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TNF-Related Apoptosis Inducing Ligand (TRAIL) Expression in Natural Killer (NK) cells and their role in Experimental Autoimmune Encephalomyelitis (EAE)

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Abstract

Title: TNF-Related Apoptosis Inducing Ligand (TRAIL) Expression in Natural Killer (NK) cells and their role in Experimental Autoimmune Encephalomyelitis (EAE)

Introduction: Multiple Sclerosis (MS) is a $CD4^+$ T cell mediated, demyelinating autoimmune disease of the central nervous system (CNS). TRAIL, a 281amino acid homotrimeric member of the TNF/NGF superfamily is expressed as soluble and membrane-bound form by activated T cells, B cells, macrophages and NK cells both in human and mouse and induces caspase-mediated cell death in transformed and non-transformed cells. [1,2] However, it has been shown that TRAIL acts not only as an apoptotic ligand but exerts also an immunoregulatory function.

In the animal model of MS, the EAE, it has been demonstrated that, when applied intracerebrally, TRAIL induces neuronal death and exacerbates the disease.[3] However, when applied in the periphery, it reduces severity by inhibiting autoreactive T cells. [4] So far, the cell population which mediates the modulation of EAE through TRAIL is undefined.

In MS patients, an increased amount of soluble TRAIL in the peripheral blood has been linked to clinical response (stable EDSS score) to IFN-β treatment at the relapsing remitting (RR-MS) form of the disease during the first year of treatment. [5] Additionally, TRAIL induction on NK cells of MS patients after in vitro culture with IFN-β is less pronounced compared to healthy controls (Infante-Duarte, unpublished data).

NK cells are members of the innate immune system, but also capable of regulating cells of the adaptive system as autoreactive T cells and dendritic cells. [7] Furthermore, a reduced functional activity of NK cells has been linked to MS relapses and NK cell depletion has been shown to exacerbate EAE. [7-9]

Aims:

1. To investigate which factors, e.g. IFN-β, are able to modulate TRAIL-expression in murine NK cells.
2. To investigate the role of TRAIL-expressing NK cells in modulating the clinical course and histopathology of EAE.

Methods: Induction of TRAIL on NK cells of wild-type (wt) C57BL/6 mice by IFN-β was investigated at the gene level by Taq-Man analysis and at the protein level through antibody detection using flow cytometry (FACS).
EAE was induced in wt C57BL/6 mice through subcutaneous (s.c.) injection of Myelin Oligodendrocyte Glycoprotein (MOG_{35-55}) peptide in Complete Freund's Adjuvant (CFA).
Shortly after immunisation, "naive" NK cells or TRAIL-expressing NK cells (which were pre-incubated with IFN-β) were injected to the aforementioned mice. Clinical course, severity of disease and histopathological landmarks of mice receiving either NK cells or TRAIL expressing-NK cells were compared with those of control mice (injected with PBS alone).

Results: IFN-β induced an increase of TRAIL mRNA expression on murine NK cells at concentrations of 500U/ml and 1000U/ml. An induction of surface TRAIL expression on NK cells with the same concentrations of IFN-β was also confirmed at the protein level.

The optimal concentration and incubation time of 1000U/ml for 18 hours was used for TRAIL induction on ‘naïve’ NK cells.

In C57BL/6 mice, which received the TRAIL-expressing NK cells intravenously (i.v.), a reduced incidence of EAE induction was observed compared to mice, which received untouched NK cells or PBS alone.

Discussion: NK cells represent a putative target of IFN-β treatment in EAE. IFN-β-induction of TRAIL in NK cells may be an important mechanism of action of this drug. TRAIL-expressing NK cells could play a regulatory role during the induction phase of the disease by influencing the priming of T cells or neuron survival in the CNS. Thus, generation of NK cells with immunoregulatory properties may represent a therapeutic strategy for EAE and MS.

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Abbreviations

Aa	Amino acid
AAF	Interferon-a activating factor
Ab	Antibody
ADCC	Antibody dependent cell cytotoxicity
ADEM	Acute disseminated encephalomyelitis
Ag	Antigen
AICD	Activation-induced cell death
AIF	Apoptosis-inducing factor
AP-1	Activator protein 1
APAF-1	Apoptotic protease activating factor-1
APC	Antigen-presenting cell
APL	Altered peptide ligand
BBB	Blood-brain barrier
BCR	B cell receptor
BDNF	Brain derived neurotrophic factor
BM	Bone Marrow
BSA	Bovine Serum Albumin
Caspase	Cysteine aspartyl-specific protease
CD	Cluster of Designation
CDK	Cyclin dependent kinase
CFA	Concentrated Freunds adjuvant
Clr-b	C-type lectin related
CNS	Central nervous system
ConA	Concavalin A
CRAC	Calcium release-activated calcium
CRD	Cystein rich domain
CTLA-4	Cytotoxic T-lymphocyte antigen
CX3CR1	CX3chemokine ligand
DA	Dark agouti
DAG	1,2-Diacyl glycerol
DC	Dendritic cells
DIABLO	Direct IAP binding protein with low pI
DISC	Death inducing signalling complex
DMDs	Disease modufying drugs
DR	Death receptor
dsRNA	Double stranded RNA
e.g.	exempli gratia
EAE	Experimental autoimmune encephalomyelitis
EDSS	Expanded disability status score
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin gallate
et al.	et alii (and others)
FACS	Fluorescence activated cell sorting
FADD	Fas-associated death domain protein
FB	FACS buffer
FBS	Fetal Bovine Serum
Fc	Constant fragment of immunoglobulin molecule
FITC	Fluorescein-isothiocyanate
FLICE	Fas-associated death domain–like IL-1b–converting enzyme
FPP	Farnesylpyrophosphate
FSC	Forward scatter
GA	Glatiramer acetate

GAS	Interferone-g activated site
GDP	Guanosine-5`-diphosphate
GFAP	Glial fibrillary acidic protein
GM-CSF	Granulocyte macrophages colony stimulating factor
GTP	Guanosine-5`-triphosphate
h	Hours
HLA	Human Leukocyte Antigen
i.e.	id est
i.p.	intraperitoneously
i.v.	intravenous
IAP	Inhibitor of Apoptosis Protein
ICAM-1	Intercellular adhesion molecule-1
IFN	Interferon
IFNAR	Interferone A receptor
IgG	Immunoglobulin G
IL	Interleukin
iNOS	Inducible nitric oxide synthase
Iono	Ionomycin
IP_3	Inositol 1,4,5-triphosphate
IRF	Interferon regulatory factor
ISGF	Interferon stimulated gene factor
ISGs	Interferon stimulated genes
ISRE	Interferon stimulated response element
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
IVIG	intravenous immunoglobulin
IκB	Inhibitory κB protein
IκK	IκB kinase
JAK	Janus kinase
JNK	N-terminal c-Jun kinase
kDa	kdalton
KIR	Killer immonoglobulin like receptors
LFA-1	Lymphocyte function–associated antigen 1
LLT-1	Lectin like transcript 1
LN	Lymphnodes
LPS	Lipopolysaccharide
LT	Lymphotoxin
MACS	Magnetic associated cell sorting
MAP	Microtubule associated protein
MAPK	Mitogen Activated Kinase
MAPKK	Mitogen Activated Kinase Kinase
MB	MACS buffer
MBP	Myelin Basic Protein
MHC	Major Histocompatibility Complex
min	Minutes
Mio	Millions
MM	Mouse medium
MMP	Metalloproteinase
MOG	Myelin oligodendrocyte protein
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
n	Number of experiments
NaN_3	Sodium azide
NF-κB	Nuclear factor κB

NGF	Nerve growth factor
NK	Natural killer
NKC	Natural killer complex
NKp	Natural killer protein
NKP-R1	Natural Killer receptor
PBL	Peripheral blood leukocytes
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PFA	Parafolmadehyde
PI3K	Phosphoinositide 3-kinase
PKC	Protein Kinase C
PKR	Protein kinase R
PLP	Proteolipid protein
PMA	Phorbol 12-myristate 13-acetate
PP-MS	Primary progressive multiple sclerosis
PR-MS	Progressive-relapsing multiple sclerosis
PTKs	Protein Tyrosine Kinases
rev.	Review
RNA	Ribonucleic acid
RPMI 1640	Roswell Park Memorial Institute 1640
RR-MS	Relapsing-remitting multiple sclerosis
RT	Room temperature
s	seconds or soluble
s.c.	subcutaneous
S1P	Sphigosine-1-phosphate
SDS	Sodium dodecylsulphate
SEM	Standard Error of Mean
SHP-1	SH2 domain phosphatase
SMAC	Second mitochondria-derived activator of caspase
SP-MS	Secondary progressive multiple sclerosis
SSC	Sideward scatter
STAT	Signal transducer and activation of transcription
SYK	Spleen tyrosine kinase
TAP	Transporter associated with processing
TCR	T cell antigen receptor
TGF	Tumour growth factor
TH	T helper cells
TLR	Toll like receptors
TNF-α	Tumour Necrosis Factor -α
TR	TRAIL receptor
TRAIL	TNF-related apoptosis-inducing ligand
Tyr	Tyrosine
WM	Washing medium
wt	wild type
ZAP	ζ chain associated protein

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1. Introduction

1.1 Multiple Sclerosis

1.1.1 Clinic and epidemiology

Multiple sclerosis (MS) is the most common chronic inflammatory disease of the central nervous system (CNS) and usually begins between 20 and 40 years of age. It leads to substantial disability through deficits of sensation (impaired vision, sensory deficits), motor (spastic paralysis), autonomic (bladder, bowel, sexual dysfunction), and neurocognitive functions. The disease is usually not life shortening, but its socio-economic importance is second only to trauma in young adults in the USA (rev. Sospedra et al., 2004).

MS presents generally one of four clinical courses, each of which might be mild, moderate, or severe:

a) A **relapsing-remitting** course (RR-MS), characterized by partial or total recovery after episodes of reversible neurological dysfunction (also called exacerbations or relapses). This is the most common form of MS. Approximately 85% of patients initially begin with a RR-MS. It affects women about twice as often as men.
b) A relapsing-remitting course that later becomes steadily progressive is called **secondary-progressive MS** (SP-MS). Attacks and partial recoveries may continue to occur. Of 85% of the patients, who start with RR-MS, more than 50% will develop SP-MS within 10 years; 90% within 25 years.
c) A progressive course from onset without any attacks is called **primary-progressive MS** (PP-MS). The symptoms that occur along the way generally do not remit. 10% of people with MS are diagnosed with PP-MS, although the diagnosis usually needs to be made when the person has been living for a period of time with progressive disability and no acute attacks.
d) A progressive course from the onset, with obvious, acute attacks along the way, is called **progressive-relapsing MS** (PR-MS). This course is quite rare, occurring in only 5%-15% of people with MS.

It is not clear which factors are responsible for the different courses of the disease and for the heterogeneity in morphological alterations of the brain detected by magnetic resonance imaging (MRI) (Mc Farland et al., 1999) or histopathological evaluation (Luchinetti et al., 2000). Unknown are also the factors determining the clinical presentation, e.g., which CNS system and areas are primarily affected and whether a patient responds to treatment. They may include a complex genetic trait that translates into different immune abnormalities and/or increased vulnerability of CNS tissue to inflammatory insult or reduced ability to repair damage (rev. Siffrin et al., 2007).

The general population prevalence of MS varies between 60–200/100,000 in Northern Europe and North America, and 6–20/100,000 in low risk areas such as Japan. Population, family, and twin studies show that the prevalence is substantially increased in family members of MS patients. First-degree relatives of affected individuals have an approximately 20- to 50-fold (2%–5%) higher risk to develop MS, and concordance rates in monozygotic twins vary between 20% and 35% in different studies, with the most recent studies placing it at 25% (Dyment et al., 2004).

Most people with MS have a normal life expectancy. A few patients with very severe disability may die prematurely of infectious complications (such as pneumonia) so that the overall life expectancy is 95% of normal.

1.1.2 Pathophysiology

The clinical syndrome is believed to be caused by an autoimmune attack against components of the myelin sheath. Histopathological hallmark is the demyelinating plaque characterized by a complex picture of inflammation, demyelination, remyelination, axonal/neuronal damage (neurodegeneration). Plaques are typically found in subcortical (optic nerves, periventricular white matter), in cortical brain tissue and in brain stem, cerebellum and in spinal cord white matter, often surrounding one or several medium-sized vessels.

In principle, all aspects of immune reactions can be identified in MS lesions. Typical features of inflammatory plaques are $CD4^+$ and $CD8^+$ T cells, activated macrophages and microglial cells, and antibody and complement deposition.

Additionally and early at the course of the disease, axons and their parent cell bodies are a major target in the CNS and this orientates MS research to new therapeutic targets (Figure 1). Early axonal pathology can be found in MS patients, correlating with the number of infiltrating immune cells and, in MRI studies, focal cortical thinning and cortical lesions have been reported in line with frequently observed cognitive impairment (rev. Siffrin et al., 2007).

The course of events that lead to the attack of the immune system against self-antigens, demyelination and axonal damage during MS is not yet fully understood but research has focussed on the following stages of autoimmune reaction leading from the periphery to the CNS:

A. **Activation**: Peripheral activation of T cells specific for myelin antigens and T helper (T_H) 1-type differentiation (rev. Sospedra et al., 2004). How autoreactive T cells become activated in the periphery is still unclear. The existing hypotheses for explaining these processes include: **i) Molecular mimicry**: antigen epitopes of some infectious agents resemble epitopes of myelin proteins and thus induce the corresponding T_H cells to attack myelin. ii) **Bystander activation** of T cells due to increased expression of co-stimulatory molecules in an inflammatory environment (infection). iii) **Incomplete destruction of autoreactive T cells** in the thymus, which has been doubted as autoreactive cells can also be found in healthy individuals.
B. **Transmigration through the blood-brain barrier (BBB)**: Some activated antigen-specific T cells that have escaped regulatory mechanisms transmigrate through the blood-brain barrier under specific conditions that influence BBB permeability, such as changes in adhesion molecules, cytokines, chemokines, leukocytic enzymes, cerebrovascular endothelium, and the parenchymal cells (rev. Sospedra et al, 2004).
C. **Reactivation in CNS**: Once in the CNS, myelin-specific T cells encounter their target autoantigen on antigen presenting cells (APCs), presumably microglial cells and astrocytes, and, on further stimulation, secrete cytokines, which contribute to the local effector mechanisms by creating an inflammatory environment (rev. Zipp et al., 1999).

Evidence now indicate that the T cells critical for inflammation and disease, at least in the EAE model, are characterized by the production of interleukin (IL-)17 ($CD4^+$ T_H17 cells) and require IL-23, IL-6 and TGF-β for their development and

differentiation (Mc Kenzie et al., 2006; Iwakura et al., 2006). Furthermore, the involvement of $CD8^+$ T cells cannot be dismissed. It can be speculated that the MS lesion is initiated by $CD4^+$ T cells but the amplification and damage is mediated by $CD8^+$ T cells. $CD8^+$ T cells are present at the lesion edge as well as in perivascular regions, whereas the $CD4^+$ T cells are generally present only at the lesion edge (rev. Mc Farland et al., 2007). There is also data showing that $CD8^+$ cells can, in some conditions, directly attach to and damage axons (Medana et al., 2001).

The aforementioned mechanisms of demyelination are more complex in reality as different populations of cells and different types of molecules of the immune and other systems are influencing activation, transmigration and reactivation of T_H1 and T_H17 cells and vulnerability to damage of myelin and neurons. These complex mechanisms are investigated step by step in MS research so that the different stages of this process can be targeted to improve the clinical outcome and delay clinical disability of MS patients.

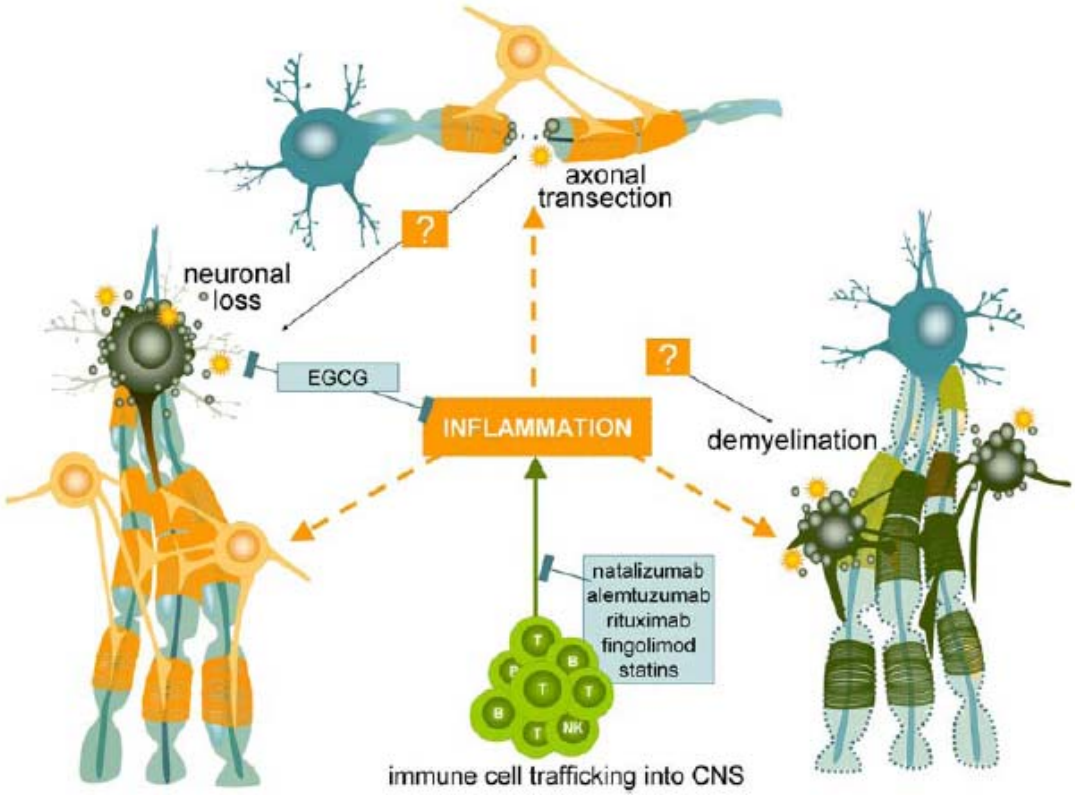


Figure 1 Current understanding of the pathogenesis of MS and potential therapeutic targets

Autoreactive myelin specific cells already activated in the periphery reach the CNS under conditions that influence BBB trafficking and become reactivated causing oligodendrocyte damage and demyelination. On the other hand, axonal transection and neuronal loss are early hallmarks of MS. Both neuronal and myelin damage are only partly mediated by inflammation. Therefore new experimental compounds have been developed such as EGCG, which provide a promising basis for drugs regulating inflammation and at the same time blocking neuronal degeneration. Other experimental drugs such as statins and fingolimod target primarily inflammation and trafficking of lymphocytes outside the LNs. (Infante Duarte et al., 2008)

1.1.3 Treatment of MS. The importance of IFN-β

General considerations

Corticosteroids are the mainstay of symptomatic relief for an acute relapse of MS. They exert immunomodulatory and anti-inflammatory effects, restore the blood-brain barrier and reduce cerebral oedema. They may also improve axonal conduction. Corticosteroid therapy shortens the duration of acute relapses and accelerates recovery. However, corticosteroids have not been shown to improve the overall degree of recovery or to alter the long-term course of MS (rev. Calabresi et al, 2004).

Disease modifying treatments (DMDs) are a group of drugs that impact the course of MS by slowing the progression of the disease and decreasing the number of relapses.

Together with IFN-β that will be described in more detail below, there are several DMDs used as first line of therapy including:

1. Glatiramer Acetate (GA) (Copaxone): This drug is a polypeptide mixture that was originally designed to mimic and compete with MBP. The mode of action of GA is by initial binding to MHC molecules and consequent competing with various myelin antigens for their presentation to T cells. A further aspect of its action is potent induction of specific suppressor cells of the T_H2 type that migrate into the brain and lead to in situ bystander suppression. Furthermore, the GA-specific cells in the brain express the anti-inflammatory cytokines IL-10 and TGF-β, in addition to BDNF, whereas they do not express IFN-γ.
2. IV immunoglobulins (IVIG): Most consistent beneficial results with a reduction of relapse rates and a slowing of disability have been obtained in RR-MS including clinically isolated syndromes. They might serve to suppress an increased recurrence of relapses immediately after delivery. Consequently, IVIG treatment may be considered as second line option for these indications as there is still uncertainty regarding the actual mechanism(s) of action and optimal dosage of this treatment
3. Mitoxantrone (Novantrone): An antineoplastic agent used for worsening forms of RR-MS and SP-MS to slow the progression of SP-MS and extend the time between relapses.
4. Natalizumab (Tysabri): Humanized monoclonal antibody against alpha-4 (α4) integrin, the first drug developed in the class of selective adhesion molecule inhibitors. α4-integrin is required for white blood cells to move into the CNS therefore its mechanism of action is believed to be the inhibition of these immune cells from crossing BBB to reach the CNS (Goodin et al., 2002).

The following paragraphs will be dedicated to introduce properties and mechanisms of actions of IFN-β.

Interferon (IFN)-β

Interferons (IFNs) are a large family of multifunctional secreted proteins involved in antiviral defence, cell growth regulation and immune activation. IFN-β belongs to the type I IFNs, which are produced in direct response to virus infection and consist of the products of the IFN-α multigene family, which are predominantly synthesized by leukocytes, and the product of the IFN-β gene, which is synthesized by most cell types but particularly by fibroblasts. Interferons spread to the nearby cells where they activate interferon-stimulated genes (ISGs), which are responsible for the establishment of an "antiviral state" by preventing viral replication and also alert the immune system (Decker et al., 2005). IFN-β uses the type I IFN receptor for signal

transduction (IFNAR), which comprises two subunits, IFNAR1 and IFNAR2. Briefly, ligand induced stimulation of the receptor complex, IFNAR, results in the activation of the receptor-associated Janus protein tyrosine kinases (JAK PTK), JAK1 and TYK2 PTK. Following the activations of these JAK PTK, tyrosine phosphorylation of signal transducer and activator of transcription 1 (STAT1) and STAT2 is induced, and this is followed by the formation of two transcription factor complexes, IFN- α -activated factor (AAF) and IFN-stimulated gene factor-3 (ISGF3). AAF is a dimeric form of STAT1, and it binds to the IFN- γ -activated site (GAS). ISGF3 consists of STAT1, STAT2 and IRF-9 (p48/ISGF3 γ), and it binds to the IFN-stimulated response element (ISRE) to activate various IFN-inducible genes (Darnell et al., 1994).

IFN-β has become a worldwide standard in treatment of MS. IFN-β has been shown to reduce relapses by about one third and is recommended as first line therapy or for use in glatiramer-intolerant patients who have RR-MS (PRISM Study Group 2001). In randomised double-blind placebo-controlled trials (Paty et al, 1993; Simon et al., 1998) the use of IFN-β resulted in a 50-80 % reduction in inflammatory lesions visualized on brain MRI scans. There is also evidence that this drug improves the quality of life and cognitive function (rev. Calabresi et al., 2004).

Table 1 Overview of commercially available IFN-β products

i.m. IFN-β-1a (Avonex)
s.c. IFN-β-1a (Rebif)
s.c. IFN-β-1b (Betaseron)

Despite the well documented efficacy, it remains unclear how IFN-β influences the clinical course of multiple sclerosis. IFN-β exerts its immunological effects antigen-independently and putative mechanisms of action include:

- Inhibition of T-cell proliferation (Goodin et al, 2002)
- Regulation of many cytokines (Wang et al, 2000)
- Blocking of BBB opening via interference with cell adhesion, migration (Stone et al, 1995), and through reducing the secretion of proteolytic matrix metalloproteinases (MMP) that mediate the migration of T cells across biological barriers (Stuve et al., 1996; Leppert et al., 1996) as well as possibly down regulating MHC class II on various antigen presenting cells (APC) (Hall et al., 1997).

In the following table (table 2) the main immunological actions of IFN-β are summarized:

Table 2 Main immunological actions of IFN-β

Immune mechanism	IFN-β
Proliferation of T cells in vitro	Antigen independent suppression of T cell proliferation
B cell activation	No known effect
Regulation of MHC expression	Reduction of IFN-γ-induced down-regulation
MHC binding	No known effect
T cell migration	Reduction of T cell migration
Adhesion molecule expression	- Increase of soluble adhesion molecules (sICAM-1, sVCAM-1) - Reduction of surface expressed adhesion molecules (VLA-4)
Matrix Metalloproteinase expression	Reduction of expression of MMP-9
Chemokine and chemokine receptor expression	- Reduction of secretion of chemokines - Reduction of expression of chemokine receptors
Cytokine shift in peripheral blood leukocytes (PBL)	Induction of T_H2 cytokines and reduction of T_H1 cytokines in PBL
Altered peptide ligand (APL) effect on CNS specific T cells	No known effect
Effects on APCs	Reduction of IFN-γ induced Fcγ RI expression in monocytes
Neuroprotection	Positive clinical and MRI effects on disease progression

Taken from: (Neuhause et al., Journal of the Neurological Sciences 2005)

On the other hand, many patients under IFN-β therapy develop of neutralizing antibodies after a period of treatment and some of them do not respond at all to the treatment (Wandinger et al., 2004). It is therefore imperative to understand how IFN-β acts in MS, in order to develop markers allowing prediction of therapy response. The aim is to recognise patients who will benefit most from treatment even before clinical worsening. Furthermore, elucidating the drug's mechanisms of action might result in more effective future therapeutic strategies.

1.2. Experimental Autoimmune Encephalomyelitis (EAE)

EAE is a model of the immune system response to priming with CNS - restricted antigens and it serves as an excellent prototype of post-vaccinal encephalitis, acute disseminated encephalomyelitis (ADEM) and many aspects of MS.

In mice, EAE can be induced actively or by adoptive transfer of encephalitogenic T cells (passive EAE). **Active EAE** is induced in mice by active priming with whole myelin proteins or specific myelin peptide epitopes in adjuvant, to induce a pro-inflammatory environment. The specific myelin epitopes able to induce EAE vary with the strain of mouse used (see Table 3). The main effector populations causing the disease are shown to be T_H1 and T_H17 $CD4^+$ T cells as their infiltration in the CNS is associated with the clinical symptoms. **Passive EAE** is induced by adoptive transfer of in vitro activated myelin-specific $CD4^+$ T cells. In this case, the cells act directly in the CNS and destroy the myelin sheath.
The major histological characteristics of the EAE in common with MS are:

- the destruction of the myelin sheaths of the nerve fibres
- the relative sparing of the other elements of the nervous tissue, such as axis cylinders, nerve cells and supporting structures
- the presence of multiple CNS lesions distributed in time and space, generally being more pronounced in the brain stem and spinal cord
- the predominantly perivascular location of lesions
- the temporal maturation of lesions from inflammation through demyelination, to gliosis and partial remyelination
- the presence of immunoglobulins in the CNS and cerebrospinal fluid (rev Baxter et al., 2007).

The sings of EAE in mice vary and mimic different clinical manifestations seen in human. The disease can be monophasic, involving an acute paralytic episode followed by complete recovery; relapsing-remitting, which involves multiple cycles of attack interspersed by full or partial recovery; or chronic, where disease symptoms of the initial attack either stabilize at peak levels or gradually worsen over time (see Table 3). In the monophasic and relapsing-remitting forms, recovery from disease is associated with clearance of inflammatory infiltrates from the CNS (rev. Ercolini et al 2006). Susceptibility to either the monophasic or relapsing-remitting subtypes has been mapped to distinct genetic loci whereas in humans these two MS disease subtypes are also suggested to be genetically distinct entities (Olenrup et al., 1989).

Table 3 Models of EAE in different mouse and rat strains and their relevance to human disease

Model	Similarities to human disease	Differences from human disease	Further comments
Lewis rat Active EAE (CNS myelin, MBP, MOG, PLP)	T-cell inflammation and weak antibody response	Monophasic, little demyelination	Reliable model, commonly used for therapy studies. With guinea-pig MBP little demyelination
Adoptive-transfer EAE (MBP, S-100, MOG, GFAP)	Marked T-cell inflammation. Topography of lesions	Monophasic, little demyelination	Homogeneous course, rapid onset. Differential recruitment of T cells/macrophages depending on autoantigen
Active EAE or AT-EAE + co-transfer of anti-MOG antibodies	T-cell inflammation and demyelination	Only transient demyelination	Basic evidence for role of antibodies in demyelination
Congenic Lewis, DA, BN strains Active EAE (recombinant MOG aa 1–125)	Relapsing–remitting disorders, may completely mimic histopathology of multiple sclerosis and subtypes	No spontaneous disease	Chronic disease course, affection of the optic nerve, also axonal damage similar to multiple sclerosis
Murine EAE (SJL, C57BL/6, PL/J, Biozzi ABH) Active EAE (MBP, MOG, PLP and peptides)	Relapsing–remitting (SJL, Biozzi) and chronic-progressive (C57BL/6) disease courses with demyelination and axonal damage	No spontaneous disease	Pertussis (toxin) required for many strains, whilst it is often not needed for SJL and some Biozzi EAE models. Higher variability of disease incidence and course, often cytotoxic demyelination in C57BL/6. With rat MBP inflammatory vasculitis with little demyelination
Murine EAE in transgenic mice or knockout mice (mostly C57BL/6 background)	Specifically addresses role of defined immune molecules/neurotrophic cytokines/ neuroanatomical tracts	Most results obtained with artificial permanent transgenic or knockouts	Extensive backcrossing (>10 times) on C57BL/6 background required. Future work with conditional (cre/loxP) or inducible (e.g. Tet-on) mutants

Taken from Gold et al., 2006

Epitope spreading in EAE

A primary hallmark of the relapsing-remitting and chronic subtypes of EAE that resembles MS is the phenomenon of epitope spreading, which is the diversification of the initial immune response, secondary to acute myelin destruction, to include reactivity to endogenous CNS determinants. Spreading can occur to different epitopes within the same myelin protein which initiates the disease (intramolecular spreading) or to epitopes within a different myelin protein (intermolecular spreading). For example, there is a sequential and hierarchical order of epitope spreading seen in the relapsing-remitting disease of SJL mice primed with PLP $_{139–151}$ (proteolipid protein) (McRae et al., 1995).

It has been shown that MS patients with long-term disease recognized more myelin epitopes than those with recent-onset disease, but there was no certain correlation between number of epitopes recognized and disease severity (Davies et al., 2005).

1.3 Natural Killer Cells

1.3.1 NK cells: more than killer cells

The immune system has classically been thought to consist of two distinct arms, the innate arm and the adaptive arm but it is becoming increasingly clear that these two arms are tightly interwoven.

Innate immunity is evolutionary older than antigen specific immunity and is crucial for its effector function. The **innate immune system** comprises the cells and mechanisms (e.g. anatomical barriers, secretory molecules) that defend the host from infection by other organisms, in a non-specific manner. This means that the cells of the innate system recognize, and respond to pathogens in a generic way, but unlike the adaptive immune system, it does not confer long-lasting or protective immunity to the host. Innate immune systems provide immediate defence against infection, and are found in all classes of plant and animal life. The cellular components of the innate immune system include basophiles, eosinophils, mast cells, neutrophils, monocytes, macrophages, dendritic cells (DCs) and NK cells.

The **adaptive immune system** is composed of highly specialized, systemic cells and processes that eliminate specifically pathogenic challenges. Its cellular components are B cells and αβ T cells (the majority of T cells, whose T cell receptor (TCR) consists of one α- and one β-chain). The characterization of γδ T cells, whose TCR is made up of one γ-chain and one δ-chain and are found primarily in the gut mucosa, provided the first evidence of a lymphocyte subset that could interface both innate and adaptive immunity, a property that can now also be ascribed to other cells, such as $NK1.1^+$ T cells (known as NK-T cells) and $CD4^+CD25^+$ regulatory T cells (rev. Degli et al., 2005). However, multiple studies in the literature provide evidence that cells within the innate immune system have the potential to inhibit autoreactive $CD4^+$ T cells from mediating autoimmune disease and foreign antigen reactive $CD4^+$ T cells from inflicting collateral damage to healthy tissues. In addition to so called "suppressor" myeloid cells (Nagaraj and Gabrilovich et al., 2007; Serafini et al., 2006) and resting or "homeostatic" $CD205^+$ dendritic cells (Hawiger et al., 2001), NK cells are emerging as key participants in the immunomodulatory circuitry.

First identified in 1975 by virtue of their ability to rapidly kill tumour cells without previous 'priming', NK cells are no longer considered simple 'killing machines', as they have gained recognition for their abilities to secrete cytokines that influence the differentiation of adaptive immune responses, to combat viral and parasitic infections, and to promote vascularisation of implanting embryos during pregnancy (rev. Segal et al., 2007).

NK cells are distinct from T cells or B cells with unique morphologic, molecular, phenotypic and functional properties. **Morphologically**, most NK cells are large granular lymphocytes and in **molecular** terms, NK cells are defined as lymphocytes that lack expression of the antigen receptors that are expressed by B cells and T cells, the B-cell receptor (BCR) and the T-cell receptor (TCR), respectively.

Human NK cells are traditionally characterized by being $CD3^-$ and $CD56^+$. They are distinct from NK-T cells which express CD3 and rearrange their germline DNA T cell receptor genes (though with a limited repertoire) (Orange et al., 2006). NK cells can be divided into $CD56^{dim}$ and $CD56^{bright}$ NK cell subsets, which differ in their homing properties (Cooper et al., 2001). Around 90% of peripheral blood and spleen NK cells are $CD56^{dim}CD16^+$ and express perforin. These $CD56^{dim}$ NK cells are cytotoxic and produce IFN-γ upon interaction with tumour cells in vitro (Anfossi et al., 2006). In contrast, most NK cells in LN and tonsils are $CD56^{bright}CD16^-$ and lack

perforin (Ferlazzo et al., 2004). These cells readily produce cytokines such as IFN-γ in response to stimulation with IL-12, IL-15 and IL-18 (rev. Vivier et al., 2008).

In mice, NK cell markers appear to be dependent on the mouse strain. In C57BL/6, NK cells are characterized as $NK1.1^{+}CD3^{-}$ cells whereas in SJL/J mice, as $DX5^{+}CD3^{-}$. In the mouse, three subsets of NK cells differing in expression of CD11b and CD27 have been described (Hayakawa et al., 2006). NK cells differentiate from $CD11b^{dull}CD27^{+}$ NK cells (found predominantly in bone marrow and lymph nodes) (Kim et al., 2002), by way of $CD11b^{+}CD27^{+}$ double-positive NK cells, to the most mature $CD11b^{+}CD27^{dull}$ NK cells (found in blood, spleen, lung and liver). Double-positive and $CD11b^{+}CD27^{dull}$ NK cells show comparable capacities to kill target cells and to secrete IFN-γ in a broad range of in vitro stimulation conditions, but $CD11b^{+}CD27^{dull}$ NK cells are in replicative senescence (Hayakawa et al., 2006; rev. Freud et al., 2006; rev. Vivier et al., 2008).

1.3.2 NK cell functions

NK cell functions can be classified in three categories, cytotoxicity, cytokine production and co-stimulation:

1. Cytotoxicity: The triggering of NK-cell cytotoxicity versus the absence of killing activity reflects a delicate balance or a 'dynamic equilibrium' between activating and inhibitory signals that are delivered by cell-surface receptors which are regulated in a spatial and temporal fashion.

The genes that encode many of the NK-cell receptors are present in a locus known as the NK complex (NKC), which is located on chromosome 6 in mice and chromosome 12 in humans. Numerous genes in the NKC encode type-II C-type lectin-like molecules, many of which are involved in target-cell recognition by NK cells (Yokoyama et al., 2003).

Inhibitory Receptors**:** The first proposal that, unlike T cells, NK cell activity is controlled by inhibitory receptors specific for MHC class I molecules was made by Klaus Kärre in 1986 and is now referred to as the 'missing self' hypothesis. NK cells use inhibitory receptors to gauge the absence of constitutively expressed self molecules on susceptible target cells for example MHC class I–deficient haematopoietic cells. (Kärre et al., 1986) In that way they ensure tolerance to self (healthy cells with normal expression of MHC I) while allowing toxicity towards stressed cells with deficient MHC I expression.

The MHC class I–specific inhibitory receptors include the killer cell immunoglobulin-like receptors (KIRs) in humans, the lectin-like Ly49 dimers in the mouse and the lectin-like CD94-NKG2A heterodimers in both species (Figure 2) (Parham et al., 2005). Intracellular signal transduction from these receptors is mediated by one or two conserved intracytoplasmic inhibitory signalling domains called immunoreceptor tyrosine-based inhibition motifs (ITIMs) (Vivier et al., 2004). The phosphorylated ITIMs then recruit and activate the cytoplasmic tyrosine phosphatase SHP-1. KIRs and Ly49 receptors are an outstanding example of convergent evolution. That is, mice and humans evolved different receptors to serve the same function of preventing NK cell activation upon encounter with self MHC (rev. Vivier et al., 2008).

MHC class I is not the only constitutive self signal detected by NK cells, as other inhibitory receptors (e.g. mouse NKR-P1B, human NKR-P1A and mouse 2B4) that recognize non-MHC self molecules (e.g. Clr-b, LLT-1 and CD48, respectively) also regulate NK cell activation (Kumar et al., 2005).

Activating receptors: T and B cells possess a single antigen receptor that dominates their development and activation. Signals initiated through these antigen receptors are augmented by co-stimulatory molecules. In contrast, NK cells do not possess one dominant receptor, but instead rely on a vast combinatorial array of receptors to initiate effector functions.

Positive signals for killing are provided by ligation of several **activating receptors** — which include CD2, NK-cell receptor protein 1 (NKR-P1), 2B4 (also known as CD244), NK-cell protein 30 (NKp30), NKp44, NKp46 and NKG2D — with ligands, the expression of which is constitutively expressed or induced (for example, following infection, cellular transformation or stress) at the surface of target cells (Figure 2) (Lanier et al., 2005).

NK cells also express the low affinity Fc receptor CD16 (FcγRIII) which enables them to detect antibody-coated target cells and to exert antibody-dependent cell cytotoxicity (ADCC) as well as Toll like receptors (TLRs). In vitro exposure of NK cells to TLR ligands induces IFN-γ production and enhances cytotoxicity. However, this process is more efficient when accessory cells are present in the environment of NK cells, suggesting that the role of TLRs in NK cells might be indirect in vivo (Sivori et al., 2004; Hart et al., 2005; Gerosa et al., 2005). Some of these activating receptors possess intracellular immunoreceptor tyrosine-based activation motifs (ITAMs).The protein tyrosine kinases SYK (spleen tyrosine kinase) and ZAP70 (ζ-chain-associated protein kinase of 70 kDa) function downstream of NK-cell activating receptors, which contain ITAMs or associate with adaptors that contain ITAMs. These protein kinases phosphorylate and mobilize multiple downstream proteins, resulting in activation of NK cells and initiation of granule exocytosis (Glimcher et al., 2004).

Ways of killing: NK cells possess relatively large numbers of cytolytic granules, which are secretory lysosomes containing perforin and various granzymes. Upon contact between NK cell and its target, these granules traffic to the contact zone with the susceptible target cell (the so-called immunological synapse), and the contents are extruded to effect lysis. Perforin-dependent cytotoxicity is the major mechanism of NK cell lysis, although NK cells can also kill in a perforin-independent manner utilizing FAS ligand, TNF or TRAIL (Orange et al., 2006).

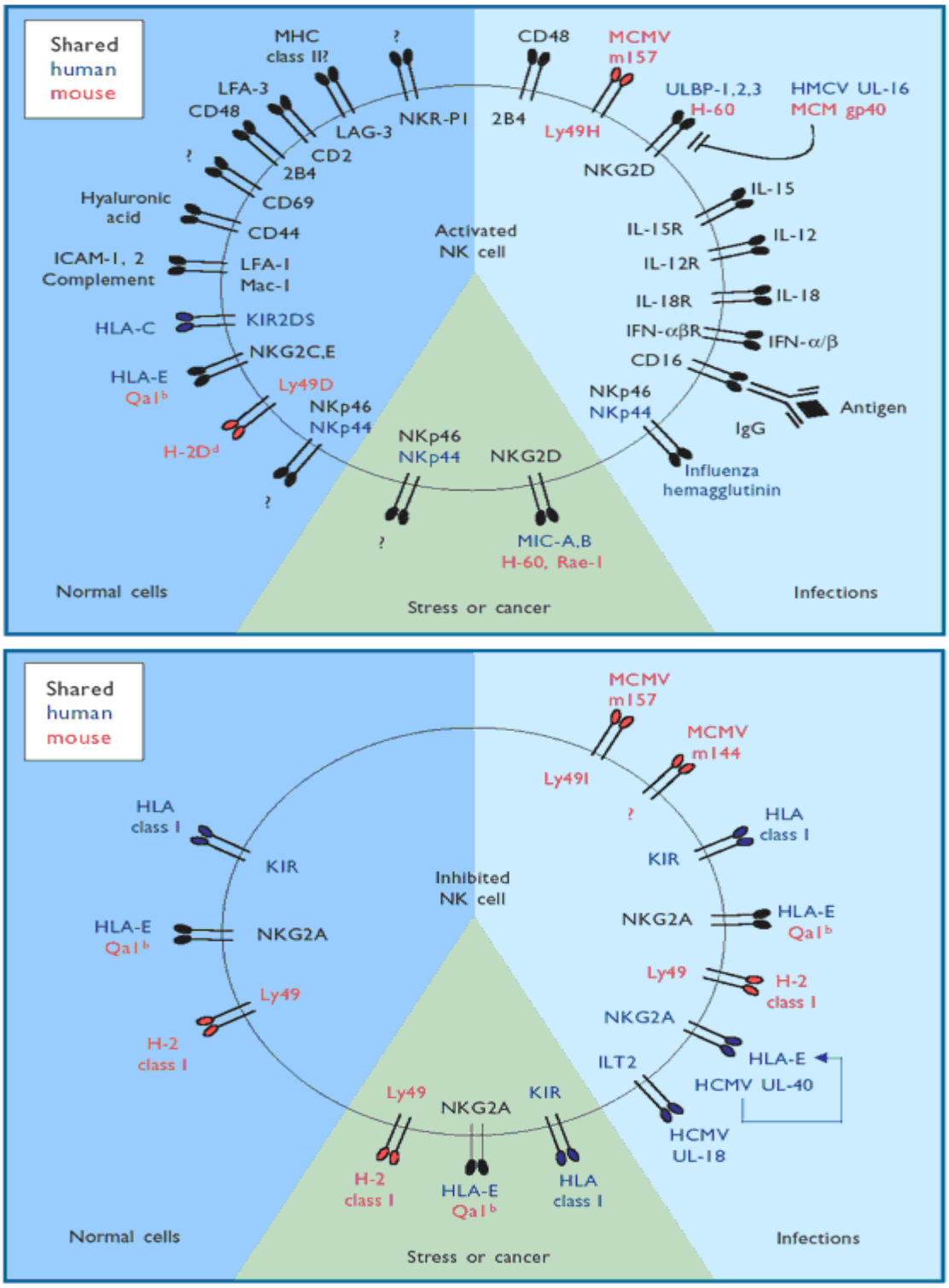


Figure 2 Activating and inhibitory NK receptors
Molecules shared by humans and mice are shown in black, whereas species-specific molecules are indicated in blue (human) or red (mouse). (a) NK cells can be activated upon contact with normal, stressed or cancer cells and during infection. The boundaries in real-life are not so well defined. For example, cytokines may activate cells during antitumor immunity as well as during infection. (b) Classical MHC class I molecules (HLA in humans, H-2 in mice) and nonclassical MHC molecules (HLA-E in humans, $Qa1^b$ in mice) inhibit NK cells upon contact with normal or diseased cells. (rev. Colucci et al., 2002)

2. Cytokine and chemokine secretion: NK-cell-mediated cytokine release following NK-cell activation is mediated through signalling molecules (such as PI3K) and transcription factors (such as T-bet) through receptors such as NKG2D (Jiang et al. 2000; Jiang et al., 2002). The cytokine that is released in the largest quantities by NK cells is IFN-γ. NK cells can also produce various other cytokines, including TGF-β, TNF, LT-α, GM-CSF, CC-chemokine ligand 3 (CCL3; also known as MIP1α), IL-1, IL-2, IL-3, IL-5, IL-10, IL-13 and CXC-chemokine ligand 8 (CXCL8; also known as IL-8) and RANTES (Dorner et al., 2004) all of which have potent immunoregulatory functions (Biron et al., 1999).

Similar to T_H0 cells, NK-cell precursors can differentiate into NK cells that produce either type 1 (NK1) or type 2 (NK2) cytokines. NK2 cells produce the type 2 cytokines IL-5 and IL-13 (but not IL-4) and are generated in the presence of IL-4. By contrast, NK1 cells, which produce IFN-γ, are generated when IL-12 dominates in the local cytokine milieu. NK1 cells kill susceptible target cells in a CD95 ligand-, perforin- and granzyme-dependent manner, whereas NK2 cells kill susceptible target cells by a mechanism that depends on the expression of TRAIL (Loza et al., 2001).

3. Contact-dependent cell co-stimulation: NK cells express several co-stimulatory ligands including CD40L (CD154) and OX40L, which allow them to provide a co stimulatory signal to T cells or B cells (Zingoni et al., 2004). Thus, NK cells may serve as a bridge in an interactive loop between innate and adaptive immunity. DCs stimulate NK cells which then deliver a co-stimulatory signal to T or B cells allowing for an optimal immune response.

Consistent with their function as innate sentinels, NK cells are widespread throughout lymphoid and non-lymphoid tissues. In most tissues, NK cells represent a minor fraction of total lymphocytes (from 2% in mouse spleen to 10% in mouse lung and from 2% to 18% in human peripheral blood) (rev. Gregoire et al., 2007). Human NK cell turnover in blood is around 2 weeks (Zhang et al., 2007), consistent with data in the mouse (Walzer et al., 2007; rev. Vivier et al., 2008).

1.3.3 Recruitment of NK cells to target organs and EAE

Three types of cell surface receptors are involved in NK cell trafficking in the mouse. The chemokine receptors CCR2, CCR5, CXCR3 and CX3CR1 regulate NK cell recruitment upon inflammation (Gregoire et al., 2007). NK cell entry to LN from blood is dependent on CD62L (Chen et al., 2005) and on sphingosine 1-phosphate (S1P) receptor, S1P5, which is selectively expressed on NK cells in LN and is acquired with maturation in both humans and mice (Walzer et al., 2007). In humans, CCR7 is expressed on $CD56^{bright}$ NK cells and CXCR1 and ChemR, which are expressed on the $CD56^{dim}$ human NK cell subset are likely to regulate their homing to LN (Kim et al., 1999) and into peripheral inflammatory sites (Parolini et al., 2007).

CX3C-chemokine ligand 1 (CX3CL1; also known as fractalkine) attracts NK cells specifically to the heart and CNS during cardiac-allograft rejection (Hashell et al., 2006) and EAE (Huang et al., 2006), respectively. It has been shown that markedly fewer NK cells were present in the inflamed CNS of CX3C-chemokine receptor 1 (CX3CR1)-deficient mice with EAE than in wt mice with EAE, whereas the recruitment of T cells, NK-T cells and cells of the monocyte–macrophage lineage to the CNS during EAE did not require CX3CR1. Impaired recruitment of NK cells in CX3CR1-deficient mice was associated with increased EAE-related mortality, non-remitting spastic paraplegia and haemorrhagic inflammatory lesions (Huang et al.,

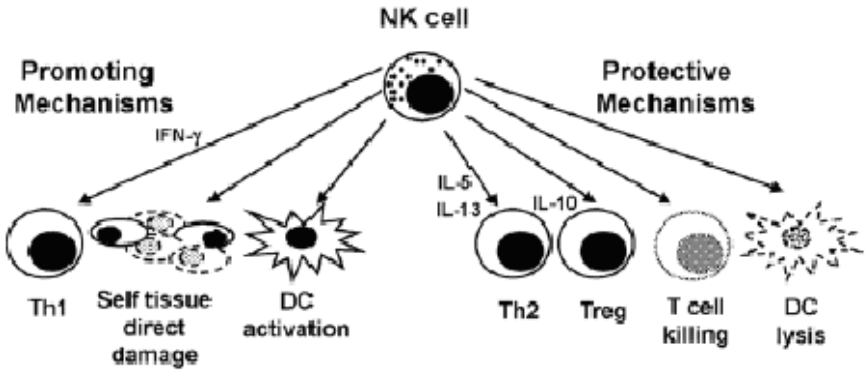
2006). These results show that there is a relationship between the specific anatomical locations of NK cells and their unique roles in modulating CNS inflammation and autoimmunity.

1.3.4 NK cells in MS and EAE

NK cells seem to mediate both beneficial and detrimental effects during the progression of autoimmunity. Patients with autoimmune diseases often show reduced NK cell numbers and impaired NK cell function. Some data indicate that such NK cell dysfunction is a secondary event caused by the IL-21 released by autoreactive CD4+ T cells (Liu et al,. 2006). However, other data indicate that NK cell dysfunction is a primary event capable of precipitating autoimmune disease. Patients harbouring mutations in the molecule transporter associated with antigen processing (TAP)-1 or TAP-2, which result in reduced surface expression of MHC class I molecules and thus might facilitate NK cell attack, suffer from chronic infections, necrotizing granulomatous skin lesions and autoimmune manifestations, specifically in pathological conditions where cytokine release may aid the breakdown of NK cell tolerance (Zimmer et al., 2001).

NK cell dysfunctions may therefore represent primary defects rather than secondary defects that cause a deleterious cascade of immunological events culminating in impaired resolution of an immunological insult. Early diagnosis of molecular and functional NK cell defects may guide appropriate prophylactic therapies for autoimmune disorders.

Figure 3 Mechanisms through which NK cells promote and inhibit autoimmunity



(Morandi et al., 2008)

In MS, NK cells might influence the magnitude of an autoantigen-specific response by direct cytotoxic effect against self-tissues and/or by immunoregulatory activity (Figure 3). **In vitro** studies show that NK cells can directly lyse neurons, oligodendrocytes, astrocytes, microglia (Backstrom et al., 2003; Morse et al., 2001; Antel et al., 1998). **In vivo**, the local inflammatory milieu in MS-lesions could provide conditions that promote NK cell-activation. In this way, NK cells would be able to bypass the inhibitory effects of self-HLA class I molecules and lyse these cells. On the contrary, a study has indicated that NK cells infiltrating the CNS express neurotrophic factors that might contribute to the rescue of mechanically injured neurons (Hammarberg et al., 2000).

Recently, other protective roles for NK cells in patients with MS have been suggested. In a longitudinal study, NK cell functional activity fell precipitously in

concert with the onset of clinical MS relapses but normalised during remissions (Kastrukoff et al., 2003). NK cells from patients in disease remission expressed high levels of Fas (CD95) and were classified as NK2 cells (Takahashi et al., 2004). A regulatory role for NK cells was further suggested by the fact that NK cells lose CD95 expression and their NK2 phenotype before disease relapse, thus presumably losing their regulatory role. It has been shown that in vitro-induced NK2 cells inhibited the induction of T_H1 cells, suggesting that NK2-like cells in vivo may also prohibit autoimmune effector T cell development (Takahashi et al., 2001). Another recent study has shown that MS patients with active disease are relatively deficient in particular subsets $CD56^+$ $CD3^-CX3CR1^+$ NK cells that are highly cytotoxic ex vivo (Infante-Duarte et al., 2005).

Stronger evidence for a role of regulatory NK cells in MS comes from data on the immunological consequences of initiating novel as well as conventional **pharmacological treatments** in patients with active disease for example IFNs and statins (Vollmer et al., 2004; Van Kärre al., 2005). In a phase II trial of daclizumab (a humanized monoclonal antibody directed against the IL-2α chain) in RR-MS patients, suppression of contrast enhancing lesions on brain MRI was significantly associated with the expansion of circulating $CD56^{bright}$ NK cells and contraction of $CD4^+$ and $CD8^+$ T cells (Bielekova et al., 2006). Furthermore, NK cells isolated from patients during, but not before, daclizumab therapy exhibited cytotoxicity towards autologous activated T cells without the need for pre-stimulation with IL-2. In a separate manuscript, newly diagnosed RR-MS patients experienced an increase in the percentage of $CD56^{bright}$ NK cells among peripheral blood mononuclear cells within 3 months of starting IFN-β treatment (Saraste et al., 2007). While these observations are intriguing, more definitive conclusions await clinical trials with agents that directly and exclusively interfere with NK cell activities.

Similarly to MS, in the EAE both a beneficial and a detrimental role of NK cells have been postulated. Initial studies with EAE depletion and adoptive transfer in different EAE mouse and rat models have shown a protective role for NK cells:

In 1997 Zhang et al. reported that treatment of $MOG_{35–55}$- sensitized C57BL/6 mice with a depleting antibody specific for NK1.1 accelerated the onset and increased the severity of clinical EAE (Zhang et al., 1997). The same observations were made for passively induced EAE with encephalitogenic $CD4^+$ T cells and for C57BL/6 β2-microglobulin deficient mice that lack $NK1.1^+$ $CD3^+$ cells. The latter indicates that depletion of conventional NK cells and not NK-T cells was responsible for the therapeutic effect of the antibody. Additionally, co-transfer of whole splenocytes, but not NK cell depleted splenocytes, ameliorated EAE induced by the injection of myelin-specific T cells into NK1.1 depleted $Rag2^{-/-}$ hosts.

Similarly, NK cell depletion **exacerbated EAE** in Lewis rats and SJL mice (Matsumoto et al., 1998; Xu et al., 1999) A down-regulatory role for NK cells in rodent EAE is further strengthened by the finding that dark agouti (DA) rat bone marrow-derived NK cells exhibited potent inhibitory effects on auto-reactive T cell proliferation to both Con A as well as the central nervous system Ag MBP (Schmeltz et al., 1999).

Matsumoto et al. also tracked the proportions of NK cells present in peripheral blood and the spinal cord in Lewis rats during the onset of, and recovery from disease. The onset of EAE was associated with a three-fold increase in the proportions of peripheral blood $NKR\text{-}P1^+$, αβ TCR2 NK cells. At that time, 17% of leukocytes infiltrating the spinal cord were NK cells, reflecting a rapid and short-lived influx of

NK cells commencing 10 days after immunisation and lasting for less than a week. Following recovery, there was a further increase in the number of circulating NK cells, but the proportion in the spinal cord had dropped to less than 1%. Other researchers have reported that NK cells account for 10–20% of the infiltrate in symptomatic C57BL/6 mice immunized with MOG $_{35–55}$ (Huang et al., 2006).

Collectively, the observations that significant numbers of NK cells accumulate within the target organ during EAE and NK depleting antibodies aggravate disease at a point past the priming of encephalitogenic T cells, implicate a role of regulatory NK cells in the effector phase of pathogenesis, possibly within the CNS itself.

A contrasting study has recently been published regarding the role of NK cell depletion in EAE (Winkler-Pickett et al., 2008). **Diminished clinical disease** was observed in C57BL/6 mice treated with anti-NK1.1, anti-asialo GM1, and selected Ly49 subtype antibodies. In addition to an increase in T cell responses to MOG, the maturational status of DCs in LNs was altered both quantitatively and qualitatively.

On day 7 following immunisation, there were significantly fewer DCs present in the NK cell- depleted EAE brain and cervical LN. Although the draining LN did not show significant changes in the absolute number of DCs, the percentage of DCs was increased. The number of brain DCs was diminished by 59%, while the percentage was almost identical. Furthermore, immunization with MOG peptide increased the number of draining LN NK cells compared with naive mice, a finding reported in another adaptive model (Martin-Fontecha et al., 2008).

NK cell depletion modified the DC composition as found in the draining LN. There was an increase in the percentage of $CD80^{+}CD86^{+}$ DCs in EAE NK cell-depleted mice, suggesting a more mature DC population compared with the EAE control mice in the draining LN, whereas immature DCs were favoured in the cervical LNs.

Finally, examination of TCR Vβ usage of the brain lymphocytes, which have been shown to be the major effector repertoire in C57BL/6 mice (Mendel et al.,) from EAE mice, indicated a spectra-type change in receptor expression in NK- depleted mice as compared with non-NK cell-depleted EAE mice (Winkler-Pickett et al., 2008).

1.4 TNF-Related Apoptosis Inducing Ligand (TRAIL)

1.4.1 TRAIL and its receptors

Apoptosis, or programmed cell death, is a genetically conserved physiological event, which plays a major role in the elimination of injured or unwanted cells in many physiological and pathophysiological conditions such as normal development, homeostasis, defence mechanism against viral infection, dysregulated immune disease, and uncontrolled cell growth. Thus, dysregulation of apoptosis might be related to serious human pathologies including neoplasia, degenerative disorders and autoimmune diseases (Nicholson et al., 2000).

In 1995, TNF-related apoptosis-inducing ligand (TRAIL), also named Apo2 Ligand (Wiley et al., 1995, Pitti et al., 1996) was identified and characterized as a member of the TNF/NGF superfamily. The initial interest for the ligand TRAIL was raised in cancer research as its ability to kill transformed cells but not at the same time exert any effects on non-transformed cells, implied a role in immunological tumour surveillance (Ashkenazy et al, 1999, Walczak et al., 1999). But in line with its main immunological function, induction of apoptosis of non-transformed human and

murine cells such as hepatocytes (Jo et al., 2000) and neurons (Aktas et al., 2004) was also attributed to TRAIL.

The open reading frame encodes 281 amino acids for the human TRAIL protein which forms a stable homotrimeric molecule (Ashkenazy et al., 1999). TRAIL is primarily expressed as a type II transmembrane protein in which the carboxyl terminus of the receptor-binding domain protrudes extracellularly. Similar to TNF-α and FasL, TRAIL can also be cleaved from the cell membrane by metalloproteases to yield a soluble and biologically active form (Liabakk et al., 2002).

Two novel TRAIL splice variants (TRAIL-β and TRAIL-γ) have been described in the human system (Krieg et al., 2003), which have a truncated extracellular binding domain because of the lack of exon 3 (in TRAIL-β) or of exons 2 and 3 (in TRAIL-γ), which probably causes a diminished apoptotic potential.

In human several TRAIL receptors have been identified (s. Table 4). However, only TRAIL-R1 and TRAIL-R2 receptors are functional as they have an intracellular signalling domain. Decoy receptors are always functionally important as they can bind TRAIL and reduce the concentration of the ligand in the extracellular space (Figure 4):

Table 4 TRAIL receptors in human

Receptors	TRAIL-R1	TRAIL-R2	TRAIL-R3	TRAIL-R4	Osteoprotegrin
Other name	DR4	TRICK2, KILLER, DR5	DcR1/TRID/ LIT	DcR2/ TRUNND	OPG
Function	Death Receptor	Death Receptor	Decoy Receptor	Decoy Receptor	Decoy Receptor
Death Domain	Yes	Yes	No	Yes (but 1/3 length of typical)	No
Highest expression organ	Peripheral blood leukocytes, spleen	Fetal liver, lung adult PBL, ovary spleen, liver, lung	PBL, spleen, lung, placenta		
CRDs	2	2	2	2	
Features			GPI anchored		Soluble, Regulates osteoclastogenesis
NF-kB activation	Yes	Yes	No	Yes	
Maps to chromosome	8p21-22	8p21-22	8p21-22	8p21-22	8p23-24
Binding affinity	High	High	High	High	Low
Bibliography	(Pan et al, 1997)	(Walczak et al, 1997)	(Degli-Eposti et al, 1997)	(Degli-Eposti et al, 1997)	(Emery et al., 1998)

Taken from Kim et al., 2003

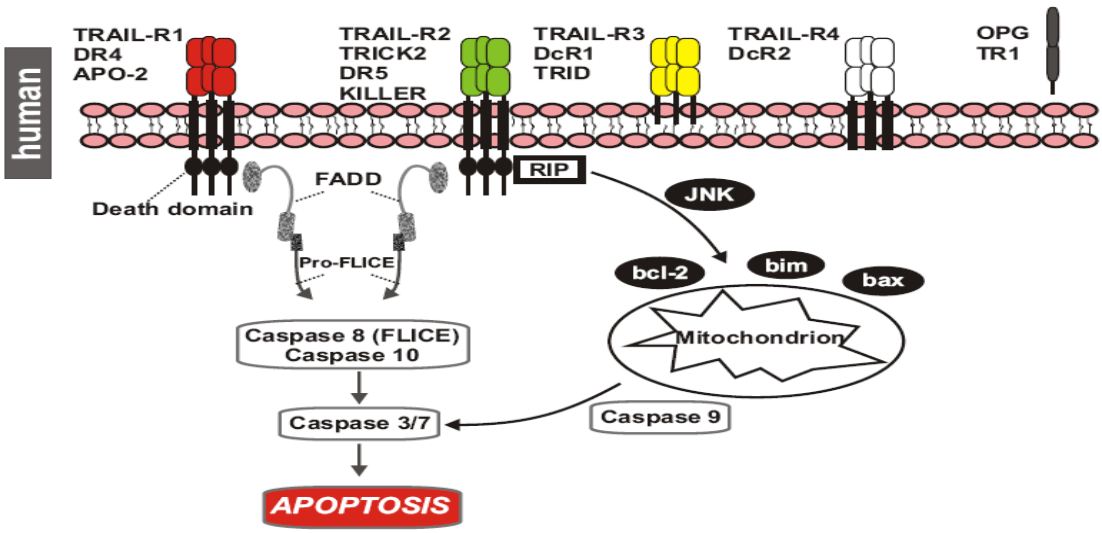


Figure 4 TRAIL receptors in human system
Taken from rev. Aktas et al 2007

In mice, two decoy receptors for TRAIL and one functional death mediating receptor, which shares homology with the human TRAIL receptor 2 have been reported (Figure 5) (Schneider et al., 2003).

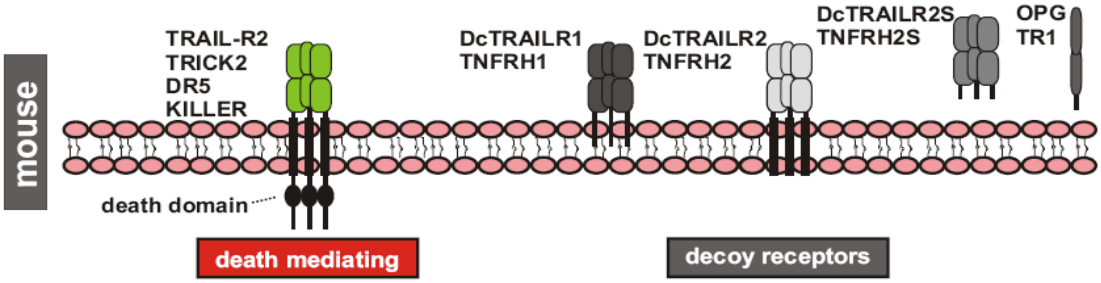


Figure 5 TRAIL receptors in murine system
Taken from rev. Aktas et al., 2007

1.4.2 Expression of TRAIL and its receptors

1. Human immune cells

Resting human $CD4^+$ T cells and $CD14^+$ macrophages express TRAIL and all TRAIL receptors including the truncated TRAIL-R3 and TRAIL-R4 (Wendling et al., 2000) whereas so far no expression in resting human B cells has been found (Zhao et al., 1999). Upon cell-type specific stimulation, human $CD3^+$ T cells, $CD19^+$ B cells and $CD14^+$ macrophages express or up- regulate expression of TRAIL (Ehrlich et al. 2003). T cell receptor stimulation of antigen-specific T cell lines with agonistic antibodies to CD3 and CD28 down-regulates TRAIL-receptors 1 and 2 whereas TRAIL is up-regulated (Wendling et al., 2000).

2. Murine immune cells and NK cells

ConA/IL-2-activated, but not resting $CD3^+$ cells expressed TRAIL in an activation-dependent fashion. Conversely, freshly isolated $B220^+$ cells displayed surface TRAIL and CD95L that were retained following activation with LPS. Restimulation with the protein kinase C activator phorbol 12-myristate 13-acetate and

the calcium ionophore ionomycin (PMA/Iono)or an agonistic anti-CD3 monoclonal antibody induced significant up-regulation of surface TRAIL and CD95L in $CD3^+$, TCR αβ cells with $CD4^+$ or $CD8^+$ phenotype (Mariani et al., 1998).

Stimulation of TRAIL-$R^{-/-}$ macrophages with Mycobacterium tuberculosis antigens and TLR-2, -3, and -4, but not TLR-9, ligands resulted in high levels of TRAIL up-regulation (Diehl et al., 2004).

According to Kayagaki et al., a remarkable level of surface TRAIL expression can be induced preferentially on $CD3^-$ $NK1.1^+$ NK cells after stimulation with IL-2 or IL-15 but not with IL-18. Furthermore, Perforin, FasL and TRAIL systems seem to contribute to IL-2- or IL-15-activated NK cell cytotoxicity against tumour cells lines as inhibition of all three of these systems was required for the abrogation of cytotoxicity (Kayagaki et al., 1999).

1.4.3. Signalling through the TRAIL receptors

TRAIL receptor mediated signalling events leading to apoptosis can be divided into two distinct but interacting pathways, involving either mitochondria (intrinsic) or death receptors (extrinsic).

Extrinsic pathway: The engagement of DRs by their ligand is followed by the recruitment of proteins to the intracellular death domain of the receptor to form a structure known as the death-inducing signalling complex (DISC) (Kischkel et al., 1995), which consists of the adaptor protein Fas-associated death domain (FADD) and the apoptosis initiator pro-caspase-8 (pro-FLICE) (Kischkel et al., 2000). Pro-caspase 8 is then auto-proteolytically activated forming caspase 8, whereas also caspase-10 can be activated and transmit an apoptosis signal in the absence of caspase-8 (Kischkel et al., 2001). Finally, activation of caspase 3 mediates caspase-activated DNase and apoptotic demise of the cell.

Intrinsic pathway: Cell-intrinsic cues such as DNA damage trigger apoptosis through a mitochondrial pathway. The Bcl-2 family member Bax translocates to the mitochondria, the mitochondrial transmembrane potential dissipates, and cytochrome c is released to the cytosol, activating caspase-9 and subsequently the effector caspases. Proapoptotic members of the Bcl-2 family are counteracted by the anti-apoptotic family members Bcl-2 or Bcl-XL (Bouillet et al., 2002) (Figure 9).

Apo2L/TRAIL induces three clusters of genes over time:

- The early cluster, induced at 1–4 h, includes primarily genes that have not been well characterised with respect to apoptosis.
- After 4 h of treatment, many NF-kB-regulated genes were induced and remained high (the intermediate cluster) as all death domains activate this anti-apoptotic transcription factor.
- The late cluster, genes up-regulated after 16 h exposure to Apo2L/TRAIL, was mainly IFN-inducible genes (Kumar-Sinha et al., 2002; rev. LeBlanc et al., 2003).

1.4.4 TRAIL and TRAIL receptor expression in the CNS

TRAIL is not expressed in the human brain under physiological conditions. Its death-mediating and decoy receptors have been detected on microtubuli-associated protein $(MAP)^+$ neurons (TRAIL-R1, -R3 and -R4), glial fibrillary acid protein $(GFAP)^+$ astrocytes (TRAIL-R1) and proteolipid $(PLP)^+$ oligodendrocytes (TRAIL-R2 and -R4) (Doerr et al., 2002). Resting $CD68^+$ microglia cells do not express

TRAIL or TRAIL receptors. However, in degenerative (e.g. Alzheimer's Disease) brain diseases and as well as brain tumours and injuries, TRAIL is induced on different kinds of CNS cell populations (Frank et al., 1999; Uberti et al., 2004). Regarding primary brain tumours in vivo and in vitro experiments imply sensitivity of malignant glioma cells towards TRAIL which can be reduced by disease-specific genomic aberrations (Li et al., 2006) but also increased by manipulating the second mitochondria-derived activator of caspase (Smac), involved in the mitochondrial pathway of apoptosis induction (Fulda et al., 2002). In a series of glioblastoma multiforme patients, TRAIL-R1 and -R2 expression on tumour cells represent independent prognostic factors for survival (Kuijlen et al., 2006).

In EAE, up-regulation of TRAIL-R2 and TRAIL itself was found in the inflamed CNS tissue of mice. TRAIL-R2 up-regulation was detected particularly on neurons, therefore neurons should be susceptible to TRAIL- mediated apoptosis in the EAE brain and this has been shown in vitro and in vivo (Aktas et al., 2005).

Sources of TRAIL in the inflamed CNS were the infiltrating and activated immune cells, among them T cells, macrophages, and microglia. This is in line with in vitro experiments that have shown that TRAIL expression can be induced in murine microglia upon stimulation with IFN-γ or lipopolysaccharide (LPS) (Genc et al., 2003). Similarly, exposure of fetal brain astrocytes to IFN-γ has been reported to result in TRAIL expression in vitro (Lee et al., 2003).

1.4.5 Role of TRAIL in EAE and MS

Recently it has been postulated that TRAIL may exert two opposite functions inside the CNS and in the periphery, by inducing neuronal apoptosis and by exerting anti-inflammatory effects, respectively.

1. TRAIL in the CNS

In MS, the importance of neuronal pathology at the initial stages of the disease, which was previously underestimated, is now coming forward. Data from the EAE model supported the conclusion that axonal damage and neuronal death are an integral part of the disease processes and occur at early stages of the pathology (Meyer et al., 2001, Diestel et al., 2003). In the human disease neuronal pathology involving axonal transection and neuronal death occurs also in the early stages as shown by the reduction of brain volume and later contributes to irreversible disability of patients (Zipp et al., 2006).

Initial in vitro studies showed that soluble TRAIL can induce death of non-transformed brain cells such as neurons (Nitsch et al, 2000) and oligodendrocytes (Jurewicz et al., 2005) and as TRAIL is up-regulated by activated human lymphocytes a putative mechanism of neuronal death is implied (Wendling et al., 2000; Doerr et al., 2002). In line with this, intracerebral injection of recombinant human TRAIL in non-inflamed CNS had no effects whereas in immunised mice it caused a deterioration of EAE symptoms. In addition, intracerebral injection of a soluble TRAIL receptor which blocks deleterious TRAIL actions, reduced the clinical score of PLP immunized SJL mice (Aktas et al., 2005). The peripheral immune response, including T cell proliferation and cytokine release, remained unchanged but a significant protection of the CNS from immune cell-mediated damage, i.e. neuronal apoptosis and myelin loss was observed. Quantitative real-time gene expression analysis for TRAIL-R2 and TRAIL showed that in EAE, both molecules were significantly up- regulated in the CNS prior to and during the peak phase of EAE

(Aktas et al., 2005) which in line with the previously mentioned findings indicate that the up- regulation of TRAIL receptors in ongoing EAE (inflammatory environment) is sufficient to mediate a significant death signal whereas the existing receptors levels in normal CNS are not.

Furthermore, transfer of **myelin-specific TRAIL-deficient T cells** into wt recipients led to a significantly attenuated disease score, indicating that brain-invading myelin-specific T cells devoid of TRAIL are significantly reduced in their capacity to induce CNS damage (Aktas et al., 2004). A similar inflammatory sensitisation of the target tissue to harmful TRAIL effects was also observed in ConA-mediated experimental hepatitis, while this study showed an up-regulation of TRAIL expression in the liver including constitutively expressed TRAIL-R2. In this model, hepatitis cell death was diminished in TRAIL-deficient mice, whereas transfer of TRAIL-expressing liver mononuclear cells was sufficient to renew hepatic cell death (Zheng et al., 2004).

2. TRAIL in the periphery

In the periphery, TRAIL does not induce apoptosis (Wendling et al., 2000), but inhibits proliferation of T cells in vitro and in vivo (Luenemann et al., 2002, Song et al., 2000). The responsible mechanism is blockage of calcium influx through store-operated calcium release activated calcium channels (CRACs), of IFN-γ/ IL-4 production, and of proliferation. The latter can be attributed to the down-regulation of the cyclin-dependent kinase 4, indicating a G1 arrest of the cell cycle (Luenemann et al., 2002). Interestingly, ligands of the TNF superfamily may themselves transmit signals to the cell after engaging their receptors.

On the other hand, i.p. injection of soluble neutralizing TRAIL receptor leads to exacerbation of murine collagen-induced arthritis (Song et al., 2000), autoimmune diabetes (Lamhamedi-Cherradi et al., 2003), and EAE (Hilliard et al., 2001). Confirming the regulatory of peripheral TRAIL, in MS, an important link between TRAIL expression in peripheral blood mononuclear cells from untreated patients and a potentially successful immunomodulatory therapy has been shown. In this study, gene expression was quantified longitudinally by real time-PCR of the peripheral immune cells of 82 patients with definite MS. The patients were treated with IFN-β for one year and evaluated clinically whereas a subgroup of them was also monitored by MRI for one year. Patients who had no further relapses and no deterioration in the EDSS score during IFN-β treatment were defined as drug first-year responders (n=20), whereas patients who continued to have one or more relapses were defined as first-year non-responders (n=19). After analysis of the data, could be shown that clinical responders to IFN-β treatment can be distinguished from non-responders by early and sustained induction of TRAIL mRNA in peripheral immune cells (Wandinger et al., 2004).

A further study reported that activated T cells from IFN-β-treated MS patients express higher levels of TRAIL than untreated patients, supporting the view that in vivo exposure to IFN-β modulates the TRAIL system in MS (Arbour et al., 2005).

Lastly, in unpublished data from our lab, it has been shown that NK cells from 15 MS patients up-regulated TRAIL expression less than NK cells from healthy donors after IFN-β incubation (Figure 6) (statistical significance: SPSS, Mann-Whitney Test: 10U/ml: p=0,040; 1000U/ml: p=0,033). This experiment may imply a role of TRAIL on NK cells in MS which was investigated in our experiments in the mouse system.

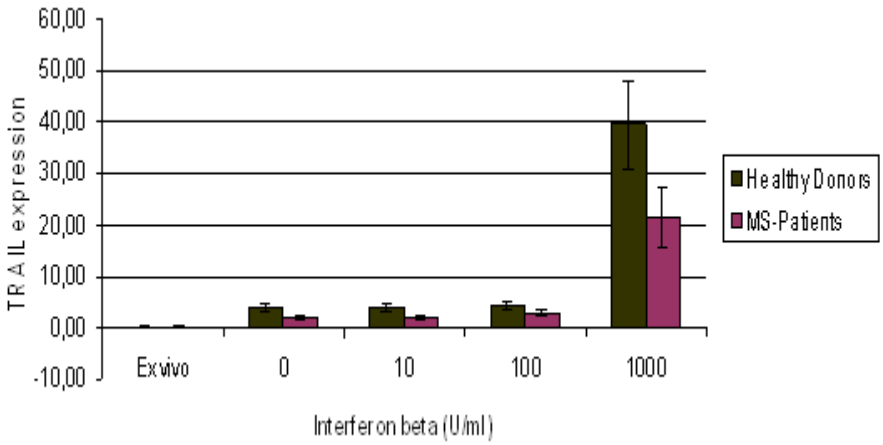


Figure 6 Comparison of TRAIL expression in NK cells of healthy donors and MS-patients
Taken from R. Horland, Diploma thesis 2005, unpublished data

2. Materials and methods

2.1 MATERIALS

2.1.1 Cell culture media and buffers

0,2M PBS (phosphate buffered saline)	0,0109M $NaH_2PO_4*H_2O$ (Merck) 0,081M Na_2HPO_4 * "H_2O(Merck) pH 7.2-7.4
Freezing medium	90% FCS (Biochrom AG) 10% DMSO (Sigma)
Mouse medium (MM)	RPMI 1640 (GibcoBRL) + 1% 1M Hepes buffer (GibcoBRL) 10% FCS 1% L-Glutamine (GibcoBRL, 200mM) 1% Penicillin- Streptomycin (Sigma, 10 mg/ml) 0.5ml β-Mercaptoethanol (β-ME) (Serva) in 500ml medium (from a 1:280 β-ME dilution)
Lysis buffer	0.15M NH_4Cl (Merck) 10mM $KHCO_3$ (Merck) 0,1M $Na_2EDTA*2H_2O$ (Merck) in distilled water pH 7.2 – 7.4
Washing medium (WM)	RPMI 1640 + 1% 1M Hepes buffer, 5% FCS 1% Penicillin- Streptomycin
MACS buffer	0.5% Bovine serum albumin (BSA) (Serva) 2 mM EDTA Buffer is kept at 4−8 °C PBS pH 7.3-7.4
FACS buffer	1x PBS 0.5% BSA
4 % Paraformaldehyd (PFA)	0,1M PBS 4% PFA pH 7.2-7.4
Defreeze medium	RPMI 1640 + 1% 1M Hepes Buffer 10% FCS 1% Penicillin-Streptomycin
Iscove's Modified Dulbecco's Media (IMDM)	GibcoBRL
DPBS GIBCO™ Dulbecco's Phosphate-Buffered Saline 1x + Mg^{2+} + Ca^{+2}	GibcoBRL

2.1.2 FACS Surface Antibodies

Antibody	Clone	Concentration	Dilution (v/v)	Source
Anti-Fc receptor	-	0,5 mg/ml	1:100	BD Bioscience
CD3e-APC	145-2C11	0,2 mg/ml	1:600	BD Bioscience
NK1.1-FITC	PK136	0,5 mg/ml	1:200	eBiosciences
NK1.1-biotin	PK136	0,5 mg/ml	1:600	BD Bioscience
Streptavidin (SA)-PerCP	-	0,2 mg/ml	1:600	BD Bioscience
TRAIL-PE	N2B2	0,5 mg/ml	1:100	BD Bioscience
CD11b-APC	M1/70	0,2mg/ml	1:1000	BD Bioscience
CD11c-biotin	H23	0,5mg/ml	1:600	BD Bioscience
CD86-PE	B7	0,2mg/ml	1:400	BD Bioscience

2.1.3 Cytokines and enzymes

Cytokine	Concentration	Source
Interferon- β	4 Million Units/ml	-
Interleukin -2	10000U/ml	Biotest
Collagenase	363U/mg stock	Sigma Aldrich Clostridium Histolyticum

2.1.4 Buffers and antibodies for Immunostaining

Blocking Buffer (BBG)

Ingredients	**stock**
5% NGS (goat)	5% NGS (normal goat serum)
1% BSA	10% BSA (bovine serum albumin)
0,2% Triton X100	10% TritonX100
PBS	1x
Filtration (0,45 um filter)	

Washing Buffer (PBS-T)

Ingredients	**stock**
1% BSA	10% BSA
0,1% Triton X100	10% Triton X100
PBS	1x

Antibodies

Antibody	**Dilution**
Hoechst staining	1:1000
Goat anti-mouse(Alexa 488)	1:500
FluoroMyelin (Invitrogen)	1:100
anti-GFP antibody (Invitrogen 3E6)	1:1000

2.1.5 Reagents and Chemicals

Reagents-Chemicals	Concentration	Source
Trypan blue solution	0.4% solution	Biochrom AG
Pertussis toxin (PTX)	50µg/ml	List Biological laboratories
Complete Freund's adjuvant (CFA)	-	Difco
Mycobacterium tuberculosis H37RA (killed and desiccated)	-	Difco
MOG $_{35-55}$ peptide (Met-Glu-Val-Gly-Trp-Tyr-Arg-Ser-Pro-Phe-Ser-Arg-Val-Val-His-Leu-Tyr-Arg-Asn-Gly-Lys)	5mg/ml	Pepceuticals
Ketamine	50mg/ml	Delta Select
Rompun (xylazine hydrochloride)	2%	Bayer
Methylbutan	100%	Roth
NaCl solution	0.9%	Braun
Ethanol	70%	Herbetta Arzneimittel

2.1.6 Consumables and instruments

Consumables	Instruments
Dako Pen	FACS Canto (BD Biosciences)
Tissue-Tek O.C.T. (Qiagen)	RT-PCR machine (Eppendorf Master Cycler)
Liquid Nitrogen	Taq Man Machine ABI Prism AB Biosciences
Alluminium foil	Microscope (Hund Wetzlar)
Neubauer Counting Chamber	Incubator (Binder)
MACS-separation columns (LS) (Miltenyi Biotec)	Pipetus (Hirschmorn Laborgeräte)
Needles (BD Microlance)	Pipettes (Gilson / Eppendorf)
Petri dishes (Becton Dickinson)	Clean Bench (Heraeus)
FACS tubes (Sarstedt)	Vortex (Bender and Hobun AG)
qPCR plate 96 well (Peqlab)	Cryostat (Leica)
Water DNAse and RNAse free (GibcoBRL)	

2.1.7 Kits and primers

1. NK Isolation Kit for mouse
Miltenyi Biotech GmbH (Order No. 130-090-864)
2. E.Z.N.A Total RNA kit I
from Omega Bio-Tek, # R6834
3. Reverse Transcriptase PCR Reaction
(Applied Bio systems) which contains:

RT PCR buffer	2x
$MgCl_2$	25mM
dNTPs Nucleotides	10mM
Random Hexamers	50mM
RNase inhibitor	20U/µL
Reverse transcriptase Multiscribe^{TM}	50U/µL

4. TAQ MAN Analysis

Buffer qPCR Master mix (+ dNTPs and MgCl2)	2x
Primers TRAIL forward/reverse	10 pmol/µl
TaqMan Probe TRAIL specific (5'end FAM fluorescence, 3'end TAMRA silencer)	5 pmol/µl

5. Primers and probes for PCR reactions (MWG Biotech AG)

The parameters for the design of the suitable pair of primers and of the probe were:

Melting temperature (Tm)	58°C-60°C (59 °C)
GC content %	30%-80%
Length of product	9-40 nucleotides
Tm of Taq Man probe	10°C higher (no guanine at '5 end)
PCR product size	50-150 nucleotides

Mouse **GAPDH** primers and probe

Forward primer	CTC AAC TAC ATG GTC TAC ATG TTC CA
Reverse primer	CCA TTC TCG GCC TTG ACT GT
Probe	TGA CTC CAC TCA CGG CAA ATT CAA CGT

Mouse **TRAIL** primers and probe

Forward primer	GAT CAC TCG GAG AAG CAA CTC A
Reverse primer	GAG AGG ACT CCC AGG ATT CAA TC
Probe	CAA TCT CCA AGG ATG GAA AGA CCT TAG GCC A

Mouse **Mx1** primers and probe

Forward primer	CCC AGC ACC TGA AAG CCT ACT
Reverse primer	CCA AAT GTT TTC AGG ATG AAG TAC TG
Probe	SYBR Green (Invitrogen)

2.1.8 Animal models

1. C57BL/6 mice

Source: Charles River Laboratories, FEM (Forschungseinrichtung für Experimentelle Medizin)

The C57BL mouse was originally derived by Little in 1921. Today, it is one of the most widely used and most popular strains in both animal research and the development of mutant mouse lines.C57BL/6 is classified as an inbred, which results from a crossing of no less than 20 consecutive generations of brother-sister mates. This allows the offspring to possess both genetic and phenotypic uniformity.

C57BL/6 has certain immunophenotypes that distinguish it from other inbred strains like BALB/c. Firstly, the immunological response to the same pathogen in C57BL/6 mice is often of an opposite spectrum compared to BALBb/c mice, namely the C57BL/6 mouse shows T_H1 and the BALB/c mouse shows a T_H2 response in response to intracellular pathogen Leishmania major, where a T_H1 response results in a resistant phenotype (since the pathogen is intracellular), whereas a T_H2 response results in a susceptible phenotype. The strains also differ in their macrophages' ability to be activated, as measured from their arginine metabolic programs when stimulated by IFN-γ or LPS or both:

- M-1 macrophages from typical T_H1 responders: C57BL/6 or B10.D2 mice, preferentially produce nitric oxide (NO) by action of inducible Nitric Oxide synthase (iNOS)
- M-2 macrophages from typical T_H2 responders: DBA or BALB/c mice preferentially produce ornithine and urea by action of arginase. (Mills et al., 2000)

2. C57BL/6 - EGFP mice

Source: F.E.M. (Forschungseinrichtung für Experimentelle Medizin)

This transgenic mouse line with an "enhanced" GFP (EGFP) cDNA under the control of a chicken β-actin promoter and cytomegalovirus enhancer makes all of the tissues, with the exception of erythrocytes and hair, appear green under excitation light. Note that mice homozygous for this transgene die within the first two weeks following birth.

2.2 METHODS

2.2.1 In vitro experiments

2.2.1.1 Mice spleen cells isolation / Counting cells

Mice were sacrificed by cervical dislocation and the fur was disinfected with 70% Ethanol. Through a medial vertical cut, the abdominal cavity was opened so that the peritoneum was visible. With new disinfected instruments, the peritoneal cavity

was opened and the spleen was removed from the surrounding fatty and connective tissues. Then, the spleen was kept in ice cold washing medium.

All procedures of cell preparation and handling, which are described from now on were performed in sterile conditions under a laminar flow hood and carried out under strict aseptic conditions.

In a Petri dish, the organ was meshed through a cell strainer. The cell strainer was rinsed 2-3 times with WM, to release any remaining cells. The resulting cell suspension was transferred to a 50ml Falcon tube and filled up to 50 ml with WM. The cell suspension was centrifuged at 500g at 4^{o}C for 5 min. Afterwards, The supernatant was discarded and the pellet was resuspended in an appropriate amount of MACS buffer.

The cells were counted was performed using a Neubauer Counting Chamber. A 1:10 dilution of the cell suspension with Trypan Blue (blue staining substance which allows the discrimination of dead and living cells as it passes through the membrane of dead cells and therefore stains them blue) was made. The chamber was placed underneath the microscope at a 100fold magnification and the living non-blue cells were counted. The formula used to calculate the cell number in the whole volume is as follows:

$$N = n * DF * V(ml) * 10.000$$

,wherein N is the resulting cell count, n the mean of cells counted in the quadrants, DF the dilution factor of the cell suspension with Trypan Blue and V the volume of the cell suspension.

2.2.1.2 Magnetic associated cell sorting (MACS)

Principles

Magnetic Associated Cell Sorting (MACS) is a method for isolating a desired cell population from a cell suspension. Its principle is based on antibody binding on cell surface molecules specific for each cell population. These antibodies can be conjugated with microbeads (which remain in the magnetic field) or with biotin. In the first case, passing the cells through a column attached to a magnet can separate a positively microbead-labelled fraction, which will be attached to the column from a second negative fraction that will pass through the column.

If the antibodies are biotin conjugated, a second labelling step will follow with an anti-biotin antibody conjugated with microbeads. Then the same magnetic separation will follow. The desired cell population can be the positive fraction (microbeads labelled cells- positive selection) or the negative untouched fraction (negative selection). The selection of one of the two methods is important and depends on the purpose of the experiment. Negative selection methods are preferred if the cells need to be ‘untouched’ or non-activated.

Method

The NK Cell Isolation Kit is an indirect magnetic labelling system for the isolation of untouched NK cells from suspensions of murine spleen and lymph node cells. Non-NK cells, i.e. T cells, dendritic cells, B cells, granulocytes, macrophages and erythroid cells are indirectly magnetically labelled by using a cocktail of biotin-

conjugated antibodies against CD4 (L3T4), CD8a (Ly-2), CD5 (Ly-1), CD19, Ly-6G (Gr-1) and Ter-119 as primary labelling reagent, and Anti-Biotin microbeads as a secondary labelling reagent. The magnetically labelled non-NK cells are depleted by retaining them on a MACS Column in the magnetic field of a MACS Separator, while the unlabeled NK cells pass through the column (negative selection).
For the isolation of NK cells from spleen or LN, the removal of red blood cells by lysis or density gradient centrifugation is not necessary since the biotin- antibody (Ab) cocktail contains a red blood cell specific antibody (Ter-119).

Procedure

1) Magnetic Labelling

The number of splenocytes was determined and the following protocol was performed:
All following procedures were performed on ice as higher temperatures lead to non-specific cell labelling.
1. The cells were centrifuged at 500g for 5 min and the supernatant was removed completely
2. The cell pellet was resuspended in 40 µL of MACS buffer (MB) per 10^7 cells
3. 10 µL of biotin- antibody cocktail were added per 10^7 cells and the suspension was mixed well and incubated for 10 minutes at 4–8°C
4. 30 µL of MB and 20 µL of anti- biotin microbeads per 10^7 cells were added, the cells were mixed well and incubated for additional 15 min at 4–8 °C
5. The cells were washed in 50mL of MB and centrifuged at 500g for 5 min. The supernatant was removed completely and the cells were resuspended in 500 µL of MB per 10^8 cells

2) Magnetic Separation

The appropriate MACS Column according to the number of labelled leukocytes and the number of total leukocytes (Table 5) was chosen and the separation was performed according to the following:

Column	Max. number of labelled cells	Max. number of total cells
MS	10^7	2×10^8
LS	10^8	2×10^9

1. The column was placed in the magnetic field of a suitable MACS separator attached to a metal stand. A pre-separation filter was placed on the column to ensure that remaining clumps will not pass through as they can interfere with the sorting process due to obstruction of the flow and reduction of the purity of the sorting procedure
2. The column was prepared by rinsing it with the appropriate amount of buffer which is 500 µL for MS columns and 3 mL for LS columns
3. The splenocyte suspension was applied onto the column
4. The unlabeled NK cells which pass through the column were collected in a 50 ml Falcon tube and the column was washed with 9 mL (for LS columns) or 1,5 mL (for MS columns) of MB. The washing steps were performed by adding buffer three times, each time once the column reservoir was empty i.e. for MS columns : 3×500 µL For LS columns : 3×3mL

The entire effluent was collected as this represents the desired enriched NK cells fraction. Lastly the cell number was determined according to the above described method.

2.2.1.3 Evaluation of MACS sorting purity

Principles

Flow cytometry is a technology that simultaneously measures and then analyses multiple physical characteristics of single particles, usually cells, as they flow in a hydro dynamically focused stream of fluid through a beam of light. The properties measured include a particle's relative size, relative granularity or internal complexity, and relative fluorescence intensity. These characteristics are determined using an optical-to-electronic coupling system that records how the cell or particle scatters incident laser light and emits fluorescence.

As cells pass through the beam of light the particles change the course and intensity of the beam which is detected by different detectors, one in line with the beam (forward scatter- FSC) and several perpendicular to the light (side scatter- SSC) and fluorescence detectors). FSC correlates with the cell volume, SSC with internal complexity (relative granularity, cell nucleus complexity). Cell type specific surface or cytoplasmic molecules can also be marked with fluorescence conjugated antibodies. Each fluorochrome has a specific absorption spectrum and a specific emission spectrum, which is detected by specific detectors and is proportional to the fluorescence intensity. In that way information about the size, relative granularity or internal complexity and surface molecules of a cell population can be acquired.

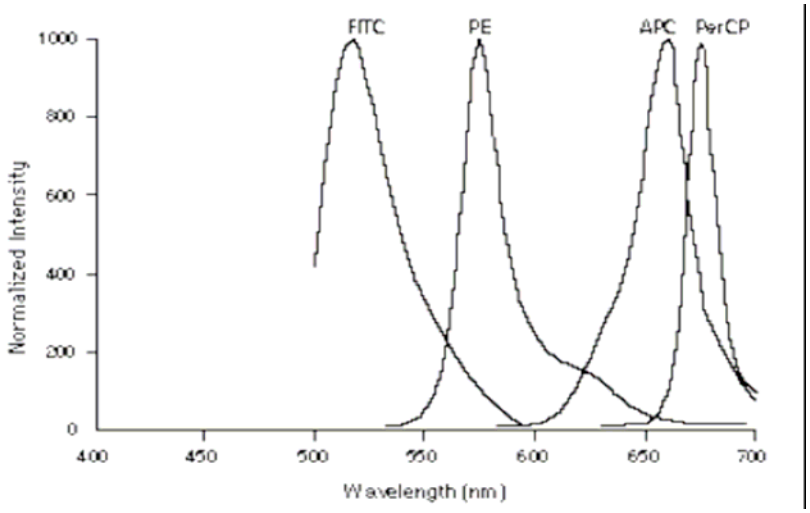


Figure 7 Emission spectra of the four most common types of fluorescence
FITC = fluorescein isothiocyanate, PE = phycoerythrin, PerCP = peridinin chlorophyll protein, APC = allophycocyanin

As is presented in Figure 7 the emission spectra of the four most common fluorescence overlap. These overlapping areas should be kept in mind in cases of multiple staining protocols because overlapping fluorescence emission spectra interfere with the accuracy of the detection method.

Method

For the evaluation of NK cell purity 200.000 sorted untouched cells were stained according to the following:

- The cells were resuspended in 1ml FACS buffer (FB), centrifuged at 550g at 4°C, for 5min and the supernatant was aspirated. This procedure was repeated once
- The cell suspension was blocked with Fc-receptor antibody for 10 min at 4°C to reduce unspecific staining
- A master mix of the NK1.1 FITC and CD3-APC in the dilutions described in the Materials FB was prepared. The end volume of incubation was 100µL for each sample
- Samples were incubated with the Ab mix for 15 min at 4°C
- Samples were washed twice with FB and each pellet was resuspended in 300 µL FB
- Samples were transferred to the FACS tubes and measured on a FACS Canto

2.2.1.4 NK cell incubation

To investigate TRAIL gene expression, NK cells were incubated in a 48 well plate (1ml mouse medium (MM) /well) at 37°C, pCO_2 5, 0% in a Binder sterile incubator with the following cytokines at a concentration of 1Mio cells/ml:

16h in culture following 2h incubation with:	18h incubation with:
no cytokines	no cytokines
500U/ml IFN-β	500U/ml IFN-β
1000 U/ml IFN-β	1000 U/ml IFN-β
1000U/ml IL-2	1000U/ml IL-2

2.2.1.5 Evaluation of TRAIL expression on lymphocytes using FACS analysis

An additional experiment was performed to investigate the surface expression of TRAIL on lymphocytes from wt C57BL/6 mice. For this purpose splenocytes were isolated and incubated at a concentration of 3millions (Mio) cells/ml according to the following layout:

18h incubation	48h incubation	4d incubation	6d incubation
no cytokines	no cytokines	no cytokines	no cytokines
IFN-ß 100U/ml	IFN-ß 100U/ml	IFN-ß 100U/ml	IFN-ß 100U/ml
IFN-ß 500U/ml	IFN-ß 500U/ml	IFN-ß 500U/ml	IFN-ß 500U/ml
IFN-ß 1000U/ml	IFN-ß 1000U/ml	IFN-ß 1000U/ml	IFN-ß 1000U/ml
IFN-ß 5000U/ml	IFN-ß 5000U/ml	IFN-ß 5000U/ml	IFN-ß 5000U/ml
IL-2 2000U/ml	IL-2 2000U/ml	IL-2 2000U/ml	IL-2 2000U/ml

Afterwards, the cells were harvested from the culture and stained according to the following:

- Cells were resuspended in 1ml FB, centrifuged at 550g at 4°C, for 5 min and the supernatant was aspirated. This procedure was repeated twice.
- The cell suspension was blocked with Fc-receptor antibody for 10 min at 4°C to reduce unspecific staining.
- A Master Mix of the NK1.1-FITC, CD3-APC and TRAIL-PE in FB was prepared calculating for 100µL as the end volume of incubation for each sample.

- Samples were incubated with the Ab mix for 15 min at 4°C
- Samples were washed once with FB and each pellet was resuspended in 300 µL FB
- Samples were transferred in FACS tubes and measured on a FACSCanto

2.2.1.6 RNA isolation and evaluation of RNA concentration

Principles

RNA Isolation methods are based on cell outer and inner membrane lysis and release of nucleic acids. RNA is then retained in the final elution by β-Mercaptoethanol (β-ME).

Method

The whole procedure was performed under a fume hood and all materials and solutions used were strictly only for RNA Isolation and each time they were disinfected with RNAse removal solution.

- NK cells were harvested after in vitro incubation, centrifuged at 550g at 4^{o}C, for 5min and the supernatant was aspirated. This procedure was repeated twice.
- 350µl lysis buffer containing 20µl/ml β-ME (7µl per sample) was added to the pellets and then they were resuspended with an insulin syringe gauge needle to ensure the membranes lysis.
- 350 µl of 70% ethanol was added and mixed thoroughly with the lysed material. Samples were applied to labelled spin columns.
- Samples were centrifuged at 14000 rpm (maximum g) for 1 minute.
- Flow through was discarded. Columns were washed with 500µl RNA wash buffer I and samples were centrifuged at 14000 rpm (maximum g) for 1 minute.
- The collection tubes were emptied. Columns were washed with 500µl RNA wash buffer II. Samples were centrifuged at 14000 rpm (maximum g) for 1 minute.
- Collection tubes were emptied and the previous step was repeated twice.
- Columns were placed on new collection tubes. Samples were centrifuged at 14000 rpm (maximum g) for 1 minute.
- Collection tubes were emptied and columns were placed on labelled 1,5ml Eppendorf tubes. 50µl Diethyl pyrocarbonate treated (DEPC), RNase free water at a temperature of 50^{o}C was pipetted onto the column and samples were centrifuged at 14000 rpm (maximum g) for 1 minute.
- Columns were discarded and RNA concentration was assessed using a Nano Drop counter

Nano Drop counter

The principle of the Nanodrop evaluating of RNA concentration is calculating the absorbance of the sample in 260nm and 280nm. According to these two measurements the RNA concentration can be calculated. The purity of the RNA sample is optimal when the fraction OD260/OD280 is between 1, 8-2,0.

For baseline setting, 2µL of the water used to resuspend RNA was placed at first on the counter. The absorbance of this sample was adjusted to 0. Subsequently, 2µl of the RNA solution were applied on the Nanodrop counter and the concentration was measured (µg/ml).

Reverse transcriptase reaction (RT-PCR) followed.

2.2.1.7 Reverse Transcriptase Polymerase Chain reactions

Principles

The isolated mRNA undergoes reverse transcription to cDNA using the viral enzyme reverse transcriptase, deoxyribonucleotides (dds) and random hexamers (a chain of 6dd, complementary to areas of the transcribed chain), from which reverse transcription begins in the presence of Mg^{2+} and RNase inhibitors to protect RNA from degradation.

Method

A Master Mix was created for all samples plus one according to the table, which corresponds to one sample when RNA quantity is 100ng and the total volume of each sample is 25μl. The above characteristics were adjusted according to the quantity and concentration of the RNA sample available.

10x Puffer	2,5 μl
25mM MgCl	5,,5 μl
dNTPs	5 μl
Random Hexamers	1,25 μl
RNase Inhibitor	0,5 μl
RT	0,625 μl
Total	25 μl
+	
total RNA =	100 ng

Initially, the master mix volume was placed in a 1,5 ml Eppendorf tube and then the desired quantity of RNA sample was added so that the final concentration for all samples is the same.

The samples were placed in an Eppendorf Mastercycler Gradient and the following program was used:

Step	Temperature	Time
1	25°C	10 minutes
2	48°C	45 minutes
3	95°C	5 minutes

cDNA was stored at -20°C until further processing.

2.2.1.8 Quantitative Polymerase Chain reaction

Principles

PCR reaction exploits the 5′ nuclease activity of AmpliTaq Gold DNA polymerase to cleave a Taq-Man probe during PCR.

The Taq-Man probe contains a reporter dye at the 5′ end of the probe and a quencher dye at the 3′ end of the probe. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence.

During the reaction, if the target of interest is present, the probe specifically anneals between the forward and reverse primer sites. The cleavage of the probe by the polymerase separates the reporter dye and the quencher dye, which results in increased fluorescence of the reporter. The probe fragments are then displaced from the target, and polymerization of the strand continues. The 3′ end of the probe is blocked to prevent extension of the probe during PCR.

This process occurs in every cycle and does not interfere with the exponential accumulation of product. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye. Figure 5 below

shows the forklike-structure-dependent, polymerization associated 5´ to 3´ nuclease activity of Ampli-Taq Gold enzyme during PCR.

A Taq-Man plate reader both initiates the reaction and reads the signal resulting from that. With every cycle the amount of cDNA multiplies and the cumulative signal from the probes intensifies until it reaches a threshold level where it can be distinguished from background fluorescence. From then, the signal intensifies rapidly until a plateau is reached. The data used from a quantification assay is the cycle number of the moment the threshold lever is reached, the so called Cycle threshold (Ct) (Figure 8).

Using this, an absolute or relative expression can be calculated. Relative expressions are determined between the target gene and a housekeeping gene.

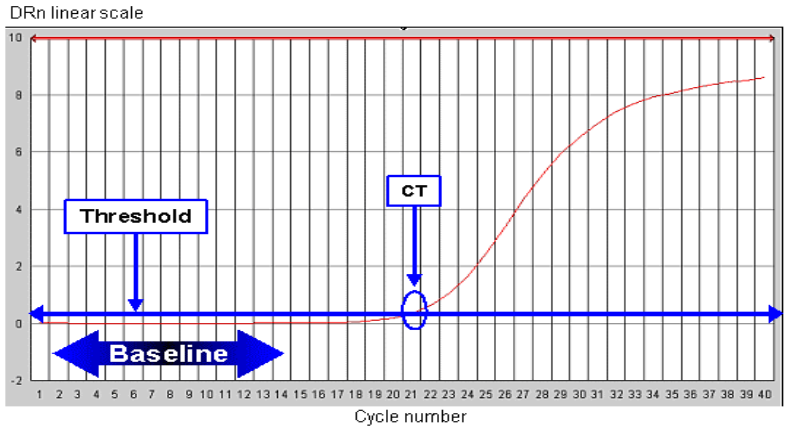


Figure 8 Graph of signal increase for one sample in a Taq-Man PCR

Method

Taq-Man PCR was performed on cDNA to calculate relative gene expression. Every sample was detected for each gene in duplicate.

-The housekeeping gene with a standard expression in all cells, which was used for the comparison to TRAIL expression, was Glycerinaldehyd-3-phosphate-Dehydrogenase (GAPDH). GAPDH is a glycolytic enzyme, which catalyses the reaction of the Glycerinaldehyd-3-Phosphate to 1, 3-Bisphosphoglycerate.

-Mx1 gene is known to be induced by IFNβ but not by IL-2 and it was used as a positive control of IFN-β treatment.

Per gene a Master Mix was made for the amount of reactions performed according to the following tables. For TRAIL and GAPDH the quantities used were the same whereas for Mx1 they were different. For every reaction, 20µL (TRAIL/GAPDH) or 16 µl (Mx1) Master Mix was pipetted into a qPCR 96 well plate under sterile conditions and with filtered tips. 4 µl cDNA were added.

Reagents (TRAIL/GAPDH)	**µL**	**Reagents Mx1**	**µL**
Buffer	12,5	Buffer	5
Forward Primer TRAIL or GAPDH	1	Forward Primer Mx1	1
Reverse Primer TRAIL or GAPDH	1	Reverse Primer Mx1	1
TaqMan probe TRAIL or GAPDH	1	SYBR Green	5
Water	4,5	Water	4
Total volume pro sample	20	Total volume pro sample	16

The plate was centrifuged and then run in an AbiPrism 7000 Sequence Detection System from Applied Bio systems with the following program:

Step	Temperature	Time
1	50°C	2 minutes
2	95°C	10 minutes
3 (×40 times)	95°C	15 seconds
4	60°C	1 minute

After the run was completed, Cycle threshold (Ct) and baseline were set according to the following:
- Manual baseline was set in an effort to minimize the distance between the two graphs for each sample. The minimum cycle has to be 2 cycles before the start of the straight horizontal line and the maximum cycle should be 2 cycles before the start of the straight climbing line in the graph.
- Ct threshold was set at a different level for each gene which corresponds to the point at which the graph of the signal begins to increase

As each sample exists as a duplicate, this ensures the accuracy of Ct calculation.

To calculate the relative expression, data was exported to a Microsoft Excel file at a compatible format and further analyzed according to the following calculations:
Each two housekeeping Ct values were subtracted from every two Ct target gene values, creating four different ΔCt values, each of which is transformed into a relative expression value. The Mean of those values is then graphically plotted.

$$\Delta Ct = Ct\ target - Ct\ household$$

$$Relative\ expression = 2^{-\Delta Ct}$$

2.2.2 In vivo experiments

2.2.2.1 EAE induction and mice scoring

Principles

Experimental autoimmune encephalomyelitis (EAE) is a $CD4^+$ T cell–mediated autoimmune disease characterized by perivascular $CD4^+$ Thelper cell and mononuclear cell inflammation, subsequent primary demyelination of axonal tracks in the central nervous system (CNS) accompanied by neuronal loss and leading to progressive tail, hind-limb and front limb paralysis.

EAE provides a powerful model for the study of the pathogenesis and immune regulation of $CD4^+$ Th1/Th17–mediated tissue damage and is generally considered to be a relevant model for the human immune mediated demyelinating disease multiple sclerosis.

Actively induced EAE (**active EAE**) consists of an induction phase and an effector phase. The induction phase of the disease involves the priming of myelin epitope–specific $CD4^+$ T cells following immunisation with myelin proteins or peptides in complete Freund's adjuvant (CFA). The effector phase consists of multiple stages:
(1) Migration of activated myelin-specific T cells to the CNS, which involves extravasation of the T cells across the tight endothelial junctions comprising the blood-brain barrier;
(2) Elaboration of chemokines and cytokines by the myelin-specific T cells, which induce the influx of peripheral mononuclear phagocytes into the CNS parenchyma;
(3) Activation of peripheral monocytes/macrophages and CNS-resident microglial cells by T_H cell–derived cytokines; and
(4) Demyelination of CNS axonal tracts by the phagocytic activity of activated mononuclear cells and by the inflammatory and cytotoxic effects of cytokines (e.g., IFN-γ, LT/TNF-β, IL-17, TNF-α and NO) released from activated $CD4^+$ T cells and monocytes.

The effector phase of the disease is modelled by the adoptive transfer model of EAE (**passive EAE**) in which disease is induced by the peripheral introduction of a preactivated population of myelin epitope–specific $CD4^+$ T cells to a naive mouse.

In the SJL (H-2s haplotype) mouse, the disease is characterized by a relapsing-remitting course of paralysis, which allows assessment of the efficacy of various immunoregulatory strategies in a progressive autoimmune disease setting. In C57BL/6 (H-2b haplotype) mice, the disease displays a chronic-progressive clinical course. EAE is induced by immunisation with MOG_{35-55} in CFA. In this model the disease does not follow a diffuse pattern as in its human counterpart as the spinal cord and not the cortex is primarily damaged. The clinical symptoms of the disease are initially deficits of the long pyramidal motor neurons that control tail muscles followed by axonal muscles and lastly by hind and frontal leg controlling neurons.

Method

EAE was actively induced in C57Bl/6 mice. Mice received a s.c. injection in the four flanks (total volume 200µl/mouse) of an emulsion containing the MOG_{35-55} peptide (250µg/mouse, stock: 5000µg/ml) and Mycobacterium tuberculosis antigen **(**H37Ra, 800µg/mouse) in CFA and PBS.

On the same day, 4-6 hours after immunisation, the mice were injected i.p. with 400 ng of Pertussis Toxin (PTX) per mouse (stock 50µg/mL) in 200µl of PBS and this treatment was repeated 48 h later. Onset of paralysis was anticipated to occur 12-16 days after immunization. The mice were starting to recover from their symptoms after day 23 as they were in the chronic phase of the disease.

Following immunisation, from day 7 on, mice were monitored daily for clinical signs of EAE and given a score according to the following:
A numerical score is given according to the following:

Table 5 Clinical scoring of EAE mice

Clinical score	Clinical signs
0	no clinical signs
0,5	TPA or RRW
1	TPL
1,5	TPL and HPA or TPL and RRW
2	TPL and HPA and RRW
2,5	TPL and HPA and RRW and FPA
3	TPL and HPL and RRW
3,5	TPL and HPL and RRW and no clear FPA
4	TPL and HPL and RRW and a clear FPA
4,5	TPL and HPL and RRW and FPL
5	death

Clinical index used for the scoring of EAE mice
Abbreviations: TPA tail paresis, RRW* righting reflex, TPL tail plegia, HPA hind limb paresis, HPL hind limb plegia FPA frontal limb paresis, FPL frontal limb plegia

*Righting reflex: Any of the various reflexes that tend to bring the body into normal position in space and resist forces acting to displace it out of normal position. It can be provoked if the animal is being placed on its back or side whereas it returns to sternal recumbency. A normal reaction is dependent on normal vestibular, visual and proprioceptive functions.

2.2.2.2 MACS check and of i.v. injection

NK cells were sorted from transgenic EGFP C57Bl/6 mice as mentioned above for the in vitro experiments.

To check the purity of MACS sorting procedure 200.000 cells were stained according to the following:

- Cells were resuspended in 1ml FB, centrifuged at 550g at 4°C, for 5min and supernatant was aspirated. This procedure was repeated twice.
- The cell suspension was blocked with Fc-receptor antibody for 10 min at 4°C to reduce unspecific staining.
- A master mix of NK1.1-biotin and CD3-APC antibodies in FB was prepared calculating for 100µL as the final volume of incubation for each sample.
- Samples were incubated with the Abs mix for 15 min at 4°C
- Samples were washed once with FB
- A second incubation with Streptavidin (SA) PercP in a 1:600 dilution for 15 min in 4°C was performed. Four molecules of streptavidin conjugated with PercP, bind to each molecule of the biotinylated antibody NK1.1-APC which results to the amplification of the signal.
- Each pellet was resuspended in 300 µL FB
- The samples were transferred in the FACS tubes and measured on a FACS Canto

Half of the sorted NK cells were incubated with 1000U/ml IFN-β and the other half without IFN-β for 18 hours 37°C and 5% pCO_2 in a 48 well plate (1 ml/well) at a maximum concentration 1 million cells/ml.

After 18h the cells were transferred to a 15ml tube, filled up with 10ml PBS and centrifuged at 500g for 5min at RT. This procedure was repeated once. Then the

cells were resuspended in PBS at a concentration of up to 1million cells/100μL and 100 μL were injected intravenously in the tail vein of a mouse with an insulin syringe (27G needle).

2.2.2.3 Organ preparation

Induction of anaesthesia

On day 34-36 the mice were injected i.p. with 1 ml of a mix of the following substances:

- Ketamine (NMDA receptor antagonist inducing dissociative anesthesia and partly analgesia) 1,6%

- Rompun 2% (Xylazine, α_2 receptor agonist with a sedative, analgesic and antispasmodic effect); 0,83%

in NaCl 0.9% solution (for the dilution)

Removal of primary and secondary lymphoid organs and FACS analysis

Spleen and paraxial, inguinal and mesenterial lymph nodes were removed and the cells were isolated according to the procedure described for the in vitro experiments. For the spleen cells an extra lysis step was added as there is a high concentration of erythrocytes. Following a washing step the pellet was resuspended in 10 ml of lysis buffer and then 5ml of washing buffer were added. The suspension was centrifuged at 500g for 5min at 4 ^{o}C and one additional washing step with washing buffer followed as described above. Then the cells were counted.

Subsequently, spleen and lymph node cells were subjected to FACS analysis with the following antibodies, to investigate the immune status of the animals and check the presence of EGFP positive cells in these organs procedure. The staining procedure with CD3-APC and NK1.1-biotin was the same as the one followed for MACS check of NK cell purity.

For the DC staining performed only in the third transfer experiment the procedure was the same also with the difference that the antibodies used at the first staining step were CD11b-APC CD11c-bio and CD86-PE. The second step of staining was performed also with SA-PercP.

Perfusion technique and brain and spinal cord preparation

To enable the histological analysis of brain and spinal cord of the EAE mice that received the NK cells the mice were perfused in 4% Paraformaldehyd (PFA) solution after the removal of spleen and lymph nodes. PFA stops the autolytic process, stabilises the structures and kills all micro-organisms.

The thoracic cavity was opened by two vertical cuts upwards 3mm at the left and right of sternum. Then the sternum was fixated or cut so that the thoracic cavity remained open so that a needle could pass in the left ventricle of the heart and after making sure that the needle was at the right position a small cut on the right atrium was made. 40 ml of PBS were slowly injected in the left ventricle so that they removed the blood out of the vessel compartment of the whole body through the right atrium opening. Thereafter, 40 ml PFA 4% was passed slowly through the left ventricle to fixate all tissues.

Subsequently, the fixated brain and spinal cord (cervical, thoracic and lumbar area) were isolated from the surrounding tissues following a caudal approach and kept in 4% PFA overnight. The following day they were transferred in 1x PBS where they remained for up to one week and then they were dehydrated in 15% sucrose solution

for 24 hours and thereafter in 30% sucrose solution for another 24 hours. Afterwards the tissues were frozen.

2.2.2.4 Freezing of tissues

Cylindrical aluminum moulds were prepared for brain and envelope-like ones for spinal cord. The spinal cord was divided in a lumbar and thoracic-cervical part. The tissues were subsequently covered with Tissue-Tek O.C.T., a substance used for embedding tissue after fixation and before cryo-sectioning, which also maintained them in a straight and horizontal position. Tags containing information about the type of tissue and the mouse it came from were attached to the above moulds. The tissues were positioned in a glass containing 2-methylbutan and placed onto dry ice. The alcohol containing glass was cooled in liquid nitrogen to acquire a very low temperature and this step had to be repeated after 2-3 tissues. The tissue was held into the 2-methylbutan until the Tissue-Tek was completely frozen and then put on dry ice until it was stored in -80^{o}C.

Tissues were cut to 10μm slices for histological staining with the help of a cryostat.

2.2.2.5 Spleen digestion

Principles

Spleen digestion with collagenase was followed for the third experimental EAE because of its usefulness in the acquisition of dendritic cells from the collagen-stroma of the spleen.

Methods

The usual method of transcardial perfusion was followed as described above and then the spleen was removed and placed in 5ml of IMDM medium in falcon tube. Then the tube was placed in the water bath at 37^{o}C for 2-3 min so that the spleen could acquire the optimal temperature for collagenase. Subsequently, 50μl Collagenase was injected (5mg, 363U/mg stock) in 2-3 places in the spleen in a petri dish with some IMDM medium and then the spleen was cut into pieces and placed again in the tube. The mix was incubated for 30min at 37^{o}C whereas it was mixed every 5min.

After the incubation time, the spleen pieces were passed through a cell strainer for the acquisition of the cells and washed with WM. The normal procedure of spleen cells lysis was followed afterwards and then the cells were counted and stained with DCs markers.

2.2.2.6 Immunostaining

The slices of the brain and spinal cord of the mice were stained for GFP, myelin and nucleus proteins according to the following protocol:

Initially two 2ml aliquots of Blocking Buffer (BBG) and washing buffer (PBS-T) were prepared and frozen at -20^{o}C.

1. Tissue preparation:

- Tissues were dried for 1-12h in RT (all incubations were performed in the dark).
- The tissues were post-fixated for 5 min in 4% PFA also in RT on a shaker.
- Thereafter, the tissues were blocked for unspecific staining 1-2h with BBG

2. GFP staining:
   - 75µL/section of the mouse anti-GFP antibody was used in a 1:2000 dilution with PBS-T and for 3h in RT on a shaker.
   - The tissues were washed 3 times with PBS-T, each time for 5 min.
3. Secondary antibody, nucleus staining and co-staining with myelin were performed in the dilutions described in Materials in PBS-T
   - The tissues were incubated with 75µL/section (or overnight in 4°C) on a shaker.
   - Thereafter they were washed 3 times PBS each time for 5min.
4. Finally the slices were examined under the microscope

2.2.2.7 Confocal Laser Scanning Microscopy (CLSM)

Confocal Laser Scanning Microscopy (CLSM) is an optical microscopy technique that is based on conventional wide-field fluorescence microscopy. The use of modern technology (laser, photomultiplying detectors) in CLSM gives this method a lot of advantages. The laser beam is focussed into the sample and by using electronic lenses and apertures only the fluorescence light that comes directly from the confocal plane is detected by a photomultiplier. Interfering intensity from outside this plane is cancelled out by a pinhole. More precisely, the detected fluorescence intensity comes from a small space, the confocal point, and thus the sample plane has to be scanned to get a whole fluorescence image. Therefore it is possible to get spatial resolution and additionally very detailed intensity information not only in xy-direction but also in the z-direction. Furthermore the resolution of a CLSM is much better than of a common wide-field microscope.

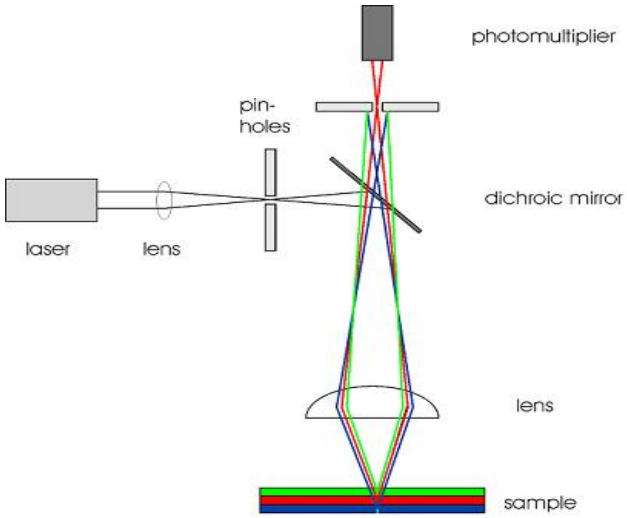


Figure 9 Setup of a confocal laser scanning microscope

3. Aims of the study

NK cells are becoming a focus of immunology research as they are proven to play an additional regulatory function apart from their direct effect on infectious agents and tumour cells. Their role in maintaining the balance between immune defence and autoimmunity in MS is implied by evidence linking their dysfunction to the clinical relapses (Takahashi et al., 2001) and by the fact that effective drugs such as daclizumab modulate their subpopulations (Bielekova et al., 2006).

TRAIL is an important effector and regulatory molecule for EAE, as it can directly attack neurons during inflammation in the CNS (Aktas et al., 2005) and inhibit proliferation of autoimmune T cells in the periphery (Zhang et al., 1997), respectively. Furthermore, in MS patients, an elevated level of serum TRAIL has been linked to the response to IFN-β treatment (Wandinger et al., 2004) whereas it seems that NK cells from MS patients express less TRAIL than healthy controls after stimulation with IFN-β (Infante-Duarte et al., unpublished data).

Therefore, this study aims to investigate the involvement of TRAIL and NK cells in the animal model of MS, which is simpler and easier to manipulate than its human counterpart. Firstly, we aim to prove whether the murine system presents equivalents to the human concerning TRAIL expression and regulation on NK cells. If similar expression patterns are identified, we will further elucidate the role of TRAIL expressing NK cells in influencing the course of EAE.

More specifically, this thesis consists of two parts.

1. The initial in vitro investigation aims to investigate which factors e.g. IFN-β, are able to modulate TRAIL-expression in murine NK cells at the gene and protein level. IFN- β is the worldwide standard for the therapy of MS and has been also proven effective for EAE. However the exact mechanisms of actions are still unknown.

2. Subsequently, the system of TRAIL expressing NK cells will be applied in vivo in an effort to investigate the role of transferred TRAIL- expressing $EGFP^+$-NK cells in EAE mice in modulating:

a) the clinical course (e.g. incidence, severity of disease)
b) the immune system populations in the lymphoid organs of mice and
c) the histopathology of EAE (presence of transferred cells in CNS or periphery, inflammatory lesions in CNS)

4. Results

4.1 In vitro Experiments

In the following experiments, we aim to investigate in vitro how TRAIL expression is regulated at the RNA and protein levels on NK cells. The only cytokines which according to publications are able to induce protein TRAIL on NK cells are IL-2 and IL-15 (Kayagaki et al., 1999). Therefore, for the first experiments we chose to establish a positive control with IL-2 that will serve as reference for the experiments assessing whether IFN-β affects TRAIL also in the murine system.

4.1.1 IL-2 induces TRAIL gene expression on splenocytes of C57BL/6 mice

IL-2 is a typical four - helix cytokine and is produced primarily by activated $CD4^+$ T cells, although expression by naive $CD8^+$ T cells, dendritic cells, and thymic cells has also been reported.

TRAIL protein has been detected and found out in vitro to mediate FasL independent cytotoxicity on Con-A/IL-2 activated T cells (Mariani et al., 1998) and on IL-2 (500U/ml) activated NK cells against tumour cells lines. On NK cells, TRAIL was up-regulated after 2 days and reached a peak after 5 days of incubation with IL-2 (Kayagaki et al., 1999).

Initially and according to the aforementioned evidence, up-regulation of the TRAIL gene by IL-2 was investigated after a shorter incubation time with two different concentrations (500U/ml and 1000U/ml). Splenocytes, enriched NK cells (MACS sorting, 66 % purity) and NK-depleted splenocytes fraction (non-NK cells) from wt C57BL/6 mice were incubated for 2 hours and 18 hours with 500U/ml and 1000U/ml of IL-2. Relative TRAIL gene expression compared to the GAPDH was investigated by Taq-Man analysis as described in the Material and Methods.

- ☞ As shown in Figure 10 IL-2 induces TRAIL gene expression on splenocytes at a concentration of 1000U/ml after 2h whereas there is a four fold increase after 18h. Different subpopulations of splenocytes up-regulate TRAIL whereas interactions between them may influence the extent of the induction.
- ☞ IL-2 was shown to induce TRAIL after 18h at a concentration of 1000U/ml on NK-depleted splenocytes fraction (non-NK cells) by 8-fold (Figure 11). In this fraction T cells B cells and macrophages could up-regulate TRAIL as has been reported before (Mariani et al., 1998).
- ☞ On the NK-enriched fraction of splenocytes an induction of TRAIL was seen at all concentrations and for both incubation periods used (Figure 12). The most potent concentration of 1000U/ml induced TRAIL by 5-fold after 2h incubation and by 5-fold after 18h incubation.

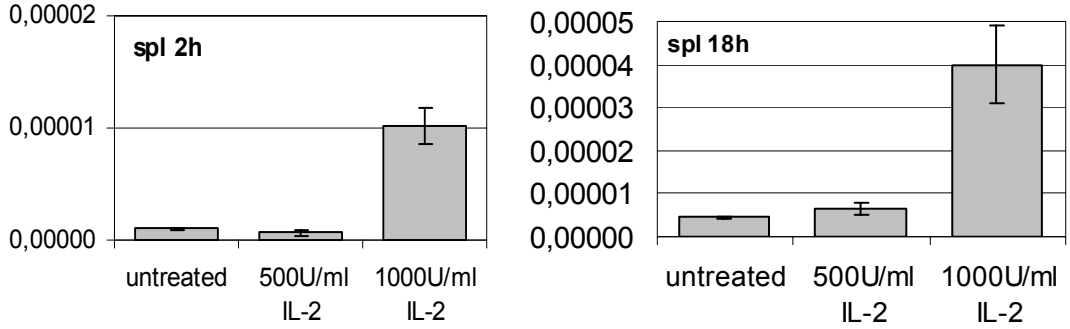


Figure 10 TRAIL gene induction on splenocytes after incubation with IL-2
Data derived from Taq-Man analysis. The values on the y axis represent relative expression of TRAIL gene to GAPDH. The error bars represent SEM. An induction of TRAIL is seen on splenocytes after 2 hours incubation with 1000U/ml IL-2 (spl 2h) and after 18 hours with the same concentration (spl 18h).

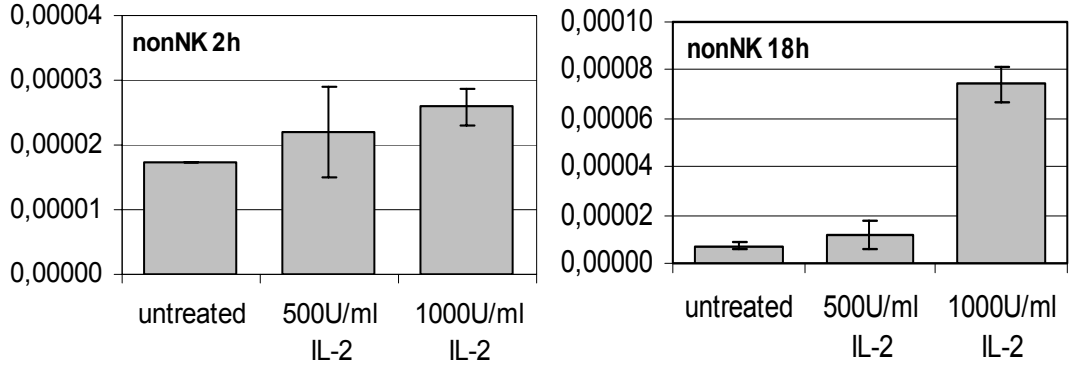


Figure 11 TRAIL gene induction on NK depleted splenocytes after incubation with IL-2
Data derived from Taq-Man analysis. The values on the y axis represent relative expression of TRAIL gene to GAPDH. The error bars represent SEM. An induction of TRAIL gene induction is seen only after 18 hours incubation with 1000U/ml IL-2 (non-NK 18h) whereas no induction is seen after 2 hours (non-NK 2h).

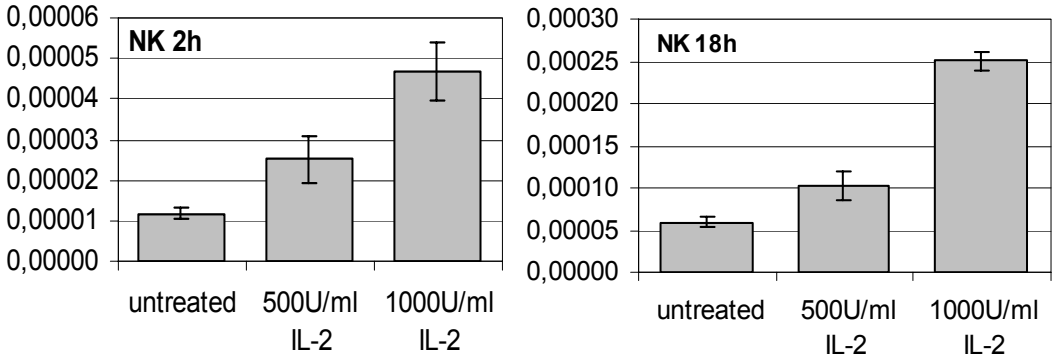


Figure 12 TRAIL gene induction on enriched NK cells after incubation with IL-2
Data derived from Taq-Man analysis. The values on the y axis represent relative expression of TRAIL gene to GAPDH. The error bars represent SEM. An induction of TRAIL gene is seen by both concentrations of IL-2 used after 2 hours (NK 2h) as well as after 18 hours (NK 18h).

4.1.2 IL-2 induces TRAIL protein expression on NK cells

Secondly, the induction of TRAIL at the protein level with IL-2 was investigated using FACS analysis with a PE conjugated TRAIL antibody. For this analysis, splenocytes were isolated and incubated for 2h and 18h with 2000U/ml. This higher concentration as in the gene expression experiments was used to check its effects. Then the cells were stained with CD3-APC, NK1.1-FITC and TRAIL-PE antibodies and FACS analysis followed (Figure 13).

The NK-1.1$^+$ antibody reacts with the NK-1.1 surface antigen encoded by the Klrb1c/NKR-P1C gene expressed on NK cells in selected strains of mice (eg, C57BL SJL) and the antigen encoded by the Klrb1b/NKR-P1B gene expressed on SJL mice, but not on C57BL/6. CD3$^+$ is a mature lymphocyte marker and forms a complex with TCR.

Of the whole splenocytes population depicted on FSC-SSC dot plot, the lymphocytes were gated (P1 gate) and their subpopulations are depicted on CD3-APC-NK1.1-FITC dot plot. The quadrants were set for the three different subpopulations: CD3$^+$ cells (mature lymphocytes) NK1.1$^+$ (NK cells) and CD3$^+$NK1.1$^+$ cells (NK-T cells). For each subpopulation the TRAIL expression was depicted in relation to cell count. An example of a graph is shown in Figure 13 for NK cells (P2 gate). The Geometric mean of each distribution was calculated as this measure enables the calculation of distributions with negative values of expression. Geometric mean is similar to the arithmetic mean, except that instead of adding the set of numbers and then dividing the sum by the count of numbers in the set, *n*, the numbers are multiplied and then the *n*th root of the resulting product is taken.

Therefore, as it can be seen in the two graphs, there is a shift of the distribution to the right (more cells with a higher expression of TRAIL) after incubation with IL-2 compared to the untreated cells. The geometric mean can be used as a numerical expression of this shift as presented in Figures 14, 15 for all the four different subpopulations.

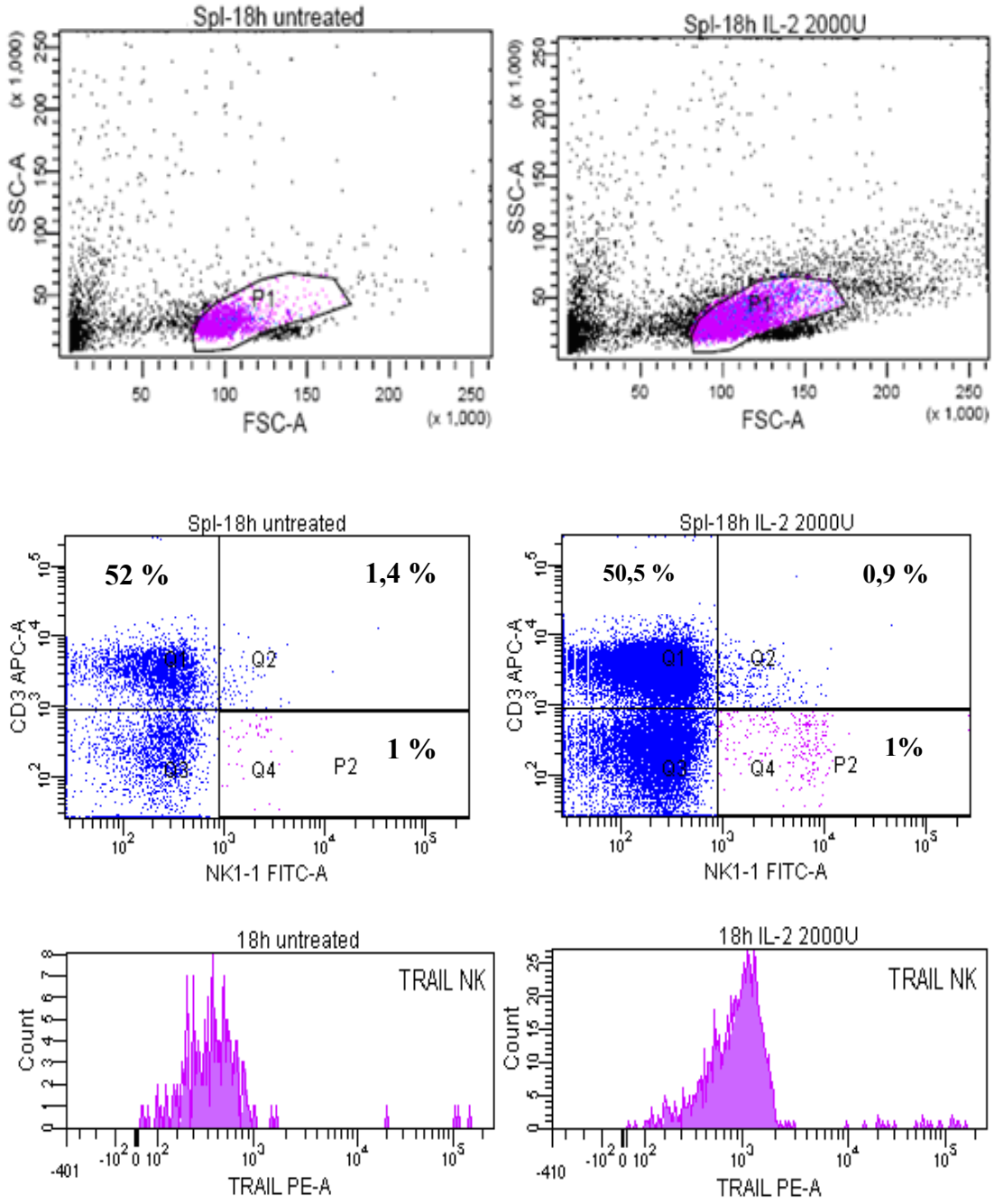


Figure 13 TRAIL surface expressions on untreated NK cells and on NK cells incubated with 1000U/ml of IL-2.

An example of the FACS analysis of TRAIL expression on wt C57BL/6 splenocytes after incubation for 2 and 18 hours with 2000U/ml IL-2 is depicted. The cells were stained with CD3-APC, NK1.1-FITC and TRAIL-PE antibodies. On the FSC-SSC dot blot (upper two plots) the lymphocyte population was gated and the percentages of three subpopulations (T cells, NK cells, NK-T cells) were calculated in the middle two plots for the cells that were kept in culture without and with IL-2 respectively. Each subpopulation was gated and the distribution of TRAIL expression in relation to the cell number is presented in the lower two graphs for the NK cells (P2 gate). There is a clear shift of the distribution to the right indicating a higher TRAIL expression after 18h incubation with IL-2. The geometric mean can be used as a numerical expression of this shift as presented in Figures 14 and 15.

In Figure 14 and Figure 15 the results of this analysis are depicted for each subpopulation analysed.

- ☞ After 2h with 2000U/ml IL-2 an induction of TRAIL was seen only on NK-T ($NK1.1^{+}CD3^{+}$) cells.
- ☞ After 18h with 2000U/ml IL-2 an induction TRAIL was seen for NK as well as for NK-T cells. Regarding NK-T cells the geometric mean of the untreated cells after 18h incubation was higher then that of the untreated cells after 2 h incubation which may imply an effect of the culture on TRAIL expression.

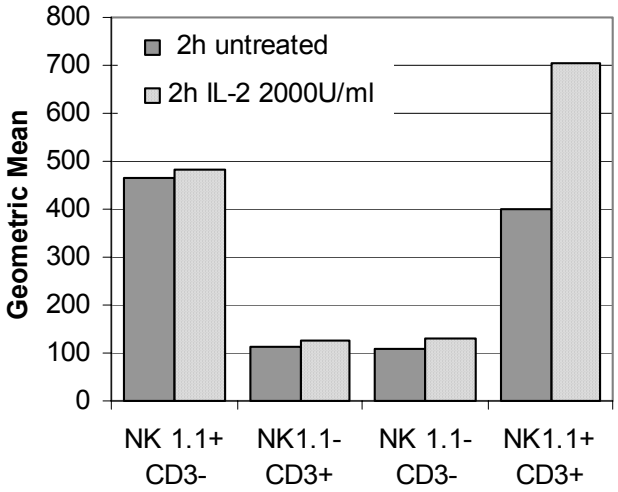


Figure 14 TRAIL surface protein expression after 2 hours incubation with IL-2
Data derived from FACS analysis. An increase of TRAIL protein is seen after 2 hours incubation with IL-2 on NK1.1+CD3+ cells.

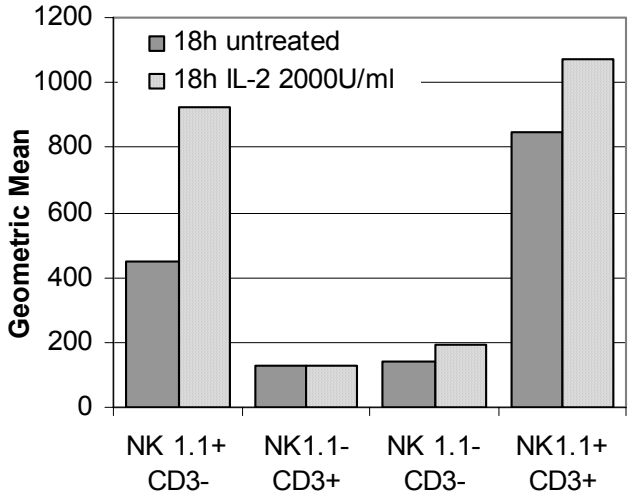


Figure 15 TRAIL surface protein expression after 18 hours incubation with IL-2
Data derived from FACS analysis. An increase of TRAIL protein is seen after 18 hours incubation with IL-2 on NK1.1-+CD3- cells and on NK1.1+CD3+

☞ Conclusively, from the aforementioned experiments it was shown that IL-2 induces TRAIL surface expression on NK cells at the gene level after 2 and 18h and at the protein level after 18h, which is in agreement with previous research reports (Kayagaki et al., 1999).

4.1.3 IFN-β induces TRAIL gene expression on NK cells

Once the positive control has been established, the expression of TRAIL on NK cells was investigated after IFN-β incubation. For these experiments the NK cells were isolated using MACS sorting from wt C57BL/6 naïve mice. As the magnetic beads and antibodies used for this procedure had no effect on them, they can be considered "untouched", un-activated, naïve NK cells.

The purities of the NK cells achieved for these series of two experiments were between 87-90%. An example of the evaluation of the purity of the sorted NK cells using FACS analysis is shown in Figure 16.

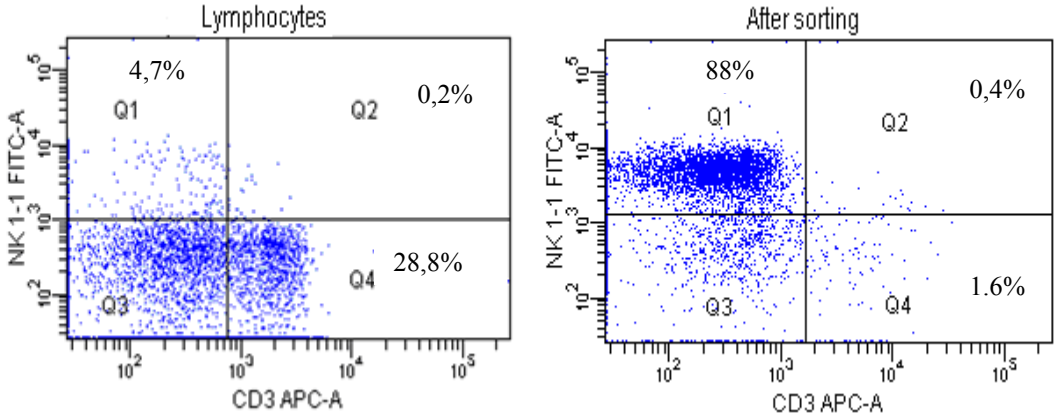


Figure 16 Example of FACS analysis of the sorted NK cells for purity check (in vitro) At the left part of the figure, FACS analysis of the lymphocytes before sorting and at the right part of the sorted NK cells is depicted. The cells were stained in both cases with CD3-APC and NK1.1-FITC. Before sorting, the NK cells comprise 4,7 % of the total lymphocytes of the spleen as seen in the Q1 part of the left plot whereas after sorting NK cells comprise 88% of the sorted population (Q1 part of the right plot).

The total yield of a NK cell sorting is very low as from five spleens used for each experiment (in vitro and in vivo) a maximum of 10 million NK cells were isolated. Therefore for all the experiments concerning NK cells incubation, the cells pro condition were around one million.

The sorted NK cells were incubated with different concentrations of IFN-β and with 1000 U/ml of IL-2 for 2 hours or 18 hours and with the following experimental details:

☞ For the 2h incubation, the NK cells were kept in culture for 16 hours and then IFN-β 500U/ml or 1000U/ml or IL-2 1000U/ml was added. This experimental planning was designed to rule out any possibilities of an effect of culture on the expression of TRAIL.

☞ For the 18 hours incubation, the NK cells were taken up in culture after the sorting procedure.

A table of this experimental layout is presented below:

16+2h incubation	18h incubation
untreated	untreated
IFN-ß 500U/ml	IFN-ß 500U/ml
IFN-ß 1000U/ml	IFN-ß 1000U/ml
IL-2 1000U/ml	IL-2 1000U/ml

TRAIL gene induction was then analysed by Taq-Man analysis in comparison to GAPDH and the results as relative expression are presented in Figures 11 and 12.

☞ IFN-β induced TRAIL gene after 2h with both concentrations used whereas IL-2 failed to induce TRAIL in this experiment (Figure 17), which is contrast with our previous experiment. The difference between these two Taq-Man analysis experiments could be attributed to the different purity of the NK cells used or to the different incubation layout used (16+2h versus 2h).

☞ After 18h with IFN-β TRAIL gene was induced by 2-fold approximately by both concentrations used and also by IL-2 (Figure 18).

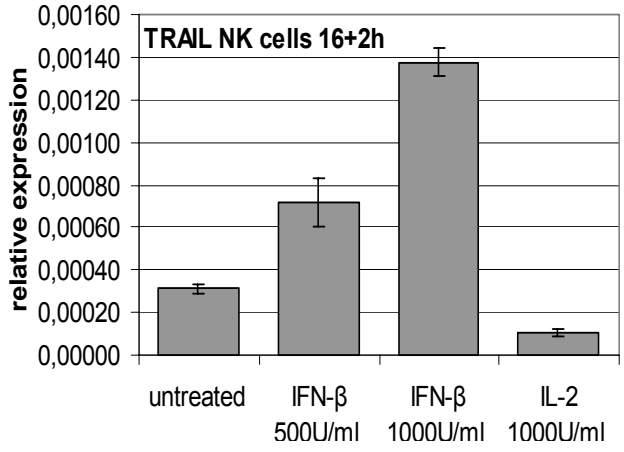


Figure 17 TRAIL gene induction on NK cells after 16+2 hours with IFN-β
Data derived from Taq-Man analysis. The values on the y axis represent relative expression of TRAIL gene to GAPDH. The error bars represent SEM. NK cells were kept in culture for 16 hours and then incubated with IFN-β or IL-2 for 2 hours. An induction of TRAIL gene is seen by both concentrations of IFN-β used.

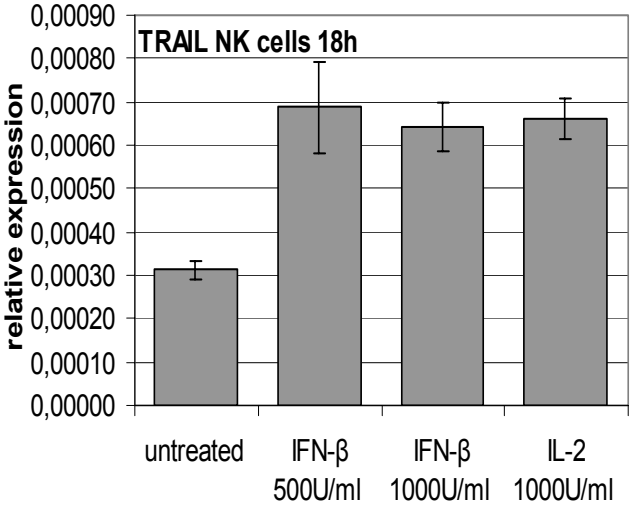


Figure 18 TRAIL gene induction on NK cells after 18 hours with IFN-β
Data derived from Taq-Man analysis. The values on the y axis represent relative expression of TRAIL gene to GAPDH. The error bars represent SEM. An induction of TRAIL gene is seen by both concentrations of IFN-β used and by IL-2.

In the same experiment, Mx1 gene was used as an additional positive control of IFN-β induction because of its following characteristics. Mx proteins are guanosine triphosphate (GTP)-binding proteins with an intrinsic GTPase activity and are strongly induced by IFN-β. They seem to act indirectly against viruses by modifying cellular functions needed along the viral replication pathway. Furthermore they are not induced by IL-2 (Horisberger et al., 1995). Mx1 relative expression was also calculated in comparison to GAPDH.

The results for both genes are summarized in Figures 19 and 20.

☞ For the 16+2h schema of incubation, IFN-β increased TRAIL expression by 2-fold at a concentration of 500U/ml whereas there was a 4-fold increase by a concentration of 1000U/ml. Mx1 gene was strongly induced by IFN- β (6 and 12-fold increase approximately at the concentrations of 500 and 1000U/ml) but not by IL-2, as expected.

☞ After 18 hours TRAIL was induced by 2-fold by both concentrations of IFN-β and also by IL-2 (Figure 20). Mx1 gene was strongly induced (4 and 9-fold increase approximately at the concentrations of 500 and 1000U/ml) by IFN-β but not by IL-2, as expected.

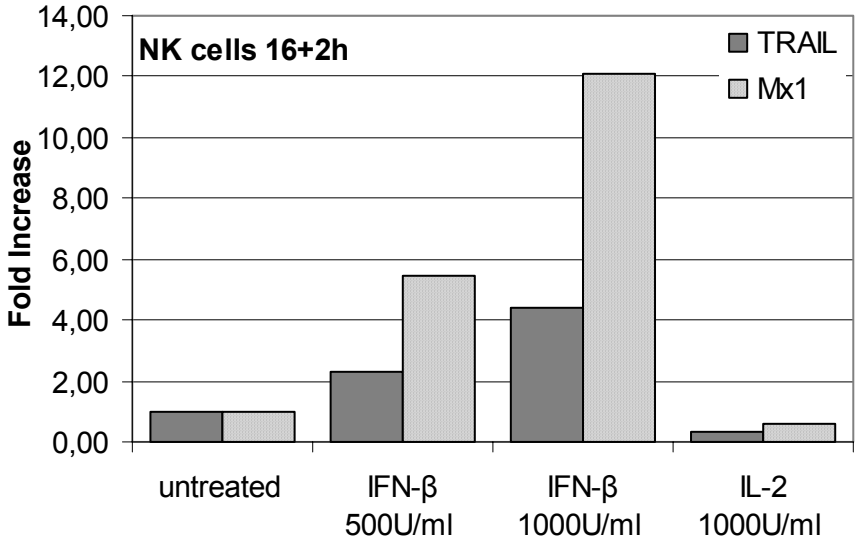


Figure 19 TRAIL and Mx1 gene induction
Data derived from Taq Man analysis and presented as fold increase. TRAIL gene induction is compared with Mx1 gene induction after 2 hours of incubation with IFN-β or IL-2. IFN-β induced TRAIL as well as Mx1 gene at both concentrations we used whereas IL-2 induced neither of them.

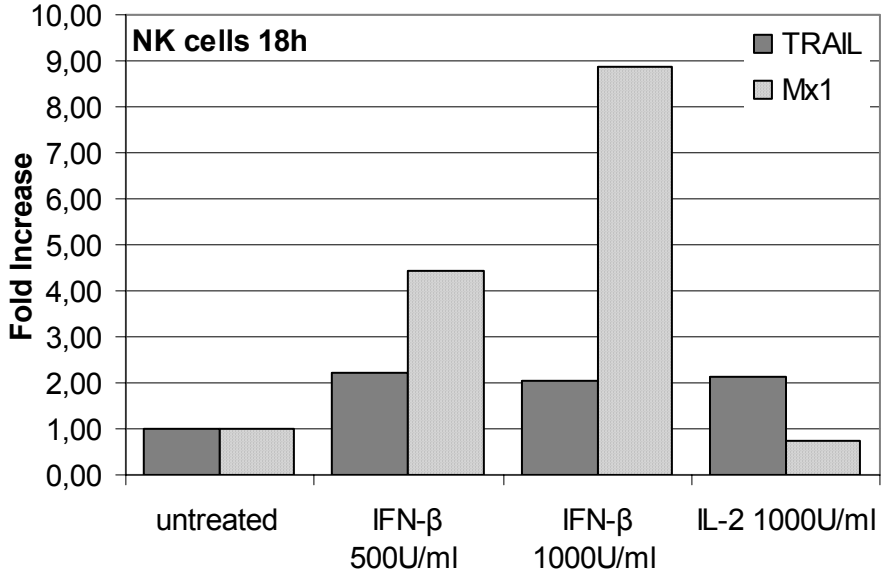


Figure 20 TRAIL and Mx1 gene induction
Data derived from Taq Man analysis and presented as fold increase. TRAIL gene induction is compared with Mx1 gene induction after 18 hours of incubation with IFN-β or IL-2. IFN-β induced TRAIL as well as Mx1 gene at both concentrations we used whereas IL-2 induced neither of them.

The experiment of TRAIL induction by IFN-β on NK cells was repeated to ensure the reproducibility of the results. The results from the second experiment are shown in Figure 21.

☞ IFN-β (1000U/ml) after 18h induced TRAIL gene on NK cells by approximately 6-fold whereas 500U/ml of IFN-β induced it by 4-fold (Figure 21).

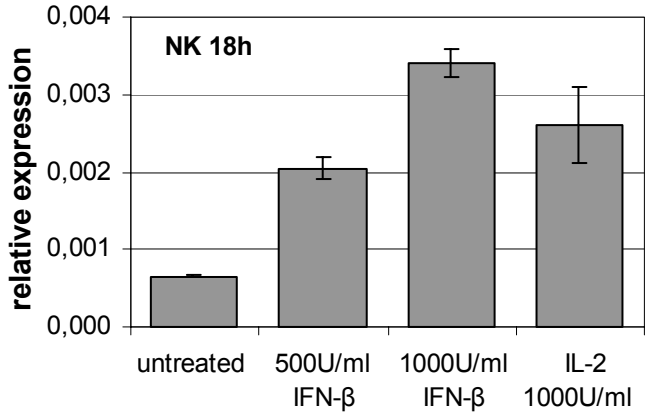


Figure 21 Relative expression of TRAIL gene on NK cells after 18 hours incubation with IFN-β in comparison to GAPDH.

Data derived from Taq-Man analysis. The values on the y axis represent relative expression of TRAIL gene to GAPDH. The error bars represent SEM. TRAIL was induced by IFN-β and IL-2 at the concentrations used

With these in vitro experiments it was shown that IFN-β can induce TRAIL on NK cells at the gene level. The optimal dose and incubation time was 1000U/ml and 18h respectively. These parameters were used for the in vivo experiments.

4.1.4 TRAIL surface protein induction by IFN-β

TRAIL induction at the gene level by IFN-β raises the question of induction at the protein level. To answer this question, surface TRAIL was measured on NK cells

by FACS analysis using different concentrations of IFN-β and a wide spectrum of incubation periods. The following experimental layout was used:

18h incubation	48h incubation	4d incubation	6d incubation
untreated	untreated	untreated	untreated
IFN-ß 100U/ml	IFN-ß 100U/ml	IFN-ß 100U/ml	IFN-ß 100U/ml
IFN-ß 500U/ml	IFN-ß 500U/ml	IFN-ß 500U/ml	IFN-ß 500U/ml
IFN-ß 1000U/ml	IFN-ß 1000U/ml	IFN-ß 1000U/ml	IFN-ß 1000U/ml
IFN-ß 5000U/ml	IFN-ß 5000U/ml	IFN-ß 5000U/ml	IFN-ß 5000U/ml
IL-2 2000U/ml	IL-2 2000U/ml	IL-2 2000U/ml	IL-2 2000U/ml

An example of the analysis of the TRAIL surface expression is given in Figure 22. The same procedure of analysis as for the investigation of TRAIL protein expression by IL-2 was followed. Splenocytes from wt C57BL/6 naïve mice were isolated and stained with CD3-APC, NK1.1-FITC and TRAIL-PE antibodies. The lymphocyte population was gated and the geometric means of TRAIL protein expression on $NK1.1^{+}CD3^{-}$ cells was calculated.

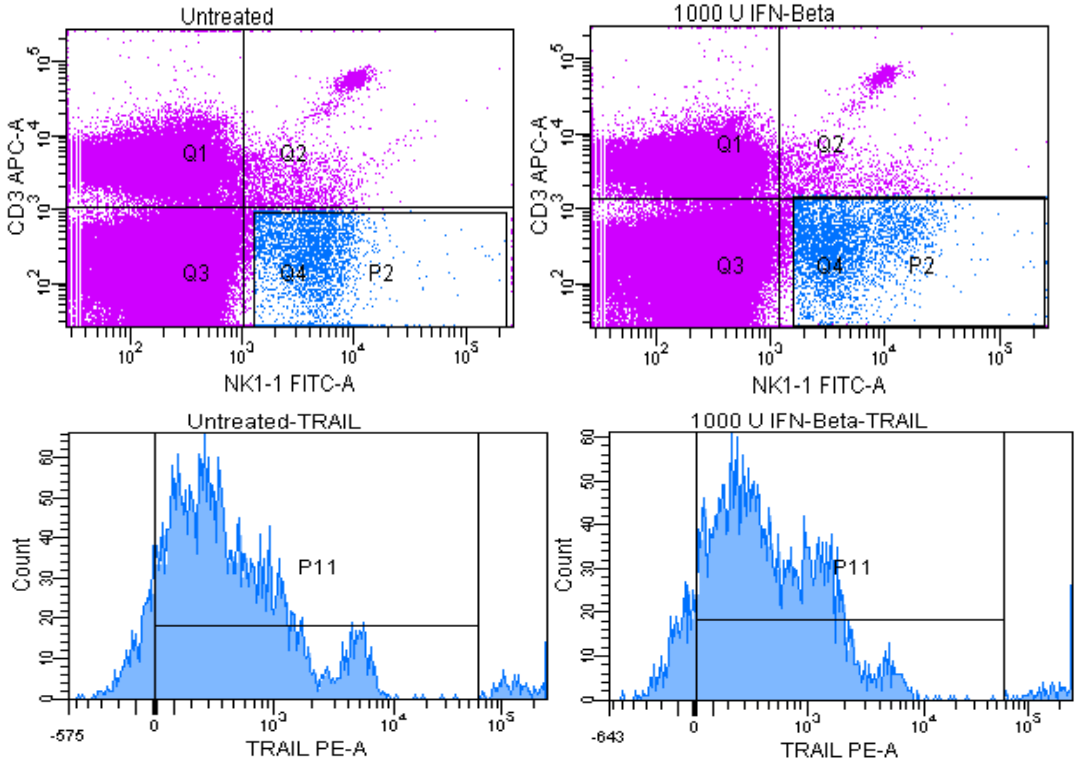


Figure 22 Example of FACS analysis of TRAIL protein induction on NK cells with IFN-β.

Splenocytes from naïve mice were isolated and stained with CD3-APC, NK 1.1-FITC and TRAIL-PE antibodies. The lymphocyte population was gated and the different subpopulations (CD3+, NK1.1 + and CD3+NK1.1+ cells) are depicted in the upper two plots. For the NK1.1+ cells (P2 gate) the distribution of TRAIL surface expression in relation to the cell count is depicted on the lower graphs. The Geometric mean for the distribution was calculated.

The concentrated results of this analysis only after 18 hours of incubation are presented in Figure 23. For this incubation period a slight induction of TRAIL was noted for the IFN-β concentrations of **100U/ml, 500U/ml and 1000U/ml** but not with IL-2.For the incubation periods of 48 hours, 4 days and 6 days with IFN-β no induction of TRAIL was observed.

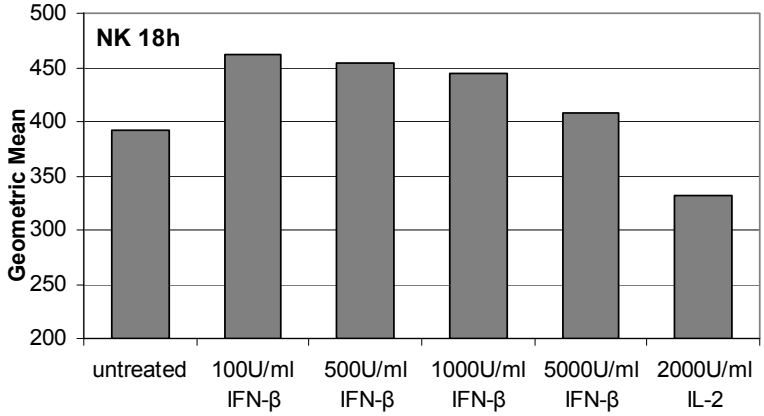


Figure 23 Surface TRAIL Expression on NK cells after 18h incubation with IFN-β
Data derived from FACS analysis. An induction of TRAIL protein was seen for IFN-β concentrations of 100U/ml, 500U/ml and 1000U/ml.

- ☞ Taking into account the in vitro experiments described until now, an induction of TRAIL on NK cells at the gene and protein level by IFN- β is confirmed.
- ☞ Moreover, an optimal concentration of IFN-β to induce TRAIL on NK cells in vitro was chosen according to the reproducibility of the results at both protein and gene level (1000U/ml IFN-β for 18 hours).

4.2 In vivo Experiments

4.2.1 Role of TRAIL producing NK cells in EAE

As established in the vitro experiments, IFN-β can induce TRAIL gene and protein expression on NK cells. At a concentration of 1000U/ml and after 18h incubation all the in vitro experiments showed a more than 2 fold increase of TRAIL gene expression whereas also the TRAIL protein expression was induced. At the specific time-point the increase of both gene and protein expression may pinpoint the existence of TRAIL protein which after stimulation is transferred on the outer membrane or an earlier induction of TRAIL gene expression which leads to the synthesis of new protein.

So far, we have established an in vitro model to induce TRAIL on NK cells. This model was further used to investigate the role of these cells in the course of EAE by adoptive transfer of these cells in mice.

Three consecutive in vivo experiments with the same experimental planning as depicted in Figure 24 were performed.

In each experiment three groups of mice were formed. Each group consisted of five female C57BL/6 mice at the age of 6-8 weeks. All mice were immunized with MOG $_{35-55}$ peptide in CFA to induce EAE as described in Material and Methods.

On day 2, NK cells were sorted from the spleens of five female transgenic EGFP-C57BL/6 mice at the age of 6-8 weeks. Following the sorting procedure, half

of the sorted NK cells were incubated with 1000 U/ml IFN-β for 18 hours whereas the other half were kept in culture without IFN-β for 18 hours.

On day 3 after immunisation (induction phase of the EAE), the NK cells treated with IFN-β were injected i.v. in the tail veins of the one group of mice (same number of cells for each mouse) whereas the other group of mice received the NK cells kept in culture without IFN-β. The third group of mice received the same volume of PBS used for the cell injection i.v..

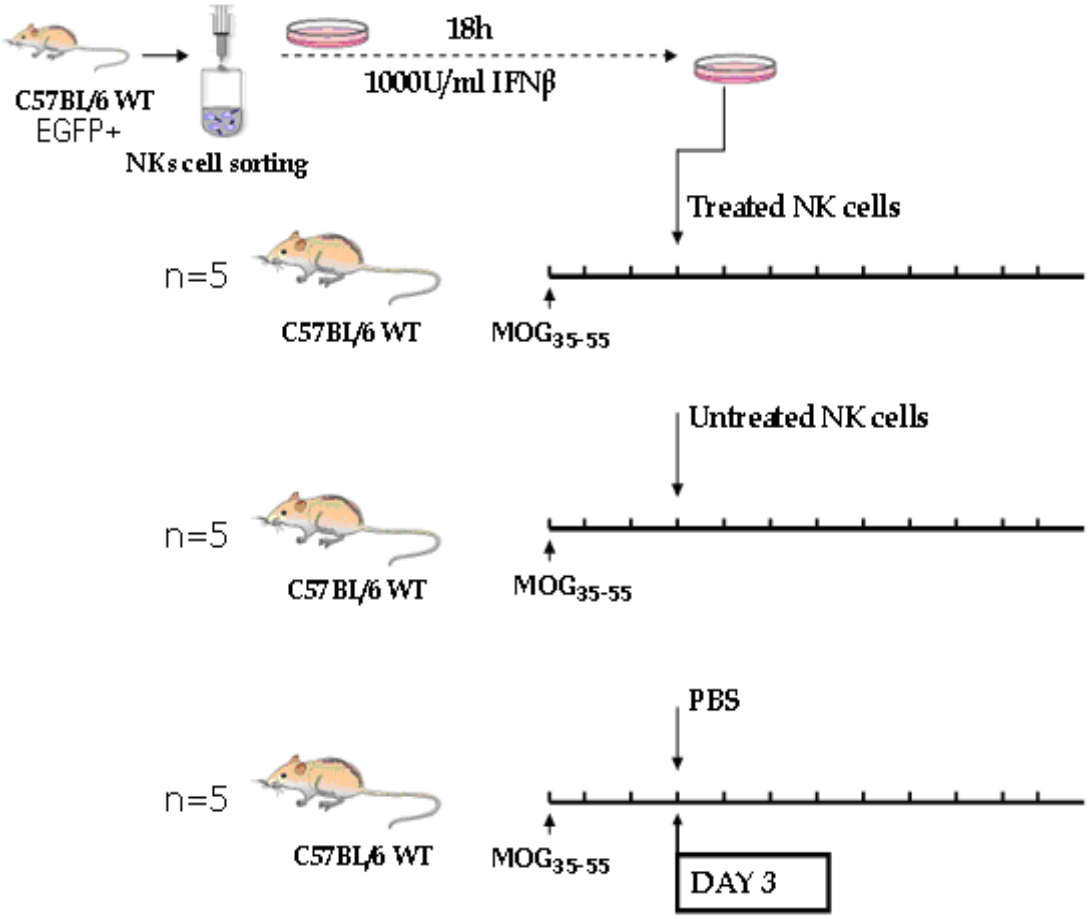


Figure 24 Experimental layout of the three in vivo experiments performed.
NK cells isolated from splenocytes of EGFP-C57BL/6 mice were incubated in vitro with IFN-β at a concentration of 1000U/ml for 18hours. Subsequently and on day three (priming phase of EAE) these NK cells were injected i.v. in one group of five C57BL/6 mice. As controls were used a group of mice which received PBS and a group of mice which received untouched NK cells which were only kept for 18 hours in culture.

The following table shows the purity of NK cells isolated for each one of the three experiments and the number of cells injected pro mouse. A representative example of the purity control using FACS analysis is shown in Figure 25.

	Purity of NK cells sorted	Number of NK cells injected
Experiment 1	85-90 %	720.000 cells/mouse
Experiment 2	82-87%	1.000.000 cells/mouse
Experiment 3	97 %	370.000 cells/mouse

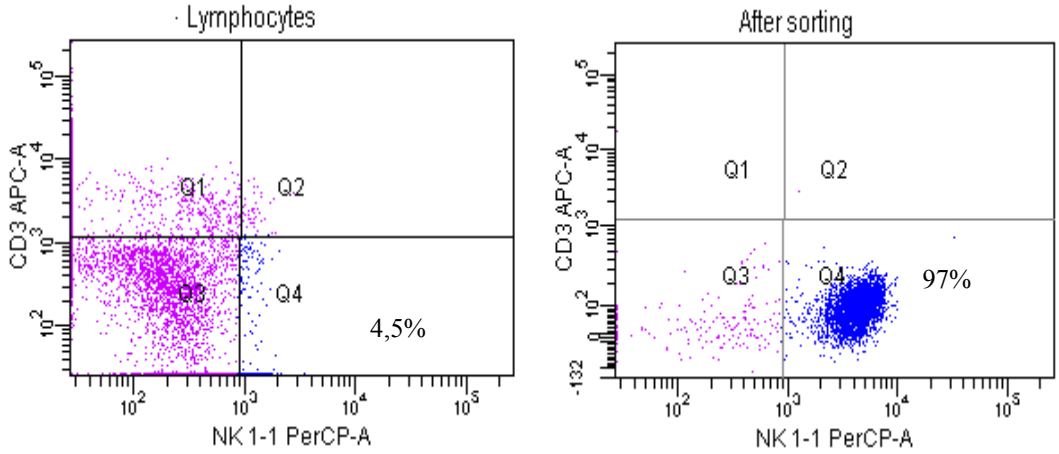


Figure 25 Example of FACS analysis of the sorted NK cells for purity check (in vivo) At the left dot plot the percentage of NK cells ($NK1.1^{+}CD3^{-}$) was estimated to be 4, 5%. At the right dot plot the isolated NK cells were analysed with the same staining and found that they were enriched to 97%. The NK1.1 FITC antibody could not be used for the in vivo experiments as the EGFP on NK cells has the same emission spectrum with FITC fluorophore.

4.2.2 Clinical evaluation of the EAE mice

Each group comprised of totally, fifteen C57BL/6 mice from the three experiments. The mice were scored daily at the same time-point starting from day 6 using the clinical scoring index shown in Table 9 (Materials and Methods).

The mean clinical score of all immunized mice (n=15 pro group) are depicted in Figure 26.

- The mean clinical scores point out a slightly worse disease course for mice that received NK cells compared to control mice who received PBS.
- After the acute course of the disease and after day 24, a classical chronic course of the disease typical for C57BL/6 mice was observed for PBS treated mice whereas for the mice, which received NK cells, a complete recovery from the disease was observed.
- Of the mice (n=15) that received IFN-β treated NK cells only one developed EAE and with a much delayed onset of the clinical disease (day = 25).

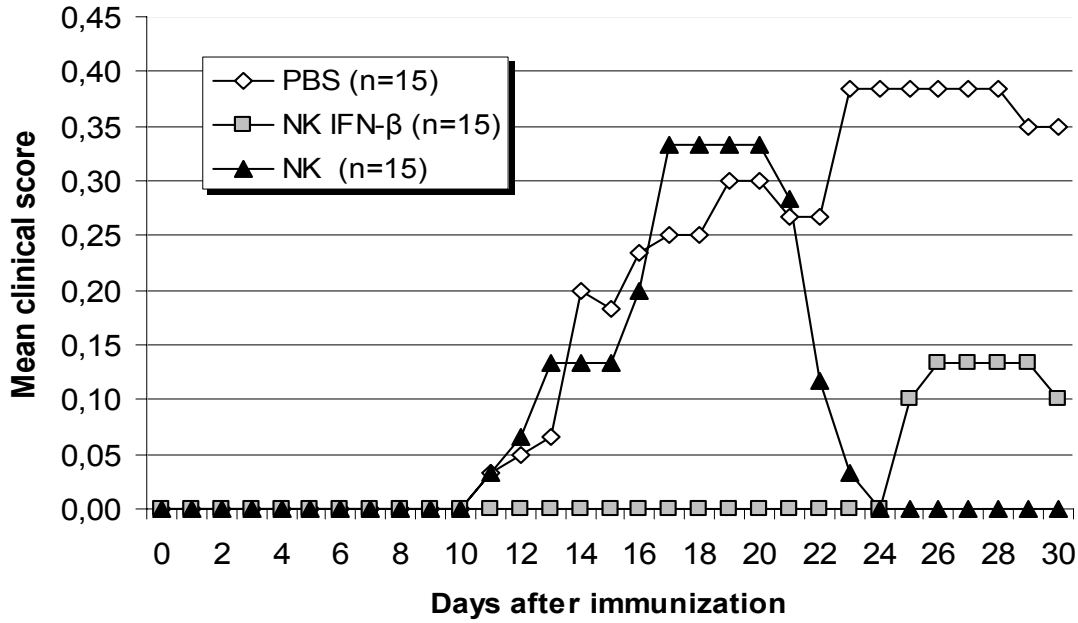


Figure 26 Mean clinical score of all MOG $_{35-55}$ immunized C57BL/6 mice
Three different groups of mice, each one consisting of 15 mice were immunized in three consecutive experiments (five mice pro group in each experiment). Of the 15 mice that received the IFN-β treated NK cells only one developed EAE with a disease onset on day 25. Mice from the other two groups developed a clinical disease on day 12 approximately and after an acute phase during the next 10 days the PBS treated mice moved to the chronic phase of the disease whereas the mice that received NK cells recovered completely from their symptoms.

Arithmetical data regarding different clinical parameters of the course of EAE of three groups of immunized mice are presented in Table 10.

	Disease Incidence	Day of onset	Max score[1]	Max mean score[2]	Duration of acute phase	Complete Recovery
PBS Group n=15	4/15 (26%)	10-22	1	0,32	10 days	No
NK Group n=15	3 /15 (20%)	13-18	1,7	0,5	10 days	Yes
NK IFN-β Group n=15	1/15(6%)	25	2	0,15	4 days	Unknown[3]

Table 6 Comparison of different clinical parameters of EAE.
[1] Maximum mean clinical score of all animals of this group which developed the disease , [2]Maximum mean clinical score of all animals in each group (n=15), [3]The one sick mouse of this group was sacrificed on day 30.

Concerning these results it was observed that the severity of the disease for mice, which received NK cells, reached the max clinical score of 1,7 whereas for the control group of PBS treated mice the score of 1. Furthermore, the disease incidence of PBS group mice was up to 26% which is lower than the incidence usually observed (60%) whereas NK group mice had an incidence of 20% and mice that received IFN-β treated NK cells 6% incidence. The days of onset were approximately similar for PBS

and NK group but the only mouse which developed signs in the IFN-β group had an initiation of clinical symptoms on day 25. Furthermore, the acute phase lasted only 5-6 days as on the last day of the EAE the mouse had already reside of the signs.

4.2.3 Cell populations in the lymphoid organs

Independently of the clinical course and incidence of the EAE, a further investigation of the lymphoid organs (spleen and LN), blood, brain and spinal cord followed. The purpose of this analysis was:

- Firstly, to track the injected EGFP positive NK cells and
- Secondly, to detect any differences in the cell populations of these organs between control mice and mice that received NK cells.

The cells populations chosen to be initially investigated were NK cells, mature T cells and therefore also NK-T cells.

Thirty days after immunization all mice that had developed an acute clinical disease were already in the chronic phase with a reduction and stabilisation of the clinical symptoms. At this time-point the mice were sacrificed and spleen and LNs (paraxial, inguinal and mesenterial) were removed. The total cell counts from spleen and of the LNs removed were calculated and 4 million cells from each organ were stained and analysed by FACS Analysis. The antibodies used were CD3-APC, NK1.1-bio and SA-PercP. Regarding the EGFP NK cells, no EGFP staining was detected in any of the organs.

All the data from the total cells counts to the percentages of these cell populations in the organs tested are presented in Figures 27,28,29,30.

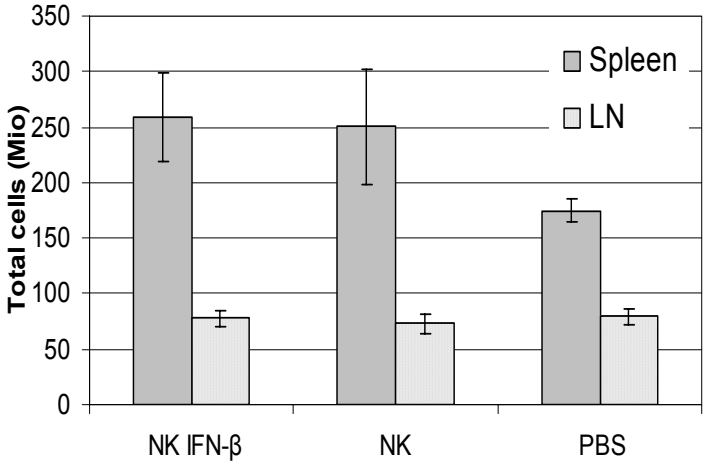


Figure 27 Mean total cell number of cells isolated from spleen and lymph nodes
Mice that received NK cells and IFN-β treated NK cells had a higher total cell number for spleen cells than PBS treated animals and this was accompanied by a splenomegaly. (n=15/group)

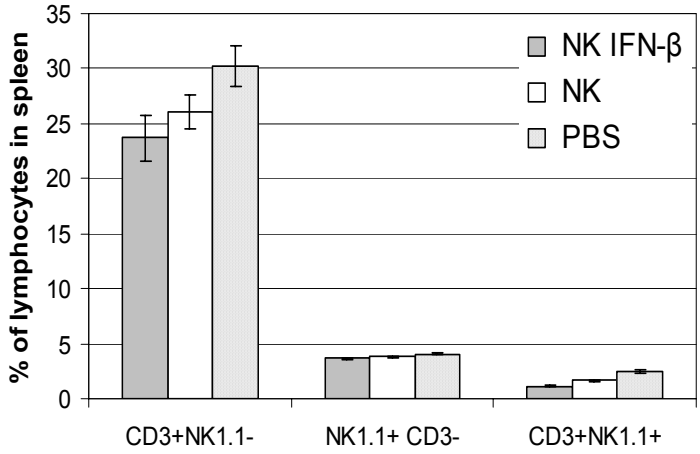


Figure 28 Mean percentages of T cells, NK cells and NK-T cells in spleens
A trend of reduction of the percentage of $CD3^+$ T cells was observed for the mice that received TRAIL-expressing NK cells whereas for NK cells no difference was detected (n=15/group)

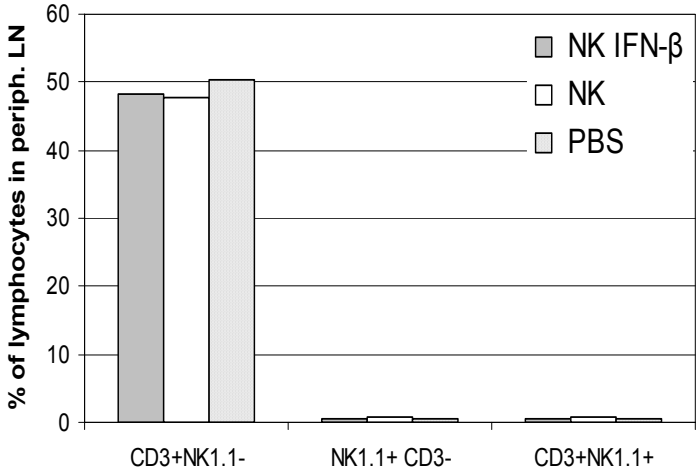


Figure 29 Mean percentages of T cells, NK cells and NK-T cells in lymph nodes
The paraxial, inguinal and mesenterial LNs were isolated. No difference for all cell populations was observed (n=15/group).

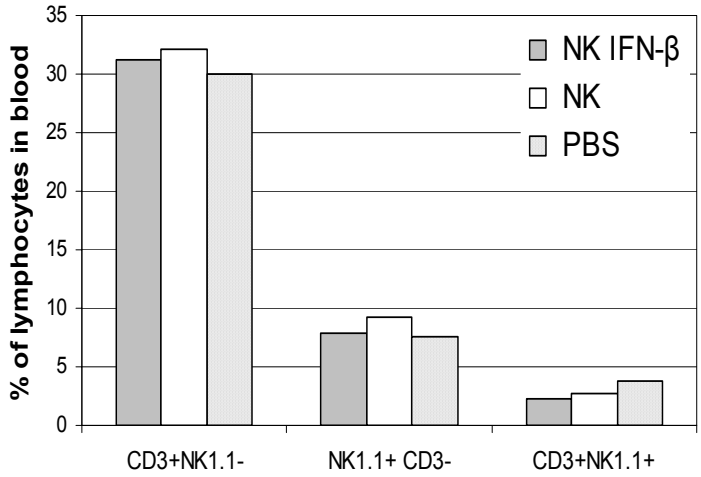


Figure 30 Mean percentages of T cell, NK cell and NK-T cell populations in blood.
Blood was taken from mice before perfusion with PFA was performed and analyzed with FACS staining. No differences between the analyzed populations were seen (n=15/group)

As can be observed in the upper graphs there is a trend of an increase of the total splenocytes in the group of mice that received IFN-β treated NK cells together with a splenomegaly whereas additionally there is also a trend of reduction of the mature T cells in the spleen of the same mice compared with the control mice.

Finally, we investigated DC populations in the lymphoid organs of the mice. The purpose of the this study was to determine whether NK cells either untreated or

pretreated with IFN-β were able to influence the frequency of DCs and monocytes population in spleens and LN of the mice. Therefore, we chose two markers, which characterise DC subpopulations ($CD11c^+$, $CD11b^+$) and monocytes ($CD11b^+$) and one DC maturation marker (CD86).

CD11c is the integrin alpha X chain protein (ITGAX) and is found in mice on DCs in lymphoid organs and blood. It is found on 1-3 % of splenocytes and on <1% in LNs of mice. The CD11b antibody which binds on CD11b/CD18 heterodimer (Mac-1, integrin α_M chain) a receptor for complement (C3bi), fibrinogen or clotting factor X. It is primarily a marker for monocytes/macrophages but is also found on myeloid DC subsets.

This staining was performed only during our last experiment and therefore the results represent the mean values of 5 mice pro group. An example of the analysis of the staining is depicted in Figure 1.

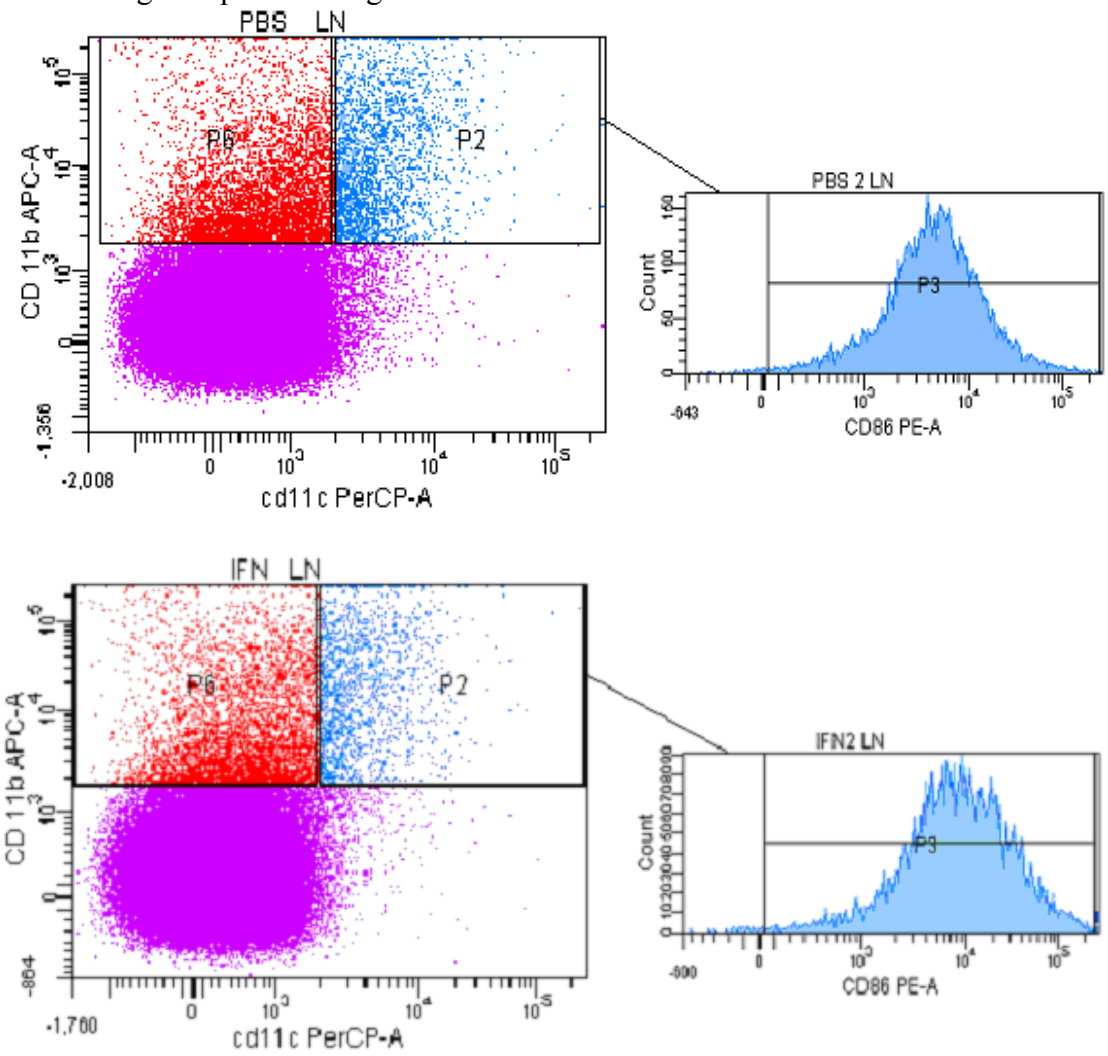


Figure 31 An example of FACS analysis for DC staining for PBS and IFN-β group in LNs. For each sample (spleen or LN) two stainings were performed. One with CD11b-APC, CD11b-PercP (not depicted here) and one with these two antibodies plus CD86-PE (depicted for PBS and IFN-β group). The populations marked on the left dot-plot are: P2 $CD11b^+CD11c^+$ DCs and P6 $CD11b^+$ monocytes. The geometric mean of the distribution of P2 population (graph) was calculated and the differences of the geometric means between double stained and triple stained sample were compared for all mice. For monocytes the percentages on account of total cells were compared.

The mean data for each group are depicted in the following graphs :

Firstly, regarding $CD11b^{+}CD11c^{+}$ myeloid DCs an increased of maturation status was observed in the LN of the mice that received NK cells pretreated with IFN-β. No difference was observed in the spleen and also no differences were observed in the percentages of this population between the groups.

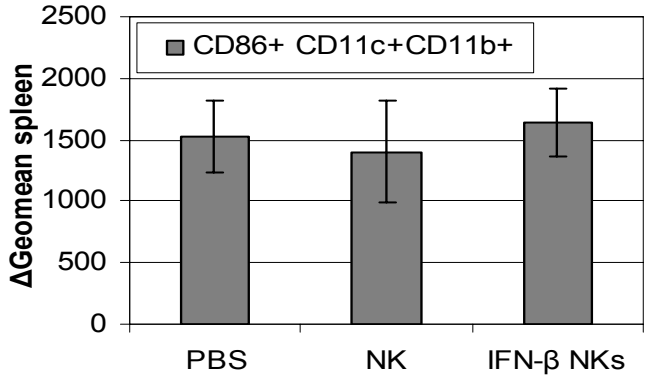


Figure 32 Maturation status of myeloid DCs in spleen
Data derived from FACS analysis. No difference is observed in the maturation status of myeloid DCs in the sleen. The y axis depicts the mean geometric mean difference of myeloid DCs distribution between a CD11b-APC, CD11c-PercP stained sample and a CD11b-APC, CD11c-PercP and CD86-PE stained sample.

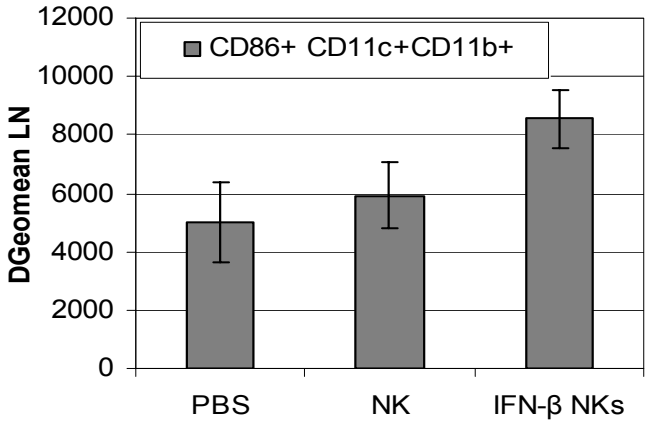


Figure 33 Maturation status of myeloid DCs in LN
Data derived from FACS analysis. More mature DCs are found in the LNs of the IFN-β group. The y axis depicts the mean geometric mean difference of myeloid DCs distribution between a CD11b-APC, CD11c-PercP and a CD11b-APC, CD11c-PercP and CD86-PE stained sample. Error bars represent SEM.

We proceeded to check the population of monocytes ($CD11b^{+}CD11c^{-}$) between the groups and we found a decreased frequency both in spleen and LN of the mice that received IFN-β pretreated NK cells.

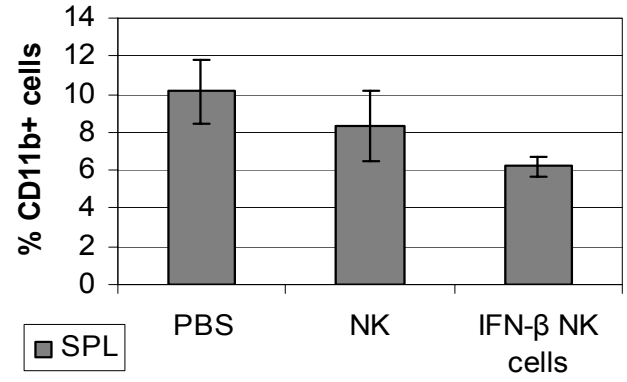


Figure 34 Monocytes populations in spleen
Data derived from FACS analysis. There is a decrease of the percentage of monocytes in spleen of IFN-β group mice. Error bars represent SEM.

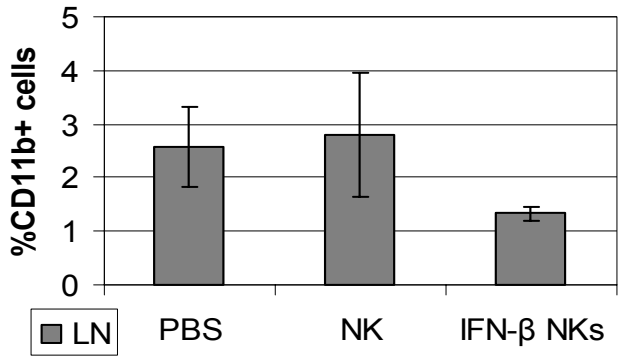


Figure 35 Monocytes populations in LNs
Data derived from FACS analysis. There is a decrease of the percentage of monocytes in LN of IFN-β group mice. Error bars represent SEM.

The potential importance of our findings collectively is discussed in the next part.

4.2.4 Immunostaining of the CNS

The brain and spinal cord frozen tissues were cut into 10μm horizontal slices with the help of a cryostat and stained as reported in the Materials and Methods for myelin in order to detect demyelination and EGFP antibody against GFP protein in order to detect any transferred NK cells existing in the brain or spinal cord parenchyma. The nuclei were counterstained with Hoechst. The sections were imaged in a Leica confocal microscope. Lesioned areas were identified by the presence of inflammatory infiltrates (perivascular and parenchymal) and lack of myelin staining. The presence of inflammatory infiltrates characterises a lesion whereas a disruption (reduction) of the myelin distribution pattern characterises demyelination.

In spinal cord sections the presence of cell infiltrates was detected at the edge of the tissue and at the lumbar level, which correlates with the clinical symptoms of tail and hind limb paresis of the sick mice. Inflammatory infiltrates were also found in the tissues of the mice that did not develop any clinical symptoms.

Regarding the brain tissue we focussed on histological examination of cerebellar white matter, choroid plexus and periventricular infiltrates GFP positive NK cells were clearly detected in lesioned areas with perivascular infiltration and rarely were associated with demyelinated axons (still under examination).

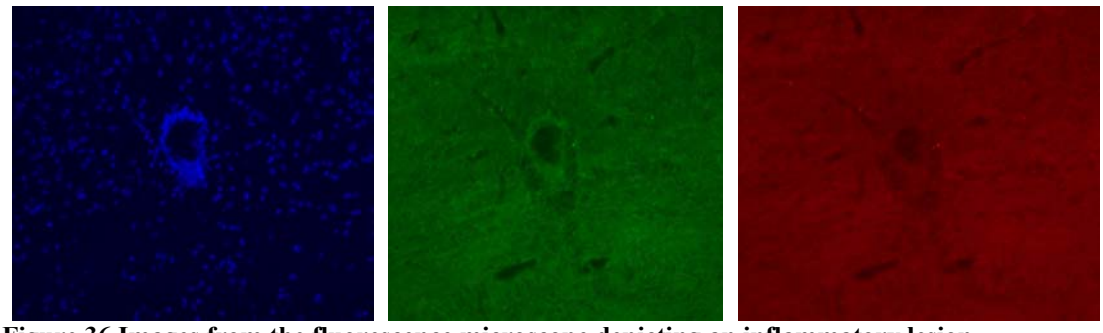


Figure 36 Images from the fluorescence microscope depicting an inflammatory lesion
With blue is depicted Hoechst staining of the nucleus, green is the staining for GFP whereas red is the staining for myelin. At the center of each picture is a blood vessel and inflammatory cells in the brain parenchyma are seen around it.

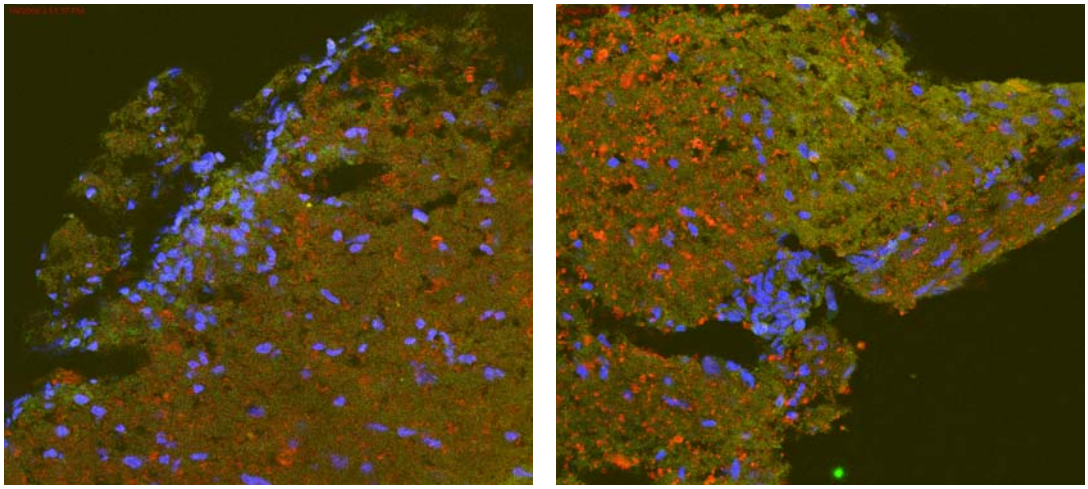


Figure 37 Confocal microscopy images depicting inflammatory infiltrates in the spinal cord These images are depicting two inflammatory infiltrates at the edge of the spinal cord (lumbar area).

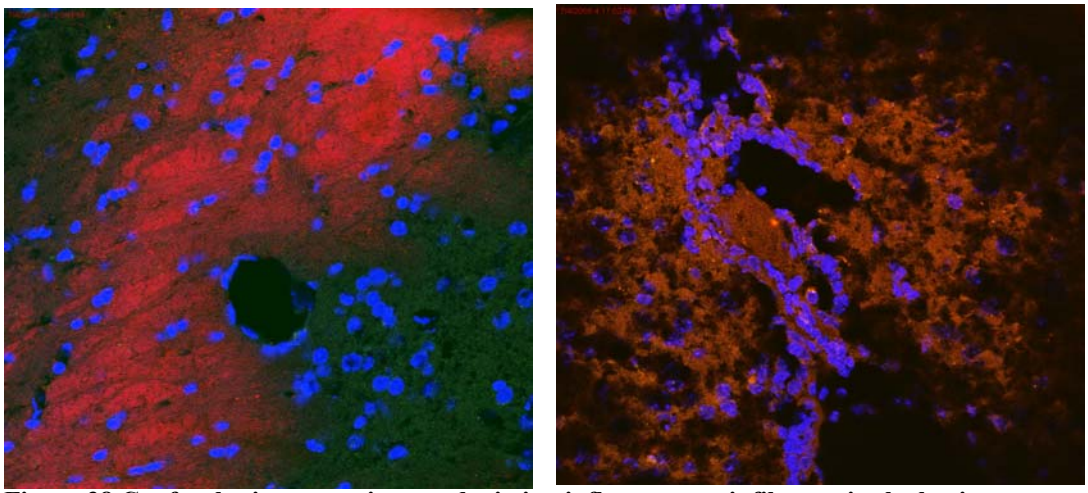


Figure 38 Confocal microscopy images depicting inflammatory infiltrates in the brain. Inflammatory infiltrates next to blood vessels and in proximity to a bandle of axons which are marked by their myelin sheaths.

5. Discussion

The main mechanism implicated in the pathogenesis of MS is the dysregulation of the innate immune system and the attack of some of its components e.g. T_H1 and T_H17 myelin specific cells against the myelin sheath. Therefore, these effector populations have been an obvious focus in MS research for years.

On the other hand, the delicate balance that controls the transition from a normal immune system to autoimmunity is guarded by regulatory mechanisms that are progressively enlightened e.g. $CD4^+CD25^+$ regulatory T cells, "suppressor" myeloid cells (Nagaraj and Gabrilovich et al., 2007; Serafini et al., 2006) and resting or "homeostatic" $CD205^+$ dendritic cells (Hawiger et al., 2001).

Among the cells of the innate immune system, the NK cells through molecules such as TRAIL could play an immunomodulatory role at both the initiation phase of autoimmunity (priming of T cells) but also during its progression by altered functionality in the inflammatory environment of the CNS. Elucidating this system could provide a better understanding of the underlying pathophysiology and new prospects of therapy.

In this Master Thesis the role of NK cells has been investigated in relation to the regulatory molecule TRAIL in EAE. It has been shown that TRAIL is induced on NK cells by IL-2 as well as by IFN-β and that TRAIL expressing NK cells have reduced the incidence of EAE in mice in comparison to control mice and have altered the populations of T cells mainly in spleens but also in the lymphnodes of the immunized mice.

5.1 Dissecting the mechanisms of regulation of TRAIL expression in NK cells

Since the identification and characterization of TRAIL (Wiley et al., 1995) this molecule has become a focus in cancer research due to its capability to induce apoptosis on transformed cells (Ashkenazy et al, 1999, Walczak et al., 1999). In this field, the focus has been set on the mechanisms that modulate the susceptibility of targets to TRAIL-induced apoptosis e.g. TRAIL receptors and intracellular signalling cascade molecules such as Smac in malignant glioma cells (Fulda et al., 2002), which can also find applications in the autoimmune research.

The interest for TRAIL was spread among immunologists as the apoptosis of non-transformed human and murine cells such as hepatocytes (Jo et al., 2000), neurons (Nitsch et al, 2000, Aktas et al., 2004) and oligodendrocytes (Jurewicz et al., 2005) was attributed to it. In this context, TRAIL is emerging not only as a weapon against transformed cells but also as a molecule capable to cause collateral damage against normal cells, especially in an inflammatory environment such as in MS (Aktas et al., 2005), hepatitis (Zheng et al, 2004) and atherosclerosis (Sato et al,. 2006) as well as regulate inflammation in the periphery (Cretney et al., 2004).

Understanding the mechanisms of TRAIL induction on immune cells is a prerequisite for the therapeutic manipulation of this system. Until now, TRAIL murine protein expression has been traced on ConA/IL-2-activated, but not resting, $CD3^+$ TCR αβ cells of $CD4^+$ or $CD8^+$ phenotype, on resting and LPS-activated B cells (Mariani et al., 1998) and on macrophages after stimulation with Mycobacterium and TLR-2, -3, and -4, but not TLR9, ligands (Diehl et al., 2004).

NK cells exploit mainly apoptosis-inducing molecules such as FasL (CD95) and perforin for their cytotoxic effects. Regarding the expression of TRAIL on NK cells two reports have so far been published. The first one by Kayagaki et al in 1999 investigated TRAIL protein expression on NK cells of C57BL/6 mice after incubation with IL-2, IL-15 and IL-18, which are all cytokines necessary for NK development and differentiation. An up-regulation of TRAIL was traced only with IL-2 and IL-15. These results are in line with the fact that IL-2 and IL-15 share two receptor components (IL-2Rb/IL-15Rb and IL-2Rg/IL-15Rg chains) and exert mostly overlapped biological activities including the up-regulation of NK activity. FACS analysis was used to detect a newly established at that time, biotinylated TRAIL antibody on NK cells. Furthermore, in this same report, TRAIL was found to contribute equivalently to FasL and perforin to the cytotoxicity against tumor cells lines (Kayagaki et al., 1999).

According to the aforementioned data, we chose to use IL-2 for TRAIL induction on NK cells in order to establish a positive control for the further investigation of TRAIL expression with IFN-β. IL-2 is produced primarily by activated $CD4^+$ T cells, although expression by naive $CD8^+$ T cells, dendritic cells, and thymic cells has also been reported. In T cells, IL-2 synthesis is tightly regulated at the mRNA level by signals from the TCR and CD28. IL-2 binds to and signals through a receptor complex consisting of three distinct subunits designated IL-2Ra (CD25), IL-2Rb (CD122), and common γ-chain (γc; CD132). Cytokines that bind to the interleukin-2 (IL-2) receptor common γ chain (γc), including IL-2, IL-4, IL-7, IL-9, and IL-15, are important for the growth and differentiation of T and B lymphocytes, NK cells, macrophages, and monocytes (Nelson et al., 2004) and also other candidate cytokines for TRAIL expression.

Both TRAIL gene and protein expression were investigated. At the gene level, TRAIL induction by IL-2 was investigated on the whole splenocytes fraction, on the enriched NK cells fraction and on the complementary non-NK cells fraction. IL-2 induced TRAIL gene expression after 18h on murine splenocytes, which is in line with the reports about the up-regulation of TRAIL on activated T cells, B cells and macrophages at the protein level (Mariani et al., 1998; Diehl et al., 2004). The NK cell depleted fraction also up-regulated TRAIL as it also consists of T, B cells and macrophages. However, the most consistent induction was seen on the enriched-NK cells fraction (up to five-fold increase) with the most effective concentration after 2h and 18h, implying that NK cells are a very potent TRAIL expressing population.

Moving to the protein level and using FACS analysis, IL-2 has been shown to induce TRAIL on NK cells only after 18 hours of incubation. In this experiment the whole splenocytes fraction was incubated with IL-2 and afterwards on the FACS analysis, the $NK1.1^+$ population was electronically gated. Therefore, here one can not exclude that the observed effects are not caused by direct activation of NK cells by IL-2 but are rather the consequence of interactions between the different cell types, which may indirectly regulate TRAIL expression on NK cells and contribute to the final outcome.

A similar induction on other subpopulations was not detected by the TRAIL-PE conjugated (N2B2 clone) antibody used in this and all following flow cytometric analysis experiments. The inability to detect TRAIL in cell populations other than NK cells appears to be an intrinsic problem of this antibody, which because of chemical/stereological reasons binds surface-TRAIL on NK cells exclusively. Unfortunately, this is the only monoclonal antibody available for cytometric detection of TRAIL.

The difference between our experiment and the one reported by Kayagaki et al regarding the period of time of TRAIL induction (18h in comparison to 2 days) can be attributed to the use of a biotinylated TRAIL antibody of the same clone by Kayagaki et al and by the different concentrations of IL-2 used (500U/ml whereas we used 2000U/ml). On the other hand, in this report, TRAIL was also detected only on NK cells and not on T cells.

Having reproduced previous experiments and established our positive control for TRAIL induction on NK cells, we focused on our cytokine of interest, IFN-β. Although being the worldwide standard for MS treatment, our understanding of the broad spectrum of the antigen-independent, immunological effects of this cytokine is not yet completed. Inhibition of T-cell proliferation (Goodin et al, 2002), regulation of many cytokines (Wang et al, 2000), blocking of BBB opening via interference with cell adhesion, migration (Stone et al, 1995), and through reducing the secretion of MMPs (Stuve et al., 1996; Leppert et al., 1996) as well as possibly down regulating MHC class II on various APCs (Hall et al., 1997) are some of the mechanisms reported.

On the other hand, plenty of evidence support that IFN-β modulates the TRAIL system in MS. Firstly, our colleagues demonstrated that clinical response to IFN-β in MS is linked to up-regulation of peripheral TRAIL protein and gene expression (Wandinger et al., 2004), indicating that this regulatory cytokine may be a mediator of the beneficial effects of IFN-β in MS patients. In addition, according to unpublished data from our lab, NK cells from MS patients produce less TRAIL protein compared to healthy individuals when incubated in vitro with IFN-β (Infante-Duarte, unpublished data), which, in turn, is indicative for a role of NK cells and, in particular, NK cell-derived TRAIL, in the beneficial cascade of IFN- β therapy. It has been also shown that activated T cells from IFN-β-treated MS patients express higher levels of TRAIL than untreated patients (Arbour et al., 2005). Therefore, IFN-β mediated effects of the TRAIL system in MS are very likely to exist, whereas the effector population is still unknown with possible candidates T cells and NK cells.

In line with the human system, IFN-β attenuates the clinical course of EAE. In a relapsing remitting model of EAE i.p. injections of IFN-β (10.000U) beginning before immunization, led to attenuation of clinical symptoms, inhibition of T cell proliferation to MBP, reduction of T_H1 (IFN-γ) and increase of T_H2 (IL-10 and TGF-β) cytokines (Yasuda et al, 1999). In another report, treatment of (SWR*SJL) F1 mice suffering from chronic progressive EAE every other day with a s.c. injection of IFN-β (5000U and 10000U) beginning from the initiation of the clinical symptoms resulted in a decrease in the mean neurological deficit and in a delay of the exacerbation onset. This was accompanied by a decrease in delayed type hypersensitivity (DTH) to $PLP_{139-151}$ but not to ex vivo T cell proliferation and an improvement of the histopathological profile (Yu et al., 1996). This last report, which resembles more the human model of treatment, indicates that IFN-β may not influence T cell profile but their migration in the CNS or other immunomodulatory populations such as NK cells.

Data supporting the effect of IFN-β on NK cells come from anti-viral research. In this report, mononuclear cells were obtained from the liver of SCID mice, which are rich in NK cells and were stimulated in vitro with 200U/ml and 2000U/ml of IFN-β for 18h. Using FACS analysis an up-regulation of TRAIL by 2000U/ml of IFN-β was detected, using the biotinylated antibody of the same clone we also used in our experiments. Furthermore, TRAIL gene expression was not shown in the same conditions but after in vivo stimulation with an i.p. injection of poly I-C, which

induces IFN-α/β and activates NK cells, by semi-quantitative RT-PCR. The induction by IFN-β was proven to be dependent on the receptor IFN-AR1 and transcription factor IRF-9 (part of interferon stimulated gene factor-3), which binds to the IFN-stimulated response element (ISRE) revealed on the murine TRAIL promoter (luciferase assay in transfected mouse lymphoma T cells with TRAIL-luc incubated with 200 and 2000U/ml of mIFN-β for 6 h) (Sato et al., 2001).

In our experiments however, untouched, naïve NK cells were isolated without previous in vivo modulation. The purity of the NK cells was subsequently confirmed with FACS analysis to be 85-90% for both gene expression experiments. The NK cells were then incubated with 500U/ml and 1000U/ml of IFN-β for 2h and 18h. TRAIL gene relative expression to GAPDH was investigated using Taq-Man analysis and compared with two different positive controls: IL-2 as another cytokine proven to induce TRAIL, and Mx1 gene as an IFN-responding but not IL-2 responding gene (rev. Decker et al., 2005).

We have therefore for the first time demonstrated that TRAIL gene is induced on a pure NK cell population by direct incubation with concentrations of IFN-β as low as 500 and 1000U/ml. The effect of culture to the expression of TRAIL has been also ruled out by adjusting the 2h incubation after 16h of culture with no cytokines. Our final results show a 2-4fold increase of TRAIL with 500U/ml of IFN-β and a 2-6fold increase with 1000U/ml of IFN-β after 18h of incubation in two independent experiments. After 2 h of incubation there was a 2 fold increase with 500U/ml and a 4 fold increase with 1000U/lm of IFN-β. The credibility of our results was furthermore, ensured with two positive controls, IL-2 and Mx1 gene. The transcription mechanism of TRAIL induction on NK cells has been already elucidated by Sato et al.

Conclusively, it can be assumed that we have established a TRAIL expressing-NK cells system

Moving to the protein level, a more extended investigation as Sato et al but with the same TRAIL N2B2 clone antibody though not biotinylated but PE conjugated was performed.

Our kinetic analysis of TRAIL protein induction on NK cells by IFN-β using FACS analysis was based on a broader spectrum of IFN-β concentrations (100U/ml-5000U/ml), a broader spectrum of incubation times ranging from 2 hours until 6 days and the use of IL-2 as a positive control of TRAIL induction. The experiment was performed by incubating splenocytes with IFN-β and using Flow Cytometric analysis the geometric mean of the distribution of TRAIL expression on NK cells was calculated. Therefore, we did not have the advantage of a pure or at least enriched NK cell population as Sato et al. As a result, interactions between the different cell types, which may indirectly regulate TRAIL expression on NK cells and contribute to the final outcome, can not be excluded. According to our results, an induction of TRAIL protein on NK cells was seen only after 18h with all concentrations of IFN-β used but not by IL-2. Our results are not to be compared with those of Sato et al because of the different populations used for incubation but regarding the time-point of induction (18h) the two experiments are in agreement whereas also lower concentrations of IFN-β as 2000U/ml used by Sato et al, have induced TRAIL in our experiment. It shall also be mentioned that detecting TRAIL protein has been proved to be a difficult application as the antibodies are dysfunctional and ELISA kits for mice do not exist.

The differences of the kinetics of TRAIL induced on NK cells by IL-2 as have been reported by Kayagaki et al and by IFN-β in our experiment are mainly the lack of TRAIL expression on NK cells at the later time-points for example after 2 days or

6 days. IL-2 is a known growth, differentiation and activation factor of NK cells therefore also contributing to their survival after prolonged periods of incubation. IFN-β contributes, on the other hand, to the activation of NK cells and as has been reported to the acute response to a viral insult. These characteristics of the two cytokines may explain the different kinetic patterns, which characterize TRAIL induction by them.

☞ Before moving to the in vivo experiments it should be mentioned that the concentration and incubation time chosen to be used for the induction of TRAIL on NK cells was 1000U/ml and 18 hours due to its efficacy to induce TRAIL both at the protein and gene level.

5.2 TRAIL-producing NK cells in EAE

Moving to the in vivo experiments in the mouse model of MS, the EAE we aim to elucidate a possible role of TRAIL expressing NK cells in the induction phase of the disease. Of particular importance is the fact that we have established in vitro a TRAIL expressing NK cells system by incubation with IFN-β. The ways to investigate the role of this system is by transferring i.v. the in vitro generated TRAIL-NK cells to EAE mice and wait for any clinical alterations. Subsequently, the question that arises is at which time-point of EAE a role of our system could be hypothesized, which can be answered by taking into account the already existing data about IFN-β, NK cells and TRAIL in EAE.

Initially, actively induced EAE can be divided into an initiation and effector phase. The main hallmark of the induction phase is the priming of epitope specific T_H1 or T_H17 $CD4^+$ cells by immunisation with MOG $_{35-55}$ in CFA. APCs of the skin (such as Langerhans cells) take up the antigen and present it to these cells thereby activating them, in the periphery or in the draining and peripheral LNs using MHC class II and co-stimulatory molecules. On the other hand, during the effector phase the primed cells become reactivated into the CNS and exert direct damage on myelin and neurons or indirectly by the secretion of cytokines and chemokines and the establishment of an inflammatory environment. Clinically, the initiation of the effector phase is marked by the initiation of the clinical signs after day 10 for most of the EAE models.

In both phases, an effect of our system has been implied. Firstly, regarding IFN-β, application of treatment beginning at the induction phase and at initiation of the clinical symptoms and lasting through the clinical course, has led to the attenuation of the clinical disease in a relapsing remitting and a chronic progressive model of EAE, respectively (Yasuda et al., 1999 Yu et al., 1996).

TRAIL on the other hand has been shown to modulate EAE also both at the effector phase in the CNS and at the induction phase in the periphery though with exactly opposite ways. Namely, it seems to cause collateral damage to neurons in the CNS, whereas on the other hand it regulates the immune response in the periphery.

In more details and regarding CNS, recombinant human TRAIL given intracerebrally on days 6 and 8 after immunisation led to the deterioration of the **whole** clinical course of active RR EAE in comparison to control mice (Aktas et al., 2004). On the other hand, when TRAIL was blocked intracerebrally on days 2, 4, 6 after a passively induced EAE, a protection from myelin loss and neuronal death (and

a reduction of the clinical score **after the initial disease** phase, days 6-25) was observed, leading us to conclude that TRAIL has a direct cytotoxic effect on neurons as they have been shown to up-regulate TRAIL-R2 receptors during inflammation. Finally, when passive EAE was induced with TRAIL KO myelin specific T cells a reduced score **at all stages** of the disease and reduced incidence of the disease was observed implying a reduced encephalitogenicity of the cells (Aktas et al., 2004).

Although with contrasting evidence regarding the exact phase of the disease, many data exist that support a protective role of TRAIL in the periphery. A neutralizing TRAIL receptor given i.p. in an active chronic model of EAE increased the clinical course only when given at days 25-46 (late effector phase) and not at days 0-16 (Hilliard et al., 2001). On the other hand, by another group it was confirmed that TRAIL blockade during the whole course of the disease (3 times weekly i.p.) exacerbated EAE in a relapsing remitting and non-remitting model whereas TRAIL KO mice develop a worse disease course. Additionally, TRAIL blockade at the disease onset (days 7-14 and not 0-7 or 14-38) exacerbates EAE in the RR EAE model. Furthermore the i.p. injection of rh-TRAIL during the whole course of the disease attenuated EAE signs. In contrast with the previous report, in the RR EAE model, when TRAIL was blocked for the whole clinical course but TRAIL was given during the same period, an attenuation of the clinical symptoms was observed but when TRAIL was given only on days 14-30 no difference was noted. These last data conclusively imply that the protective effects of TRAIL in the periphery are exerted on days 0-12. (Cretney et al., 2005)

According to the aforementioned experiments, it is clear that TRAIL can modulate both phases of EAE in different ways. In our study we chose to investigate the role of TRAIL in our system during the induction phase of EAE (injection of the cells on day 3) as it has been also shown a putative mechanism of TRAIL regulation of autoimmune cells. TRAIL has been shown to inhibit proliferation of T cells in vitro and in vivo (Song et al., 2000) and this was attributed to a down-regulation of the cyclin-dependent kinase 4, indicating a G1 arrest of the cell cycle (Luenemann et al., 2002).

Regarding NK cells their existence in inflamed CNS has been confirmed by various reports. In Lewis rats the onset of EAE was associated with a three-fold increase in the proportions of peripheral blood NK cells and at the same time 17% of leukocytes infiltrating the spinal cord were NK cells, reflecting a rapid and short-lived influx of NK cells commencing 10 days after immunisation and lasting for less than a week (Matsumoto et al., 1998). Other researchers have reported that NK cells account for 10–20% of the infiltrate in symptomatic C57BL/6 mice immunized with MOG $_{35–55}$ (Huang et al., 2006).

Before commenting on their role in EAE, we initially have to stress out that the study of NK cell function in vivo has been challenging, mainly because of the lack of mouse strains that are selectively deficient in NK cells. The most common method to study NK cell role in autoimmune diseases has been NK cell depletion with specific antibodies such as NK1.1 specific antibodies (which also deplete NK-T cells) and Asialo-GM1 specific antibodies (which also deplete cytotoxic lymphocytes). Furthermore, many knock-out mice exist, which are deficient in different IL receptors, necessary for NK cells development.

In this field there are also contradictory data published. Firstly, regarding the depletion experiments it has been shown that NK cell depletion with antibodies from the beginning of the disease exacerbates disease course in chronic and RR EAE models (Zhang et al., 1997; Xu et al., 1999). Furthermore, in the report by Zhang et al

passive EAE induced to NK cell depleted RAG2-/- mice was attenuated when the mice received RAG2-/- splenocytes on day 2 after transfer but not when they received NK depleted RAG2-/- splenocytes. The in vitro studies showed that NK cells could inhibit T cell proliferation triggered by MOG and cytokines (Zhang et al, 1997) whereas Xu et al. has shown that there is a direct cytotoxic effect on autoantigen specific encephalitogenic cells in vitro (Xu et al., 2005) implying a mechanism of the protective role of NKs in EAE.

A later study proposes a better defined protective mechanism for NK cells in relation to DCs. Galazka et al. have shown that peptides derived from brain tissue of EAE mice complexed with the chaperone heat shock protein 70 (Hsp70-pc) induce an NK-cell dependent tolerance for subsequent EAE sensitisation of SJL/J mice with PLP. Later on, they provided evidence that this tolerance involves induction of H60 ligand (MHC class I-related glycoprotein) and its interaction with the NKG2D receptor. The NK cell- mediated Hsp70-pc-induced tolerance to EAE was dependent on modulation of DCs function leading to diminished T cell reactivity to PLP. In C57BL/6 mice H60 was not expressed, and Hsp70-pc-induced tolerance was not detected (Galazka et al., 2006; 2007). In one of the experiments they transferred i.v. NK cells (300.000 pro mouse) from Hsp-70c immunized mice to SJL mice at the day of immunisation with PLP and these cells abrogated EAE. This experiment apart from implicating the mechanism of NK role in EAE it also provides us with evidence for the specific time-point of our injection, thus the induction phase of EAE.

Taken together, studies of several models of multiple sclerosis indicate that NK cells inhibit the clonal expansion of myelin-reactive T cells in the periphery probably at the induction phase of EAE, and as a consequence of eliminating the suppressive effects of NK cells; CNS inflammation and autoimmunity become more marked.

Recently though, a report has come up with contrasting results. Diminished clinical disease was observed in C57BL/6 mice treated with anti-NK1.1, anti-asialo GM1, and selected Ly49 subtype antibodies accompanied by an increase in T cell responses to MOG $_{35-55}$. The reason for these contradictory results can be attributed to the different methodology for immunisation and the different max score of the EAE observed in the two experiments as is mentioned in the latest report which reveals a problematic evaluation of the NK cell role using depleting antibodies.

To complete this confusing picture, several mouse strains that have defective NK-cell function (IL-18-deficient mice) or that are devoid of NK cells (T-bet-deficient mice) were resistant to the induction of EAE.

IL-18-deficient mice are resistant to MOG-induced EAE and mount a poor T_H1 response. IL-18 administration enhances the disease severity in wt mice and restores the ability to generate T_H1 response. This restoration was abrogated in NK cell-depleted mice; however, after transfer of IFN-γ-secreting NK cells at the induction phase, disease susceptibility returns (Shi et al., 2000). More recent data showed that administration of the cytokine IL-21 before disease induction increased the severity of EAE. These effects were not observed when IL-21 was administered after EAE induction. Interestingly, IL-21 could not promote disease in the absence of NK cells, suggesting that IL-21 might act by stimulating NK cells to become disease-promoting (Vollmer et al., 2005).

Trying to elucidate the role of NK cells and TRAIL expressing NK cells we transferred these cells in mice at the induction phase of EAE. Not many NK cells transfer experiments are reported in the bibliography probably because of the difficulty of acquiring a high amount of cells. In the few reports that exist, 350.000-

500.000 cells are transferred in mice in order to see an effect (Galazka et al., 2006, Ohira et al., 2006, Alici et al., 2007). Using similar number of cells (350.000-1.000.000) with purity over 85% the following remarks can be made regarding our results:

Regarding the incidence of the disease, it shall be mentioned that normal incidence of a control EAE in C57BL/6 mice is around 60%. In our experiments this incidence were not achieved as in the control group of PBS treated mice (as well as in the group of mice that received untreated NK cells) an incidence of 26% was observed. A factor that influenced EAE incidence in these experiments can be the stress of the continuous injections during the first three days. Apart from s.c. immunization the mice received i.p. PTX on day 0 and 2 and on day 3 the NK cells were transferred i.v. This effect should maybe be considered in following experiments.

For the PBS group, a mild chronic disease with a maximum mean score of 0,5 was observed for all mice, which developed clinical symptoms in line with the characteristics of EAE in C57BL/6 mice as a chronic model. PBS mice had also a milder clinical course than the mice which received untreated NK cells. The NK cells injected in the mice in the periphery were exposed to the inflammatory environment of the skin and draining lymph nodes and this could have altered their activation status thereby influencing the severity of the disease.

It has been shown in vitro that NK cells can directly lyse human oligodendrocytes (Morse et al., 2001; Antel et al., 1998) whereas in vivo, the local inflammatory milieu in the brain could promote NK cell-activation so that they would be able to bypass the inhibitory effects of self-HLA class I molecules and lyse these cells

Factors that could have activated NK cells can be found on their interactions with other mainly accessory cells. Ligands for NK cell activating and inhibitory receptors exist on the surface of accessory cells (CD80) and receptors for NK cells ligands (CD40) which together with cytokines (IL-18, IL-2, type I IFNs) can influence NK cells. It is therefore required that for a complete picture of the priming phase of the disease, the effects of NK cells should also be taken into account especially when effector-regulatory molecules as TRAIL are known to be induced on them by cytokines such as IL-2 or IFN-β.

An additional observation is that all mice from the NK group that developed a clinical disease recovered completely from their symptoms after the acute phase indicating that no residual damage of neurons or myelin sheath able to maintain mild symptomatology was present. The impact of this observation has to be elucidated with additional in vitro experiments concerning the role of NK cells in the inflammatory environment of the priming phase but it may be in line with the implications of NK cells in clinical relapses in MS (Kastrukoff et al., 2003) as the NK injected cells actually worsened signs but only for a short period of time.

When activated, NK cells may inhibit in the periphery autoreactive T cells. Proposed mechanisms are the lysis of dendritic cells (DCs) or T cells, the production of regulatory cytokines (such as IL-10 and/or TGF-β) or the regulation of cell-cycle progression. In addition, NK cells might inhibit T cells by the modulation of other regulatory cells, such as natural killer T (NK-T) cells and $CD4^+CD25^+$ regulatory T (T_{Reg}) cells. (rev. Shi et al., 2006)

Regarding the group of mice that received NK cells treated with IFN-β only one mouse of the 15 immunized in the 3 experiments developed a clinical syndrome.

Furthermore, with a normal initiation of clinical signs on days 12-14 the mouse in this group developed the clinical signs later on day 25. The peak of the disease lasted for 3 days and then the symptoms resided. The development of the signs is not known as the mouse was perfused to fulfill the criteria of 30 days of observation used for all the previous experiments.

The reduction of the incidence of the disease after transferring TRAIL expressing NK cells at the induction phase is in line with the aforementioned data that IFN-β through TRAIL on NK cells can alter the status of autoreactive cells in the periphery and reduce the clinical signs. Mechanisms for this effect can be the inhibition of proliferation of autoreactive T cells (Luenemann et al., 2002)

The aforementioned clinical observations are to be 'completed' by the investigation of the immunological status of the mice by FACS analysis of the splenocytes, LN cells and blood of the mice. As the primarily the clinical course of the disease was a point of investigation, the immunological status of the mice was analysed on days 30-33 when the C57BL/6 mice move to the chronic phase of the disease with only remaining signs. Therefore, acute changes of the cell populations in these organs are not expected but only more permanent changes.

Macroscopically, a splenomegaly was observed especially for the group of the mice that received TRAIL-expressing NK cells but also for mice that received NK cells especially for the two first transfer experiments. This was accompanied by an increase of the total splenocytes derived from the spleens of these mice. The difference between the experiments can be attributed to the smaller amounts of cells that the mice received in the third experiment (370.000 cells compared to 720.000 and 1.000.000 cells).

An increase of the total cell number derived from lymphnodes was observed in the peripheral lymphnodes (paraxial, inguinal and mesenterial) for the first two experiments although not to the extent observed for the spleen cells.

With FACS analysis the populations of $CD3^+$ (mature) T cells and $NK1.1^+$ $CD3^-$ NK cells were investigated. A decrease in mature T cells was observed in the spleen of TRAIL expressing NK cells transferred mice in comparison to the PBS treated mice whereas the other populations (NK and NK-T cells appeared unchanged). Therefore, this is in line of the inhibition of T cells proliferation exerted on activated T cells by TRAIL (Lunemann et al., 2002).

Blood was aspirated from the heart of the mice and the same populations were analyzed but no differences were found. For the explanation of this result the time-point of the analysis that is after the acute phase of the disease should be taken into account, and therefore no difference was expected.

In order to investigate possible alterations in DC frequency we performed a for the DC subpopulations in the last in vivo experiment. It is known that DCs and NKs interact (Degli-Esposti et al., 2007) and additionally it has been reported that some DC subsets have tolerogenic functions (Colonna et al., 2004). Immature DCs are located in peripheral organs and at mucosal surfaces, where they continuously sample the environment for foreign antigens, which they recognise through members of the TLR family (Iwasaki et al., 2004). After pathogen encounter and recognition, DCs become very efficient at stimulating naive T cells, a process that is coincident with increased expression of antigen-presenting molecules (that is, MHC class I and class II) and co-stimulatory molecules (that is CD40,CD80 and CD86). In the absence of appropriate signals, interactions between T cells and immature DCs can result in the induction of tolerance (Steiman et al., 2003). Therefore, the signals that are received

by DCs decide not only whether a response is warranted but also what type of response is induced.

DC ontogeny and DC subsets have extensively been reviewed (Heath et al., 2004; Ardavin et al., 2003; Shortman et al., 2002). The CD11c and MHC-II molecules are expressed at high levels on all mature DCs in mice and co-expression of both markers is used to define DCs phenotypically. Four other markers are currently used to further define DC subsets: CD4, CD8αα homodimer, CD11b (Mac-1) and CD205 (Wu et al., 2003). For the purpose of our discussion, mouse DCs can be broadly categorized into three subsets. Two of these subsets comprise conventional DCs, which are characterized by the expression of high levels of CD11c and the differential expression of CD8a and CD11b.

Firstly, myeloid **CD11c$^+$CD8a$^-$CD11b$^+$** DCs are mainly located in marginal zones of the spleen. DCs, which have no CD11b expression can be categorized to lymphoid-derived **CD11c$^+$CD8a$^+$** DCs, which are characterized by the expression of high levels of CD205 (also known as **DEC205)** and localize to the T-cell areas of the spleen (Shortman et al., 2002) and are required to prime cytotoxic T lymphocyte (CTL) immunity to several viruses (Belz et al., 2004). **Plasmatocytoid DCs (pDCs),** which constitute the third main subset of DCs, are distinguished by the expression of **B220, LY6C, CD45RA and intermediate levels of CD11c** and have the capacity to produce type I interferons in response to viral infection and a reduced ability to activate naive T cells (Asselin-Paturel et al., 2001; Nakano et al., 2001; Krug et al., 2003).

According to our results there is an increased maturation of myeloid DCs in the LNs of the mice that received IFN-β treated NK cells. Interactions with many different outcomes have been reported between DCs and NK cells.

One of the more intriguing aspects of DC–NK-cell interactions is that, unlike most normal healthy cells, immature DCs are uniquely susceptible to NK-cell-mediated cytolysis, whereas mature DCs are protected (Wilson et al., 1999; Carbone et al., 1999; Ferlazzo et al., 2003). These differences have been attributed to differences in the level of MHC class I expression, specifically of HLA-E, by immature and mature DCs, a concept that is supported by the involvement of signals delivered by the inhibitory receptor complex CD94–NKG2A (Dela Chiesa et al., 2003). Signals delivered by activating receptors, such as NKp30, are also important (Ferlazzo et al., 2002) and other NK-cell activating receptors might be required under appropriate circumstances. Unlike the destruction of tumour targets, elimination of immature DCs by mouse NK cells seems to be largely dependent on killing that is mediated by death receptors rather than by granule exocytosis. In mice, NK cells that express TRAIL seem to be the population of NK cells that best eliminates immature DCs (M. E.Wallace and M.J.S., unpublished observations). Notably, the expression of relevant effector molecules by NK cells is modulated by cytokines. The finding that TRAIL is up-regulated by NK cells cultured with IL-2, IL-15 or IFN-β indicates that it might be the interaction with mature DCs that are producing these cytokines that enables NK cells to kill immature DCs. Both mature and immature DCs express the TRAIL receptor DR5; however, only immature DCs are sensitive to TRAIL-mediated apoptosis, and this is not always the case (Degli-Esposti et al., 2005). It remains to be shown which factors (such as, FLIP (caspase-8 (FLICE)-like inhibitory protein) levels and MYC expression (Ricci et al., 2004)) might be important in determining the sensitivity of DCs to TRAIL-mediated cell death. Therefore these observations could explain the reduction of immature DCs in the LNs in the group of mice that received TRAIL expressing NK cells.

As well as defining the capacity of NK cells to eliminate DCs, in vitro co-culture of DCs and NK cells has revealed that the death of immature DCs is not an obligatory outcome of their interaction with NK cells. Indeed, under appropriate conditions, NK cells can induce, or at least augment, the maturation of DCs (Piccioli et al., 2002; Gerosa et al., 2002; Pan et al., 2004). Soluble factors, principally TNF and IFN-γ, and cell–cell contacts are required for effective maturation of DCs (Piccioli et al., 2002; Gerosa et al., 2002). The relative importance of cytokines compared with contact-mediated signals differs depending on the context in which DC–NK-cell interactions occur. For example, DC activation induced by NK cells that have interacted with a target in the presence of type I IFNs is independent of cell–cell contact but requires TNF and IFN-γ (Degli Eposti et al., 2005). An additional factor that determines the outcome of DC–NK-cell interactions is the ratio of the interacting partners: low NK cell to DC ratios favour DC survival and maturation, whereas high NK cell to DC ratios result in elimination of DCs and inhibition of DC maturation (Piccioli et al., 2002). These findings may explain the more mature population of myeloid DCs found in the mice which received IFN-β pre-treated NK cells.

Regarding the effect of the mature myeloid DCs on the outcome of EAE and the reduction of the disease incidence in the mice that received IFN-β pre-treated NK cells a possible tolerogenic effect could be speculated. Contrary to what was originally proposed, the capacity to polarize specific T_H-cell responses does not seem to be an intrinsic property of specific DC subsets but, instead, the outcome of flexible responses that depend on both the signals that are received by the DCs and the microenvironment where these signals are received (Boonstra et al., 2003).

The studies published linking NK cells and DCs imply a possible tolerogenic effect. In one of them, NK cells were depleted with antibodies in C57BL/6 mice and diminished clinical disease was observed. Additionally, an increase in T cell responses to MOG was observed. NK cell depletion modified the DC composition as found in the draining LN. There was an increase in the percentage of $CD80^+CD86^+$ DCs in EAE NK cell-depleted mice, suggesting a more mature DC population compared with the EAE control mice in the draining LN, whereas immature DCs were favoured in the cervical LNs (Winkler-Pickett et al., 2008).

A later study proposes a better defined protective mechanism for NK cells in relation to DCs. Galazka et al. have shown that peptides derived from brain tissue of EAE mice complexed with the chaperone heat shock protein 70 (Hsp70-pc) induce H60 ligand (MHC class I-related glycoprotein), which interacts with the NKG2D receptor. Through this interaction, the DCs are modulated in a way that leads to diminished T cell reactivity to PLP. Therefore, a subsequent NK-cell dependent tolerance for EAE sensitisation of SJL/J mice with PLP is observed. In C57BL/6 mice H60 was not expressed, and Hsp70-pc-induced tolerance was not detected (Galazka et al., 2006; 2007).

Surely, further studies are required in order to elucidate the mechanism and the DC subset that mostly interacts with NK cells in each case and whether it influences the course of EAE.

Regarding monocytes, a decrease of their percentage was observed in the IFN-β group mice. A direct interaction of NK cells and monocytes that would lead to death induction of the latter could explain this effect as TRAIL receptors exist on monocytes (Men-Yuh Len et al., 2008) and the potential neurodegenerative action of activated monocytes in the brain would explain the reduced incidence of the disease in this group of mice. More specifically, macrophages in the brain are known to produce mediators such as such as pro-inflammatory cytokines, free radicals,

glutamate and metalloproteases which harm axonal integrity and lead to neurodegeneration which is one of the hallmarks of MS (Hendriks et al. 2005).

Our results collectively unravel a new effector-regulatory system for the pathophysiology of EAE which can act both in the CNS and the periphery and possibly both at the induction and effector phase of the disease with different ways. To elucidate and manipulate this system further experiments are required which are described shortly in the following parts.

5.3 New perspectives for the experiments and future concepts

Further experiments are required to elucidate the role of TRAIL expressing NK cells in EAE at the induction phase whereas the incidence of clinical disease in the control group will achieve the usually seen 60% incidence. Furthermore, the role of the same cells at the effector phase of EAE (after day 10) as well as later during the induction phase (days 6-10) shall also be investigated. Another problem of this kind of adoptive transfer experiments is that the effective cell-number transferred is not known. Therefore, the effects on clinical scoring and cell population in relevance to the cell numbers transferred should be compared. Injection of a bigger population of NK cells may also enable tracing of the cells after the transfer. Lastly, the immune cell populations at different points of the clinical disease shall also be investigated, whereas more acute changes can be detected. A control of whether TRAIL alone is important or also the incubation of NK cells with IFN-β can be transferring of IL-2-activated NK. IL-2 also induces TRAIL, but not other IFN-inducible gene such as Mx1.

In order to enable a better controlling of the experiments adoptive transfer of cells from transgenic mice can be used. More specifically, transferring IFN-β pre-treated NK cells from TRAIL -/- mice will clarify which of parameters investigated are exclusively influenced by TRAIL. Furthermore, the course of EAE on TRAIL -/- mice has to be investigated in more details as well as a possible reconstitution with TRAIL expressing NK cells. Lastly, EAE on NK cells deficient animals and reconstitution with TRAIL expressing NK cells would also enlighten some of the effects of this molecule specifically on NK cells. The collective results from all the aforementioned experiments could enable us to unravel a possible beneficial role of NK cells and TRAIL in the mouse model, which can later lead to the development of alternative therapeutic strategies in EAE and in MS.

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