NKIS	NK cell immunologic synapse
NKT	Natural killer T cell

PacBlue	Pacific blue
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PE	Phycoerythrin
PE-Cy5.5	Phycoerythrin-Cyanine 5.5
PE-Cy7	Phycoerythrin-Cyanine 7
Pen/Strep	Penicillin/Streptomycin
PMA	Phorbol myristate acetate
PMA/Iono	Phorbol myristate acetate/Ionomycin
PP-MS	Primary progressive multiple sclerosis
pSMAC	Peripheral supramolecular activation cluster
RA	Rheumatoid Arthritis
ROR	Retinoic orphan receptor
RPMI	Roswell park memorial institute medium
RRMS	Relapsing-remitting multiple sclerosis
RT	Room temperature
RTD	Room temperature in darkness
SLE	Systemic lupus erythematosus
SLT	Secondary lymphoid tissues
SP-MS	Secondary progressive multiple sclerosis
STAT	Signal transducer and activator of transcription
TCR	T cell receptor
TGFβ	Tumor growth factor beta
Th	T helper cell
TNFα	Tumor necrosis factor alpha
Treg	T regulatory cells
UKE	Universitätsklinikum Eppendorf - University Clinic Hamburg-Eppendorf
VCAM1	Vascular Cell Adhesion Molecule 1
VLA-4	Very Late Antigen-4

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9. Curriculum vitae

Personal information

Name Angélica Cuapio Gómez
Address Vogelhüttendeich 80, 21107 Hamburg, Germany
Mobile 017649383637
Email(s) acuapio@uke.uni-hamburg.de / dracuapio@gmail.com
Nationality Mexican
Date of birth 03 December 1981

Education and training

01 November 2008 – April 2012	Dissertation "Immunophenotypical and functional characterization of CD56^{bright} NK cells in MS" University Medical Center Hamburg-Eppendorf / Institute of Immunology
01 November 2008 – 31 October 2010	Graduate Program in Molecular Biology University Medical Center Hamburg-Eppendorf /Center for Molecular Neurobiology Hamburg (ZMNH)
01 March 2008 – 01 July 2008	Voluntary research internship Project: Identification of possible cellular niches of dopaminergic differentiation in an animal model of Parkinson's disease in rats National Autonomous University of Mexico (UNAM) / Institute of Physiology
03 January 2007 – 01 February 2008	Social service in research Project: Cellular senescence in an animal model of hepatic cirrhosis in rats National Autonomous University of Mexico (UNAM) / Experimenta Medicine Unit
01 November 2001 – 01 March 2008	Medical doctor degree (MD) National Autonomous Universitiy of Mexico / Faculty of Medicine

Personal skills and competences

Organisational skills and competences	- Member of the supervisory board, representing 9 PhD students in the UEPHA*MS consortium (United Europeans for the Pharmacogenomics in Multiple Sclerosis), a project from the FP7 Marie Curie Initial Training Network. - Organizer of a weekly meeting with PhD and medical students to learn/discuss chapters of the Janeway "Immunobiology" textbook, in order to improve the background knowledge in Immunology: "Janeway club" (10-12 participants).
Technical skills and competences	Flow cytometry, cell culture, cell enrichment, cytotoxicity and suppression assays, intracellular and extracellular staining, *in vitro* cell proliferation, immunohistochemistry, induction of Parkinsonism in rats, confocal microscopy.
Computer skills and competences	Good command of Microsoft Office™ tools (Word™, Excel™ and PowerPoint™), Adobe Illustrator™, Endnote, GraphPad Prism™ and FACS Diva Software™.

Additional information

Participant in a public debate on 'Communicating science to the public' during the 60th "Nobel Laureate Meeting" in Lindau, Germany.

Publications

Swaminathan B, Cuapio A, Alloza I, Matesanz F, Alcina A, García-Barcina M, Fedetz M, Fernández O, Izquierdo G, Órpez T, Pinto-Medel MJ, Otaegui D, Olascoaga J, Urcelay E, Ortiz MA, Arroyo R, Oksenberg J, Antigüedad A, Tolosa E, Vandenbroeck K. Multiple sclerosis-associated CD6 haplotype R225W-A257V -modifies both CD6 expression in CD4+ and CD8+ naïve T cells and IFNg production. **PlosOne** 2012. *Submitted*

10. Declaration of authorship

I declare that, except when others were referred, the present thesis is the product of my own work using only the resources I have specified here.

I confirm that this work was done while in candidature for a research degree at the University of Hamburg.

Eidesstattliche Versicherung:

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