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Antigen-independent pathogenic and protective immunity in chronic neuroinflammation

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Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
APC	antigen presenting cell
BBB	blood-brain barrier
CFA	complete Freund`s adjuvant
CFSE	carboxyfluorescein succinimidyl ester
CNS	central nervous system
CSF	cerebrospinal fluid
DC	dendritic cells
EAE	experimental autoimmune encephalomyelitis
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
GM-CSF	granulocyte monocyte colony stimulating factor
GWAS	genome-wide association study
IL	interleukin
IFN	interferon
KIR	killer cell immunoglobulin-like receptors
MACS	magnetic cell sorting
MBP	myelin basic protein
MHC	major histocompatibility complex
MOG	myelin oligodendrocyte glycoprotein
MPM	multiphoton microscopy
MRI	magnetic resonance imaging
MS	multiple sclerosis
MX	mitoxantrone
NK	cell natural killer cell
NMDA	N-methyl-D-aspartate
OVA	ovalbumin
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PFA	paraformaldehyde
PLP	proteolipid protein
PPMS	primary progressive multiple sclerosis
PTX	pertussis toxin
RFP	red fluorescent protein
RRMS	relapsing-remitting multiple sclerosis
SA	streptavidin
SPMS	secondary progressive multiple sclerosis
Th cell	T helper cell

1 Introduction into the topic

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system (CNS) that primarily affects young adults and is up to three times more common in females than males (Trojano et al., 2012). The most frequent forms of MS are relapsing remitting MS (RRMS), in which acute attacks are followed by complete or partial recovery, and primary progressive MS (PPMS), characterized by disease progression from onset. More than 80% of patients show a RR-course at the beginning of the disease, which in the majority of cases converts to a progressive disease course – secondary progressive MS (SPMS) – after 10-25 years (Infante-Duarte et al., 2008).

Therapeutic options for MS have increased considerably during the past few years. However, most of the conventional treatments tend to be non-selective and have been associated with several side effects (Infante-Duarte et al., 2008). One essential limiting factor in the development of therapeutic options for MS is the incomplete understanding of the causes of the disease. To date, both disease etiology and pathogenesis remain unclear. It is presumed that MS results from the interaction between complex genetic factors and environmental influences (Comabella and Khoury, 2012). In the context of a collaborative genome-wide association study (GWAS) involving 9,772 MS patients from 15 different countries, we recently identified a further 29 novel susceptibility loci for MS. Interestingly, many MS-associated genetic variants are close to genes related to immune function, in particular to T helper cell differentiation (Sawcer et al., 2011). This study contributed to resolve one of the most important dilemmas of MS pathogenesis, i.e. the question of whether MS is primarily an immunologic or rather a neurodegerative disorder.

To date it is assumed that MS is a chronic inflammatory condition, probably of autoimmune nature (Frohman et al., 2006) in which myelin-specific T cells seem to be responsible for the orchestration and perpetuation of the autoimmune reaction that leads to oligodendrocyte damage and demyelination, as well as axonal and neuronal damage (Aktas et al., 2007) (Figure 1). The role of inflammatory autoreactive CD4-positive T helper (Th) cells, producing cytokines such as IFN-gamma and IL-17, has been extensively proven in the models of experimental autoimmune encephalomyelitis, EAE (Hofstetter et al., 2007; Ivanov et al., 2006; Langrish et al., 2005; Zamvil and Steinman, 1990). In EAE, an autoimmune response is induced by immunizing the animals with CNS - restricted antigens. In mice, EAE can be induced actively or by adoptive transfer of encephalitogenic T cells (passive EAE). Active EAE is induced in mice by immunizing with specific myelin peptide epitopes together with adjuvant. The main effector populations causing the disease have been shown to be Th1 and Th17 $CD4^+$ T cells, as their infiltration in the CNS is associated with clinical symptoms. Passive EAE is induced by adoptive transfer of *in vitro* activated myelin-specific $CD4^+$ inflammatory Th cells. EAE is an especially robust and widely accepted model of CNS inflammation, even despite the limitation that it does not recapitulate all aspects of MS. Nevertheless, EAE and MS display several histopathological features in common, including demylination, axonal and neuronal damage, 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 and the presence of immunoglobulins in the CNS and cerebrospinal fluid (Baxter, 2007).
Depending on the mouse strain and the peptide used for immunization, EAE can be, as in human, relapsing-remitting as seen in SJL mice immunized with proteolipid protein (PLP); or chronic, where disease symptoms of the initial attack either stabilize at peak levels or gradually worsen over time as observed in C57BL/6 animals immunized with myelin oligodendrocyte protein (MOG) (Croxford et al., 2011). This unequivocal model of a T cell mediated autoimmune disease is thus far the best model of MS in genetically unmanipulated animals.

Figure 1

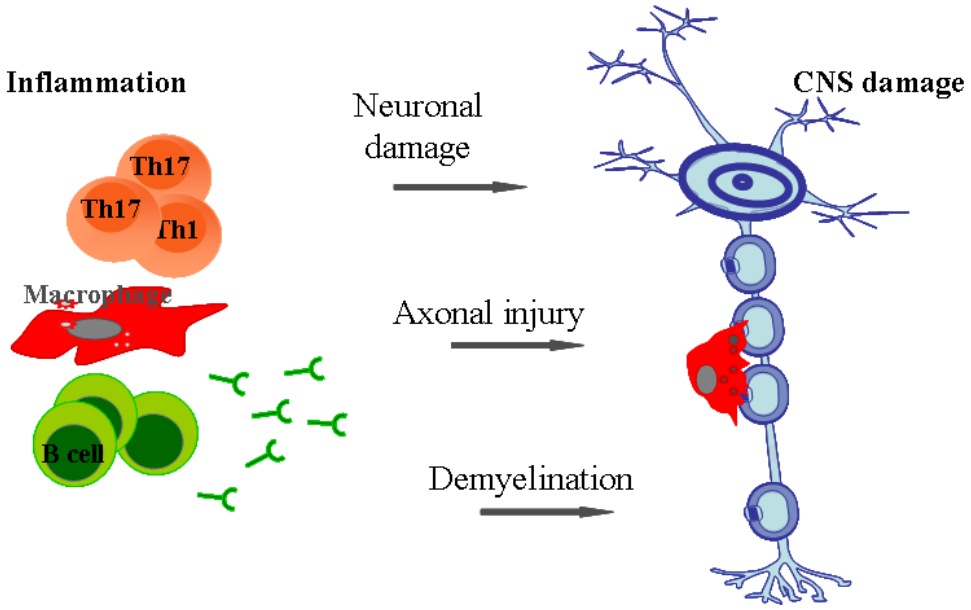

Figure 1: Schematic representation of MS pathogenesis. In MS, myelin-specific inflammatory Th1 and Th17 cells are considered to initiate and coordinate the autoimmune reaction in conjunction with other immune cells including cytotoxic T cells, B cells and macrophages. This leads to oligodendrocyte damage and demyelination, as well as axonal and neuronal damage.

In view of this pathogenic paradigm, research on MS has been intensively directed toward investigating lymphocytes of the adaptive immune system, in particular T cells specific for myelin antigens. Less attention has been paid to the role of non-CNS specific lymphocytes or of innate lymphocytes that do not express specific antigen receptors as T or B cells.

The concept of collateral damage during inflammation

It has been supposed for decades that non-CNS specific immune cells may contribute to CNS-damage. Ovalbumin (OVA)-specific activated T cells were shown to be able to damage brain vascular endothelial cells (Sedgwick et al., 1990) and to alter the blood-retinal-barrier (Hu et al., 2000). However, so far it remains uncertain whether T cells that do not recognize CNS-specific antigens may be able to enter the CNS and contribute to collateral neuronal injury.

The concept of collateral damage during an immune response describes the magnitude of tissue injury caused by activated immune cells that are not directly reactive to the target antigens. These activated cells may not only contribute collaterally to tissue damage but may also contribute to chronic inflammatory processes and/or disease exacerbation during ongoing infectious. In my PhD, I concentrated on the mechanism of bystander T cell activation in the context of chronic Lyme arthritis. I showed that lipopeptides from *Borrelia Burgdorferi* were able to induce the differentiation of naïve OVA-specific $CD4^{+}$T cells into Th1 (Infante-Duarte and Kamradt, 1997) or IL-17-producing Th cells (Infante-Duarte et al., 2000). In this context, I described for the first time the existence of a novel Th cell subset characterized by the expression of IL-17. We speculated that bystander generation of Th1 or Th17 cells during infection may contribute to disease chronicity and/or to persistent infection by promoting an immunologic milieu that protects infectious agents and/or directly causes tissue damage.

Likewise, we speculated that generation of inflammatory Th cells specific for non-CNS relevant antigens may contribute to disease pathogenesis and tissue injury also in the context of autoimmune neuroinflammation. We speculated that activation of T cells, for instance in the context of a non-CNS infection, may contribute to the development of relapses and thus to disease progression.

NK cells in neuroinflammation

NK cells were first identified in the 1970s on the basis of their ability to lyse tumour cells without prior antigen exposure (Kiessling et al., 1975). NK cells are innate lymphocytes capable of producing cytokines and discriminating between normal cells and tumor or infected cells. However, in contrast to B and T lymphocytes, NK cells provide a first rapid innate immune response, because their activity is not based on highly specialized antigen receptors produced by rearrangement of antigen receptor genes, but rather on multiple receptors that work together to induce NK cell activation (Yokoyama, 2008). However, increasing evidence indicates that NK cells are more than fast killers, and can also act as regulators of the immune response by directly interacting with dendritic cells, macrophages, endothelial cells or T cells (Vivier et al., 2008) .

In 2004, when I started my project on NK cells, human NK cells were classically categorized into two major subsets based on the expression of different levels of CD56. While the majority of the circulating NK cells are highly cytotoxic $CD56^{dim}$ cells, about 10% are immature bright CD56 expressers ($CD56^{bright}$) that seem to display imunomodulatory functions (Cooper et al., 2001).

At that time, we performed a large-scale gene expression analysis on MS patients and healthy controls and demonstrated that NK cells from MS patients display reduced expression of the chemokines receptor CX3CR1 (s. 3.2.1, Figure 3). These data indicated that expression of this receptor on NK cells may also discriminate between NK cell subsets with different functionality, which may be involved in MS pathogenesis.

In MS, disease development has been related to deficient NK cell activity (Benczur et al., 1980; Hirsch and Johnson, 1985; Infante-Duarte et al., 2005; Uchida et al., 1982), suggesting a possible beneficial effect of NK cells. However, other studies showed a relationship between NK cells and CNS pathology (Backstrom et al., 2003; Morse et al., 2001). Thus, to date both a beneficial and a deleterious role of NK cells in MS has been proposed, and it remains unclear whether these opposite effects are mediated by one type of cells, or rather by distinct NK cell subsets (Morandi et al., 2008).

2 Overall goals of the project

Taking into account the above mentioned facts, the primary goals of the studies presented here were:

- **To investigate whether and by which mechanisms non-CNS specific cells may contribute to collateral damage in the context of neuroinflammation.**

- **To further elucidate the phenotype and function of NK cells according to CX3CR1 expression and their implications in CNS inflammation.**

3 Own research projects

3.1 Collateral damage caused by non-CNS specific T cells during neuroinflammation

3.1.1 Direct impact of T cells on neurons revealed by two-photon microscopy in living brain tissue (Nitsch et al., 2004)

To investigate the capacity of CNS-specific and non-CNS specific T cells to promote neuronal injury, we used organotypic brain slices in which all components of the brain parenchyma are preserved in a proper network organization *ex vivo* (Gahwiler et al., 1997). Using two-photon microscopy, we demonstrate that activated Th cell-neuronal cell interactions can occur in an MHC-independent manner, in the absence of antigen recognition. In this study, PLP-specific as well as OVA-specific T cell lines were generated from SJL mice. Living cortical slices were obtained from SJL/J and control B10.PL mice at a thickness of 400 μm and transferred into a thermoregulated perfusion chamber for investigation with two-photon microscopy. Our data showed that activated PLP-specific T cells not only damage oligodendrocytes, which are the source of the antigen, but also cause damage to neurons, resulting in a lethal increase in intra-neuronal calcium overload. This effect appears to be independent of the antigen specificity and of MHC-restricted antigen presentation, because this effect was also observed in MHC mismatch experiments (SJL T cells in B10 slices) and, moreover, T cells directed against a non-murine antigen, OVA, were also able to induce an increase in neuronal calcium.

As expected, unstimulated T cells did not cause any damage in the organotypic *ex vivo* model. Thus, it appears that stimulation of T cells, independent of their target antigen, enables T cells to induce neuronal cell death via direct cell-cell contact. Moreover, we showed that both inhibition of perforin release and NMDA receptor blockade, or indirect inhibition via blockade of depolarizing AMPA-kainate receptors, was able to protect neurons from T cell-induced calcium increase. Because the production of excessive amounts of glutamate by T cells remains speculative, we believe rather that T cell-promoted glutamate production by other neuronal cells may be critical for the ability of T cells to promote calcium oscillations and calcium overload in neurons.

In conclusion, our data indicate that the detrimental effects of activated non-neural specific T cell on neurons can only be the consequence of bystander damage in which mechanisms other that classical MHC-mediated cytotoxicity are involved.

See publication #1:
Nitsch, R., Pohl, E. E., Smorodchenko, A., **Infante-Duarte, C**., Aktas, O., and Zipp, F. (2004)Direct impact of T cells on neurons revealed by two-photon microscopy in living brain tissue, *J Neurosci 24*, 2458-2464.

3.1.2 CNS-irrelevant T-cells enter the brain, cause blood-brain barrier disruption but no glial pathology (Smorodchenko et al., 2007)

In the previous study we demonstrated that OVA-specific T cells are able to induce collateral death of neurons in organotypic slice cultures. However, the role of non-CNS-specific T cells in BBB disruption and cellular neuropathology *in vivo* remains unclear. It was shown *in vitro* that ovalbumin (OVA)-specific activated T cells are able to damage brain vascular endothelial cells (Sedgwick et al., 1990) and to alter the blood-retinal-barrier (Hu et al., 2000). Yet, thus far no study had been performed comparing systematically T cell transmigration, localization, persistence, relation to BBB disruption, and subsequent effects on CNS tissue in transfer models of labelled activated encephalitogenic vs. non-CNS-specific T cells *in vivo*.

Here, we combined two imaging approaches, magnetic resonance imaging (MRI) and multi-photon microscopy (MPM) as well as histochemical and high-precision unbiased stereological analyses, to monitor T cell infiltration and CNS alterations in a model of T-cell transfer of OVA- and PLP-specific T cells *in vivo*. We showed that BBB alterations were present in both EAE-mice and mice transferred with OVA-specific T cells. Even if, in the latter case, BBB alterations were less pronounced, the pattern of initial cell migration into the CNS was similar for both PLP- and OVA-specific cells. However, gliosis was observed exclusively in the brains of mice transferred with encephalotigenic T cells. While mice transferred with non-neural-specific cells showed similar levels of rhodamine-dextran extravasation in susceptible brain regions, EAE mice presented massive BBB disruption in the brain stem and moderate leakage in the cerebellum.

Thus, activated CNS-irrelevant T cells recognizing non-mammalian antigen not only have the capacity to enter the CNS, but also to promote alterations in the permeability of cerebral blood vessels without promoting any neuronal pathology. Yet, in a compromised brain, such as in MS, bystander perturbations of the BBB caused by non-specific effector cells may have further pathological consequences, and may exacerbate disease, as is frequently observed in MS patients after ordinary infections.

See publication # 2:
Smorodchenko, A., Wuerfel, J., Pohl, E. E., Vogt, J., Tysiak, E., Glumm, R., Hendrix, S., Nitsch, R., Zipp, F., and **Infante-Duarte, C**. (2007) CNS-irrelevant T-cells enter the brain, cause blood-brain barrier disruption but no glial pathology, *Eur J Neurosci 26*, 1387-1398.

3.2 NK cells and their implications in neuroinflammation

3.2.1 Frequency of blood CX3CR1-positive natural killer cells correlates with disease activity in multiple sclerosis patients (Infante-Duarte et al., 2005)

To identify possible biological markers of MS, we performed a functional genome and single cell analysis in RRMS and PPMS patients as well as healthy individuals. Although we detected no significant gene regulation differences in RRMS vs. PPMS patients, we were able to identify a diverse set of genes that were significantly and differentially regulated in both groups of patients, compared to control individuals. One of these genes encodes the chemokine receptor CX3CR1, which showed an approximate 5-fold decrease in expression in both PPMS and RRMS patients, compared to healthy controls.

CX3CR1 is the only known receptor for fractalkine (CX3CL1), a chemokine that exists in both a soluble and a surface-bound form, (Bazan et al., 1997) and mediates both chemotaxis and the adhesion of leukocytes (Fong et al., 1998; Haskell et al., 1999). CX3CR1 was shown to be expressed on monocytes, NK cells and activated Th1-like cells, (Fraticelli et al., 2001) as well as on cytotoxic lymphocytes, suggesting that the receptor is involved in the migration of cytotoxic lymphocytes (Nishimura et al., 2002) and pro-inflammatory T cells (Fraticelli et al., 2001).

The reduced gene expression of CX3CR1 in patients was validated in an independent cohort by real-time RT-PCR and was also confirmed at the protein level by flow cytometry. Interestingly, from the analysed immune cell populations, we observed that the reduced expression of CX3CR1occurred exclusively on NK cells from MS patients, and was not seen in other CX3CR1-expressing lymphocytes such as CD8+ T cells. Furthermore, we demonstrated that the frequency of receptor-positive NK cells correlates with disease activity: CX3CR1 is particularly reduced in patients with a stable disease course, but increased to normal levels in patients with acute relapses and/or with gadolinium enhancing magnetic resonance lesions. This led to the question of whether this “normal” fraction of circulating CX3CR1-expressing NK cells might account for the relapse, or rather for the disease remission after acute attack.

Finally, we showed that expression of CX3CR1 correlates with the cytotoxic capacities of NK cells. $CX3CR1^{high}$ NK cells were more cytotoxic than their negative/low counterparts, providing the first evidence for potential CX3CR1-related functional differences of NK cell subsets defined by the expression of this receptor. This is an issue we investigated in our next study.

See publication # 3:

Infante-Duarte, C., Weber, A., Kratzschmar, J., Prozorovski, T., Pikol, S., Hamann, I., Bellmann-Strobl, J., Aktas, O., Dorr, J., Wuerfel, J., Sturzebecher, C. S., and Zipp, F. (2005) Frequency of blood CX3CR1-positive natural killer cells correlates with disease activity in multiple sclerosis patients, *Faseb J 19*, 1902-1904.

3.2.2 Analyses of phenotypic and functional characteristics of CX3CR1-expressing natural killer cells (Hamann et al., 2011)

We next aimed to characterize the phenotype and function of NK cells according to the expression of CX3CR1. We demonstrated that apart from the cytotoxic differences, $CX3CR1^{high}$ and $CX3CR1^{neg/low}$ NK cell phenotypes are distinct in terms of their cytokine profiles. When compared to $CX3CR1^{high}$ NK cells, $CX3CR1^{neg/low}$ expressed high amounts of type-2 cytokines (such as IL-5 and IL-13, but also IL-10) as well as GM-CSF and TNF-alpha, but expressed similar amount of IFN-gamma. Further, we showed that $CX3CR1^{neg/low}$ NK cells display a high expression of the activation marker CD25 and proliferated strongly in response to IL2.

Sorted $CX3CR1^{high}$ and $CX3CR1^{neg/low}$ NK cell also showed different effects on human monocytes. Only the $CX3CR1^{neg/low}$ NK cell fraction promoted increased CD40 expression, indicating that $CX3CR1^{neg/low}$ NK cells differ from the $CX3CR1^{high}$ subset in their ability to influence the costimulatory capacities of monocytes.

From the data obtained thus far, we found many parallels between $CX3CR1^{neg/low}$ and the well characterized $CD56^{bright}$ NK cells. However, a deeper analysis revealed that while all $CX3CR1^{high}$ NK cells were indeed $CD56^{dim}/CD16^{high}$, the $CX3CR1^{neg/low}$ fraction encompassed both $CD56^{dim}/CD16^{low}$ and $CD56^{bright}/CD16^{negative\ /low}$ NK cells. Monitoring the expression of KIRs NKG2A, NKp30 and NKp46 on $CX3CR1^{neg}CD56^{bright}$, $CX3CR1^{neg}CD56^{dim}$ and $CX3CR1^{high}CD56^{dim}$ NK cell fractions, we demonstrated that $CX3CR1^{neg}CD56^{dim}$ NK cells represented a phenotypic stage intermediate between the proliferating, cytokine-producing NKG2A/NKp30/NKp46-expressing NK cells and the non-proliferating KIR-expressing NK cells. Moreover, analysis of the expression of the differentiation markers CD27, CD62L and CD57 on the three subsets verified the intermediate character in terms of maturation of the $CX3CR1^{neg}CD56^{dim}$ NK cell fraction.

Next, we addressed here the question of how cytokines known to modulate NK cell activity such as IL-2, IL-15, IL-21, IL-12, IL-18 and TGF-beta may affect CX3CR1 gene expression. With the exception of IL-21, all tested cytokines induced a concentration-dependent downregulation of CX3CR1 gene expression. These data were also confirmed at the protein level. Thus, most cytokines inducing activation or proliferation of NK cells negatively influenced CX3CR1 expression.

See publication # 4:
Hamann, I., Unterwalder, N., Cardona, A. E., Meisel, C., Zipp, F., Ransohoff, R. M., and **Infante-Duarte, C**. (2011) Analyses of phenotypic and functional characteristics of CX3CR1-expressing natural killer cells, *Immunology 133*, 62-73.

3.2.3 Characterization of natural killer cells in paired CSF and blood samples during neuroinflammation (Hamann et al., 2013)

From our previous research, CX3CR1 emerged as an additional differentiation marker that may link NK cell maturation with the ability to migrate to different organs, including the CNS. Until now the implications of NK cells in chronic neuroinflammation had remained unclear, partly due to the lack of compelling studies on NK cell trafficking into the CNS.

To characterize NK cell trafficking into the CSF, we next examined the frequencies and phenotype of NK cells in CSF and blood from patients with MS, other inflammatory neurological diseases (IND) and non-inflammatory neurological diseases (NIND).

To assess NK cell frequencies, we analyzed a total of 62 paired CSF and blood samples, including 22 patients with definite multiple sclerosis (MS) according to the 2005 McDonald criteria (Polman et al., 2005), 11 with other inflammatory neurological diseases (IND) and 29 patients with non-inflammatory neurological diseases (NIND).

Confirming our previous data (Infante-Duarte et al., 2005), similar percentages of blood NK cells were detected in MS and IND or NIND patients. Overall, independently of the pathology, the fraction of NK cells present in CSF is reduced when compared with frequencies of blood NK cells. Moreover, we found a more pronounced decreased in NK cell frequency in the CSF of patients with MS compared to IND or NIND patients (MS: 2.7 ± 2.1 %; IND: 3.2 ± 2.1 %; NIND: 4.5 ± 2.7 %). Thus, the CSF/blood NK cell ratio is particularly reduced in MS patients, although T cell ratios remained comparable with other pathologies.

Next, we determined the phenotype of NK cells trafficking into the CSF in paired CSF and blood samples from 9 additional patients with inflammatory neurological diseases, including 6 patients with MS and 3 patients with other IND. We demonstrated that the frequency of immature NK cells defined as $CD56^{bright}CD27^{+}$ or $CD56^{bright}CX3CR1^{-}$ is highly increased in CSF of the patients (45% and 65%, respectively), while in blood the proportion of immature NK cells defined by the markers mentioned above is about 3.5 and 4 %, respectively.

Thus, in patients with inflammatory neurological diseases, CSF NK cells display an immature phenotype characterized by high expression of CD56 and CD27, and reduced expression of CX3CR1, which contrasts with the mature phenotype observed in NK cells in blood.

See publication # 5:
Hamann I, Dörr J, Glumm R, Chanvillard C, Janssen A, Millward JM, Paul F, Ransohoff RM, **Infante-Duarte C (**2013). Characterization of natural killer cells in paired CSF and blood samples during neuroinflammation. *J Neuroimmunol.* 15;254(1-2):165-9.

3.2.4 Mitoxantrone Induces Natural Killer Cell Maturation in Patients with Secondary Progressive Multiple Sclerosis (Chanvillard et al., 2012)

To clarify how NK cells exert their benefit, it is essential to examine how they are modulated during the disease course, and to which extent they may be affected by MS therapies.

Mitoxantrone (MX) is one of the few treatments licensed for use in secondary progressive MS (SPMS). However, its administration is limited due to the cumulative risk of severe adverse effects, such as cardiotoxicity (Dorr et al., 2009; Paul et al., 2007). To better understand how MX exerts its benefit in MS, we investigated its effects on the immune status of patients with SPMS in a prospective monocentric single-arm open-label study.

Clinical examination and venipunction occurred at baseline, after six months (prior to the third MX administration) and after twelve months (prior to the 5th MX administration). We compared major blood populations intra-individually (baseline versus treatment) and demonstrated that, apart from being cytotoxic for B lymphocytes, MX promoted the enrichment of peripheral neutrophils, as well as subsets of $CD8^{low}$ T lymphocytes and of NK cells.

The frequency of both $CD56^{dim}$ and $CD56^{bright}$ NK cells increased after six months of treatment although no variations of absolute cell numbers were observed, pointing to a non-specific enrichment of all NK cells at this time point. To determine the effects of MX on the NK cell maturation and activation, we analyzed different NK cell markers including CD27, CD57 and CD62L at months 6 and 12 of treatment. After six months, the NK cell phenotype appeared not to be affected, however, we did observe a long-term MX-associated reduction of CD62L expression, which is indicative of a process of maturation (Juelke et al., 2010). We also examined the expression of the maturation markers NKp30, NKp46, CD94/NKG2A and panKIR (Hamann et al., 2011). Confirming the preceding data, both NKp46 and NKp30 were decreased after treatment. No changes were detected in the inhibitory receptors CD94/NKG2A or KIR. Thus, as reflected by the downregulation of CD62L, NKp46 and NKp30, it seems that MX promoted a shift towards a more mature NK cell phenotype. It has been shown that regulation of NK cells may be associated with response to MS therapies such as daclizumab or interferon beta therapy (Bielekova et al., 2006; Martinez-Rodriguez et al., 2011). Therefore, we investigated whether changes in NK cell status may correlate with the response to MX. We demonstrated that NK cell maturation, reflected by the downregulation of CD62L, NKp4 and NKp30 was observed exclusively in responders to MX, while non-responder patients showed no significant alterations of the various NK cell markers examined here.

See publication # 6:
Chanvillard, C., Millward, J. M., Lozano, M., Hamann, I., Zipp, F., Paul, F., Dörr, J., and **Infante-Duarte, C**. (2012) Mitoxantrone Induces Natural Killer Cell Maturation in Patients with Secondary Progressive Multiple Sclerosis, *PLoS One*. 7(6):e39625

4 Discussion

4.1 Collateral damage caused by non-CNS specific T cells during neuroinflammantion

In the course of Multiple sclerosis, infections have been proposed as one of the most important factors triggering disease exacerbations (Correale et al. , 2006, Oikonen et al. , 2011, Tremlett et al. , 2008). The mechanisms by which infections can trigger relapses are thought to involve bystander activation of auto-reactive T cells, molecular mimicry or direct alterations of the BBB integrity. Here we investigated whether non-CNS specific T cells, such as those generated in the course of an infection, may also damage the CNS collaterally. To exclude mechanisms such as mimicry or bystander activation of encephalitogenic cells, we used T cells specific for an OVA peptide, and monitored their capacity to damage neuronal cells *ex vivo* utilizing organotypic brain slices from the hippocampus (Nitsch et al. , 2004), and *in vivo* using a model of adoptive cell transfer.

In the organotypic brain slices, we showed that activated PLP-specific $CD4^+$T cells not only damaged oligodendrocytes, which express the antigen, but also directly damaged the neurons in an MHC-independent faction. Moreover, we demonstrated that activated T cells specific for the non-CNS antigen OVA were also able to induce a lethal increase in intra-neuronal calcium leading to neuronal death. Importantly, we later confirmed these data in Siffrin et al. using intravital two-photon laser scanning microscopy (Siffrin et al. , 2010). Thus, activation status, and not the antigen specificity of invading T cells, seems to determine the capacity of these cells to damage neurons. Our data are consistent with previous studies indicating in human single-cell co-cultures of T cells and neurons (Giuliani et al. , 2003) as well as in a delayed-type hypersensitivity (DTH) animal model (Newman et al. , 2001), that independent of their target antigen, stimulated T cells are able to promote neuronal cell death via direct cell–cell contact.

Glutamate excitotoxicity was shown to be an important mechanism in autoimmune demyelination by inducing both damage of neurons and oligodendrocytes (Werner et al. , 2000). In our experimental set-up, neuronal calcium influx was inhibited both by blocking perforin, an effector molecule also produced by $CD4^+$T cells (Brown et al. , 2009) and via blockade of the NMDA receptor or depolarizing the AMPA–kainate receptors. The latter is in line with the finding that blockade of AMPA receptors protects against neuronal cell death and ameliorates EAE (Smith et al. , 2000). Thus, we speculated that during T cell attack, receptor-mediated glutamate toxicity and direct damage by cytotoxic granules may jointly contribute to calcium overload and cell death (Nitsch, Pohl, 2004).

Interestingly, more recent reports have also highlighted the involvement of glutamate excitotoxicity in EAE and MS (Basso et al. , 2008, Centonze et al. , 2010), helping to elucidate the relationship between inflammation and excitotoxicity. Very recently, two studies indicated that IL-1beta and changes in synaptic glutamate receptors subunit composition (Di Filippo et al. , 2013) as well as TNF-alpha induced in microglia by activated T cells and the subsequent abnormal expression and phosphorylation of AMPA receptors (Centonze et al. , 2009) may represent a link between inflammation and excitotoxicity.

Importantly, also very recently Guo et al. elegantly demonstrated that NMDA receptor expression on oligodendrocytes was not required for EAE induction, pointing to a central role of excitotoxic neuronal death in the pathology of EAE (Guo et al. , 2012).

Moreover, in the context of T cell-mediated mechanisms of neuronal apoptosis, a key role has been proposed for the death ligand TNF-related apoptosis-inducing ligand (TRAIL). My former research group demonstrated that death-mediating TRAIL receptors are found on potential target brain cells, such as neurons and oligodendrocytes, and that soluble TRAIL mediates neuronal cell death in human brain slices (Nitsch et al. , 2000). Furthermore, we demonstrated that TRAIL expressed by encephalitogenic CD4+ T cells induces collateral death of neurons and contributes to neuronal damage in a model of transfer EAE (Aktas et al. , 2005) (see Figure 2). Altogether, these findings indicate that TRAIL may be also involved in neuronal damage caused by non-CNS specific T cells – an assumption which will require further investigation to confirm.

In the *ex vivo* paradigm of living brain slices, we applied T cells directly onto the target tissue. This leads top the question of whether *in vivo*, non-CNS specific T cells could also contribute to CNS damage, even in the presence of an intact BBB. It was already shown *in vitro*, that OVA-specific activated T cells are able to damage brain vascular endothelial cells (Sedgwick et al. , 1990) and to alter the blood-retinal-barrier (Hu et al. , 2000). Here, we used magnetic resonance imaging (MRI) and multiphoton microscopy (MPM) as well as histochemical and high-precision unbiased stereological analyses to compare T cell transmigration, localization, persistence, relation to BBB disruption, and subsequent effects on CNS tissue in a model of T-cell transfer of ovalbumin (OVA)- compared to proteolipid protein (PLP)-specific T cells (Smorodchenko et al. , 2007).

We demonstrated that both T cells specific for the myelin antigen PLP, and T cells specific for the non-neural antigen OVA caused microscopic BBB alterations. In the case of OVA-specific T cells, this occurred in the absence of any antigen presentation inside the brain, since, in contrast to an earlier study, we did not administer OVA to the mouse CNS before or during T cell transfer (Westland et al. , 1999). These data were confirmed in 2008 by an independent group which also showed that antigen specificity is not necessary to cause enhanced BBB permeability. However, an antigen-specific reaction is required to induce clinical signs of EAE (Spitsin et al. , 2008). Interestingly, although BBB disruption was more pronounced in mice transferred with CNS-specific cells, the pattern of initial cell migration inside the brain parenchyma was similar for both PLP- and OVA-specific cells, which is in line with earlier data in rats (Hickey et al. , 1991, Hu, Pollard, 2000), but which contradicted a more recent study by Archambault et al. which reported that activated non-CNS-specific cells failed to migrate into the CNS (Archambault et al. , 2005). Moreover, our stereological cell quantification revealed that equal numbers of PLP- and OVA-specific T cells infiltrated the CNS up to 10 days post-transfer, but only the encephalitogenic T cells persisted over a longer period of time, confirming also the study of Kawakami et al, which showed that OVA-specific T cells were able to enter the CNS parenchyma, but only cells encountering their antigen persisted and interacted with neural cells (Kawakami et al. , 2005).

We also found T cells in brain areas with intact BBB. Thus, in agreement with a previous study on BBB alterations (Claudio et al. , 1990, Floris et al. , 2004, Koh et al. , 1993, Muller et al. , 2005, Rausch et al. , 2003), it appears that BBB alterations are in part, the consequence of, rather than the cause for T cell migration into the CNS parenchyma.

Importantly, only encephalitogenic T cells, but not non-CNS-specific T cells promoted tissue alteration, as reflected by enhanced microglial cell density, astrogliosis, and demyelination. Thus, although activated T cells have the potential to alter microglia phenotype and function in an antigen-independent way (reviewed in (Carson, 2002), *in vivo* microglia proliferation was triggered exclusively by CNS-specific T cells, perhaps as a consequence of their reactivation inside the CNS, as postulated by Becher et al. (Becher et al., 2006). However, as discussed in the original publication (Smorodchenko et al , 2007), we speculated that in a situation with a compromised BBB, such as in MS, bystander perturbations of the barrier caused by non-specific effector cells may have further pathological consequences that, in concert with leakage of pro-inflammatory mediators, may induce disease exacerbation. This could occur, for example, in MS patients suffering from a common infection. This hypothesis was tested by our former PhD-Student Magdalena Paterka (Paterka, Dissertation 2013) in the EAE model. Interestingly, she showed in her PhD thesis that even though inflammatory IL-17-producing non-CNS specific T cells were unable to induce EAE after adoptive transfer into healthy animals, non-CNS specific cells were nevertheless able to induce disease relapses in mice already suffering from chronic EAE (s. http://www.diss.fu-berlin.de/diss/receive/FUDISS_thesis_000000049934.). Thus, in the context of a pathological process affecting BBB integrity, non-CNS specific T cells may contribute to disease exacerbation and /or progression.

Figure 2

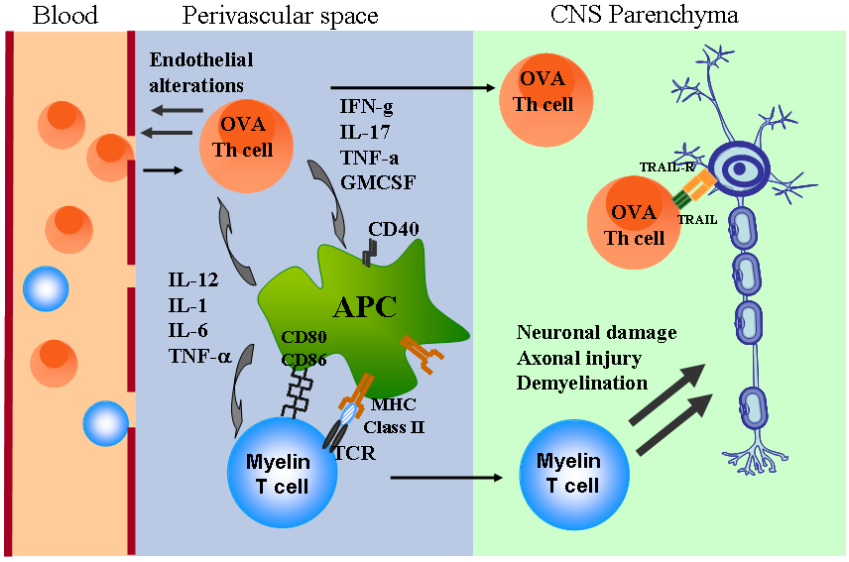

Figure 2: Antigen-independent damage mechanisms inside the CNS. Activated inflammatory non-CNS-specific T cells may enhance the inflammatory response inside the perivascular space by activating local APC and/or disrupting endothelial integrity through the secretion of pro-inflammatory cytokines such as IL-17, IFN-gamma or TNF-alpha. In the context of a disrupted BBB, activated non-CNS specific T cells may damage neurons in a bystander way, for instance via the TRAIL/TRAIL-receptor system (see (Aktas, Smorodchenko, 2005, Aktas et al. , 2007).

4.2 NK cells and their implication in neuroinflammation

Using large-scale gene expression analysis, we identified 17 genes differentially regulated in MS patients versus healthy controls. One of those genes was *CX3CR1*, which was decreased in both RRMS and PPMS when compared to healthy donors. CX3CR1 is the only known receptor for fractalkine (CX3CL1), a chemokine that exists both in soluble and surface-bound forms (Bazan et al. , 1997). and which mediates both chemotaxis and the adhesion of leukocytes through endothelial cells (Fong et al. , 1998, Haskell et al. , 1999). CX3CR1 is expressed on monocytes, NK cells and activated T cells; preferentially on Th1-like cells. The reduced frequency of cytotoxic $CX3CR1^{+}$ NK cells is in line with several reports of deficient NK cell activity in MS (Baxter and Smyth, 2002, Benczur et al. , 1980, Hirsch and Johnson, 1985, Kastrukoff et al. , 1998, Neighbour et al. , 1982, Vranes et al. , 1989). In our study, the frequency of $CX3CR1^{+}$ NK cells was increased during relapses or active disease phases, to levels comparable to those observed in healthy individuals. This may indicate either a critical role of this pro-inflammatory NK cell subset in disease exacerbation, or a contribution of cytotoxic T cells to disease remission (Infante-Duarte et al. , 2005).

In the CNS, CX3CL1 is expressed by neurons and astrocytes while the receptor is expressed on neurons, microglia and astrocytes (Harrison et al. , 1998, Hatori et al. , 2002, Hulshof et al. , 2003). However, more recently, CCL26 was identified as an additional ligand for CX3CR1 (Nakayama et al. , 2010) principally involved in migration of NK cells in allergic processes (El-Shazly et al. , 2013). CX3CL1 and its receptor are implicated in several human pathological conditions including atherosclerosis (Landsman et al. , 2009, McDermott et al. , 2001, Moatti et al. , 2001); age-related macular degeneration (Tuo et al. , 2007, Tuo et al. , 2004); allograft rejection (Cao et al. , 2006, Simeoni et al. , 2005); rheumatic diseases (Murphy et al. , 2008, Umehara et al. , 2006) and cytomegalovirus-related vascular endothelial damage (Bolovan-Fritts and Spector, 2008). In rodent models of brain pathologies, the CX3CR1/CX3CL1 axis has been related to both pathological leukocyte migration into the central nervous system (CNS) (Soriano et al. , 2002, Sunnemark et al. , 2005) as well as neuroprotective mechanisms.(Cardona et al. , 2006, Mizuno et al. , 2003). In EAE it was shown that mice deficient for the chemokine receptor CX3CR1 displayed a more severe form of the disease, accompanied by reduced infiltration of NK cells into the CNS (Huang et al. , 2006). This suggested that CX3CR1-mediated infiltration of NK cells into the CNS might be beneficial in this animal model. Along this line, we speculate that apart from their potential contributions to tissue injury, NK cells may be involved in the regulation of the autoimmune attack during MS (Flodstrom-Tullberg et al. , 2009, Morandi et al. , 2008).

To better understand the contributions of NK cells which express or do not express CX3CR1 in neuroinflammation, and to prove whether CX3CR1 expression may distinguish functionally different NK cell subsets, we next aimed to characterize NK cells according to their receptor expression (Hamann et al. , 2011). We demonstrated that $CX3CR1^{high}$ and $CX3CR1^{neg/low}$ NK cells do indeed represent distinct subsets. $CX3CR1^{neg/low}$ NK cells expressed high levels of IL-5 and IL-13, IL-10, GM-CSF and TNF-alpha, and did not differ

from CX3CR1^{high} in expression of IFN-gamma. CX3CR1$^{neg/low}$ NK cells are also characterized by high expression of the activation marker CD25 and a very strong proliferative response to IL-2 *in vitro*. Moreover, we showed that only CX3CR1$^{neg/low}$ NK cells promoted upregulation of monocytic CD40, which is in line with the results on the upregulation of CD40 on monocytes co-cultured with CD56^{bright} NK cells reported by Zhang et al.(Zhang et al. , 2007). From these first data, we hypothesized that the CX3CR1$^{neg/low}$ NK cell fraction seems to be identical to the CD56^{bright} NK cells, in terms of both proliferative capacities (Carson et al. , 1997) and monocyte modulation. However, analysis of the co-expression of CD56 and CX3CR1 revealed the existence of three distinct NK cells populations based on CD56 and CX3CR1 expression: CX3CR1^{neg}CD56^{bright}, CX3CR1^{neg}CD56^{dim} and CX3CR1^{high}CD56^{dim} NK cells.

These findings were also confirmed by examination of the expression on the three populations of different natural killer receptors, including KIR, NKp30, NKp46 and NKG2A. Thus, we showed that the chemokine receptor CX3CR1 serves as a marker to define the maturation status and migratory capacities of NK cells. Other previous studies also defined additional markers such as CD62L, CD57, CD11b, and CD27 (Bjorkstrom et al. , 2010, Fu et al. , 2011, Juelke et al. , 2010, Lopez-Verges et al. , 2010, Vossen et al. , 2008, Yu et al. , 2010) and also the NK cell receptors CD94, NKG2A and killer-cell immunoglobulin-like receptors (KIRs) (Beziat et al. , 2010, Yu, Mao, 2010) to be suitable to characterize different maturation stages of NK cells, which are associated with specific functional capabilities. Our analysis of the expression of the differentiation markers CD27, CD62L and CD57 also verified that CX3CR1^{neg}CD56^{dim} NK cells represent an intermediate phenotype, distinct from the fully differentiated CX3CR1^{high}CD56^{dim} (Hamann, Unterwalder, 2011). Thus, as indicated in Figure 3, our study contributed to the elaboration of a more complex picture of the process of NK cell differentiation.

Figure 3

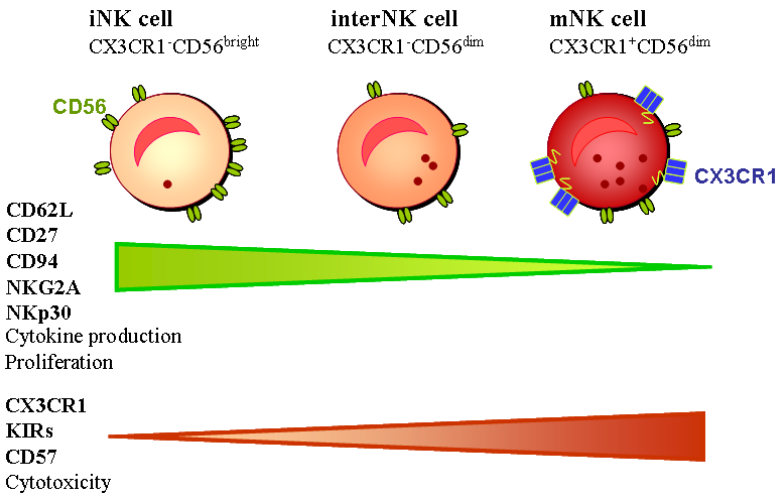

Figure 3: Maturation stages of NK cells according to the expression of the fractalkine receptor CX3CR1. CX3CR1 expression discriminates between three different NK cell subtypes: immature CX3CR1^{neg}CD56^{bright}, intermediary CX3CR1^{neg}CD56^{dim} and mature CX3CR1^{high}CD56^{dim} NK cells. This categorization was confirmed by assessing the expression of CD27, CD62L, NKp30, NKp46 and NKG2A, which decrease during maturation, and the expression of KIRs and CD57 which are known to be upregulated during maturation.

Moreover, we present here evidence for cytokine-mediated regulation of CX3CR1 in NK cells. We showed that IL-15, IL-2 and also TGF-beta downregulated the gene and protein expression of CX3CR1 on human NK cells, confirming previous data on human cells (Bellone et al. , 1995, Dunne et al. , 2001, Rook et al. , 1986, Sechler et al. , 2004) but contradicting the data published on rodents (Barlic et al. , 2003, Chen et al. , 2002). Moreover, CX3CR1 expression was suppressed by IL-12, which is known to modulate NK cell activity (Agaugue et al. , 2008), indicating that most cytokines known to induce NK cell proliferation or activation promote CX3CR1 downregulation in human NK cells.

Next, we characterized NK cell trafficking into the CSF by monitoring frequencies and phenotype of NK cells in paired CSF and blood samples from patients with MS, other inflammatory neurological diseases (IND) and non-inflammatory neurological diseases (NIND) using flow cytometry (Hamann et al. , 2013). Independent of the particular CNS disease category, we observed an overall diminished frequency of NK cells in CSF as compared to blood. The CSF/blood NK cell ratio was particularly decreased in MS patients compared to the other pathologies, which is in agreement with the reported decreased NK cell activity in CSF from MS patients, as compared to other conditions (Salmaggi et al. , 1989). Since in MS patients, the frequencies of T lymphocytes in CSF remained comparable to those observed in other neurological disorders, a reduced NK cell proportion in the CSF may represent a pathology-related hallmark of MS, and possibly other inflammatory diseases. To determine the phenotype of NK cells trafficking into the CSF, we collected paired CSF and blood samples from 9 additional patients with inflammatory neurological diseases including MS. We analyzed the NK cell subsets according to their expression of CD56 and CD16 and their maturation status by means of determining the expression of CX3CR1 and CD27 (Hamann, Unterwalder, 2011). We found that, contrary to blood NK cells, NK cells trafficking to the CSF display a phenotype characteristic of a very early maturation status. Thus, although this study included only a small number of patients, the consistency of the data suggests that the composition of CSF NK cells contrasts with the NK cell composition of the blood. CX3CL1 is known to be expressed on activated endothelial cells (Fong, Robinson, 1998), and recently it has been shown to be expressed at the choroid plexus (Hasegawa-Ishii et al. , 2013). Since receptor-ligand interaction on NK cells may affect NK cell functionality (our own unpublished data; (Pachot et al. , 2008) three potential mechanisms may explain the selective presence of immature NK cell inside the CSF (s. schematic representation in Figure 4).

Thus our data could reflect a selective migration of iNK cells from the circulation into the CSF, or a switch of the NK cell phenotype from mature to immature as a consequence of the trafficking through the blood-CSF barrier (s. Figure 4). The choroid plexus, located within the brain ventricles, is the anatomical basis of the blood-CSF barrier. In a study we published in 2010, we applied Gadofluorine M-enhanced MRI in EAE, and showed the first *in vivo* evidence for the involvement of the choroid plexus in disease development in EAE (Wuerfel et al. , 2010). Moreover, using very small superparamagnetic iron oxide particles (VSOP), we published very recently that the choroid plexus and the meninges are involved in

early inflammatory processes in autoimmune inflammation (Millward et al. , 2013). However the involvement of this structure in NK cell trafficking remains speculative.

Figure 4

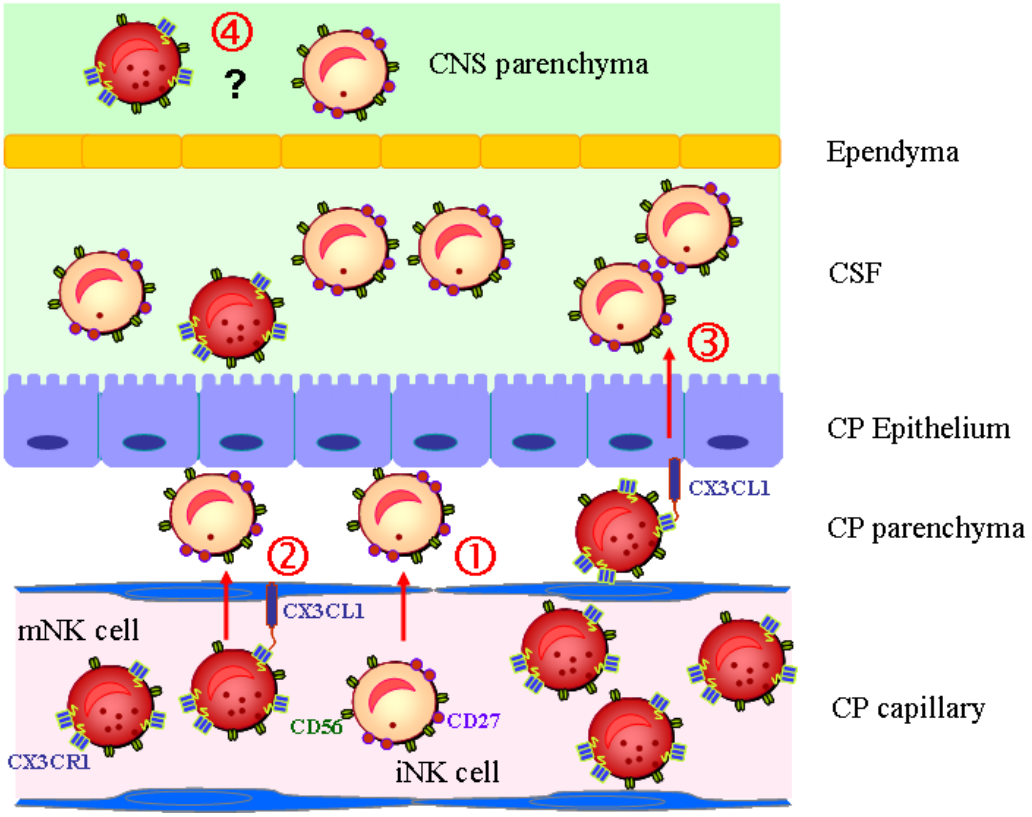

Figure 4. Enrichment of immature NK cells (iNK cells) inside the CSF of patients with neuroinflammatory disorders. NK cells may enter the CFS at the blood-CSF barrier at the choroid plexus (CP). Although mature CX3CR1^{+}CD56^{dim} NK cells (mNK cells) represent the most frequent population of circulating NK cells in patients, the majority of the CSF NK cells display a immature CX3CR1^{-}CD56^{bright} phenotype (iNK cells). Accumulation of iNK cells inside the CSF may be caused by: ① a selective trafficking of iNK cells; ②a phenotypic conversion of mNK cells into iNKcells due to the endothelial transmigration via CX3CL1; ③a phenotypic conversion of mNK cells into iNKcells due to transepithelial migration via CX3CL1.④The phenotype of the cells crossing the ependyma and reaching the human CNS parenchyma remains elusive.

Immature CD56^{bright} NK cells are known to express the homing receptors CD62L and CCR7 (Berahovich et al. , 2006, Vitale et al. , 2004), two receptors also involved in the migration of central memory T cells (Tcm) into the CSF in both patients with MS and NIND (Kivisakk et al. , 2003). CSF CCR7^{+} Tcm seem to differentiate into CCR7^{-} Tem (effector memory) and to acquire the ability to traffic into the CNS parenchyma after interacting with APC inside the subarachnoid space (Kivisakk et al. , 2009, Ransohoff et al. , 2003). Therefore, considering the parallels between NK cell and T cell activation, we speculate that CSF CD56^{bright}CX3CR1^{-}CD27^{+} NK cells might also become matured inside the subarachnoid space by interacting with APC (see Figure 5). Maturation would lead to CCR7 downregulation (Berahovich, Lai, 2006) and CX3CR1 upregulation (Hamann, Unterwalder,

2011), which would allow mature NK cells to move into and adhere to CNS tissue, and which would explain why in CX3CR1 deficient mice, NK cells are not able to enter the CNS (Huang, Shi, 2006).

Figure 5

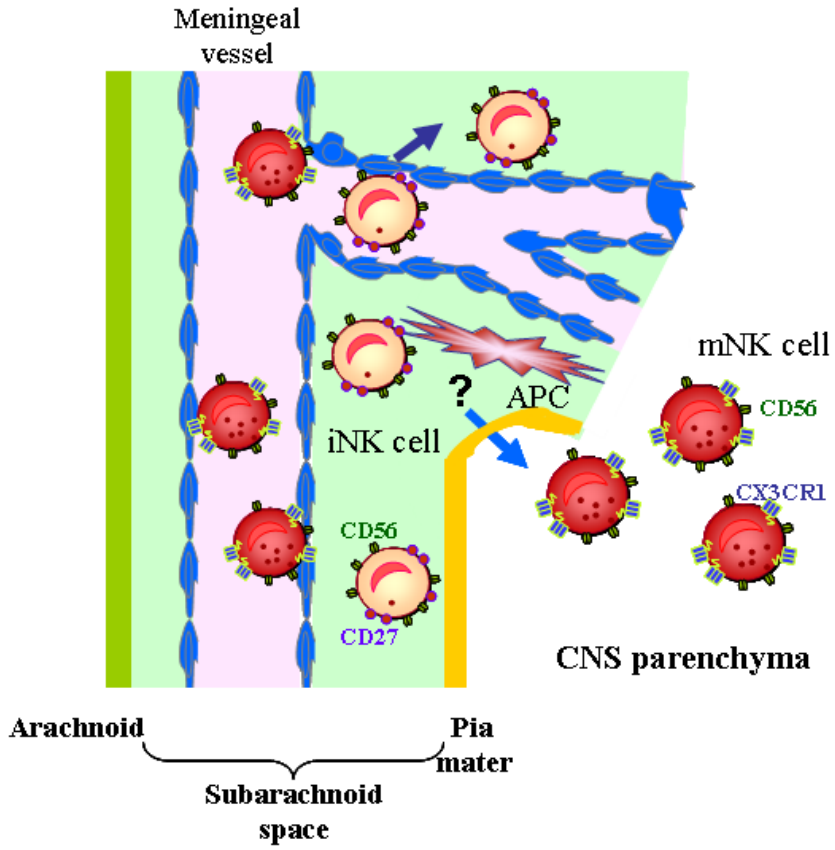

Figure 5. Maturation of iNK cells in the subarachnoid space. Immature NK cells may be converted into mature NK cells inside the subarachnoid space by interacting with APC. The mechanisms of this interaction remain unknown, but may involve CD27. Mature NK cells may then be able to migrate into the CNS parenchyma.

Finally, we investigated how NK cells are modulated during treatment with MX, and whether this modulation may correlate with clinical efficacy of the therapy. In a longitudinal study on a cohort of 15 SPMS patients, we demonstrated that, apart from the expected cytotoxicity to B lymphocytes (Gbadamosi et al. , 2003), MX promoted the enrichment of peripheral neutrophils and restored the frequency of $CD8^{low}$ T lymphocytes, shown to be reduced in untreated patients with clinically isolated syndrome and MS (De Jager et al. , 2008), to healthy levels. In addition, we observed that MX induced an enrichment of NK cells and a shift in the maturation of circulating NK cells, which was associated with clinical response to MX treatment.

We showed that at six months of treatment, both the cytotoxic $CD56^{dim}$ and the immunomodulatory $CD56^{bright}$ subpopulations were enriched, without any shift to a particular phenotype. Since changes in the frequencies of total NK cells were not accompanied by an elevation of absolute NK cell numbers, we believe that this enrichment is the consequence of

the dramatic suppression of other immune populations, primarily B cells. Importantly, we showed that prolonged MX treatment (12 months) did not further affect immune cell frequencies, but affected the proportion of mature NK cells characterized by the dowregulation of CD62L (Juelke, Killig, 2010) as well as the activatory receptors NKp46 and NKp30. This could be partly the consequence of the expected elevated susceptibility of immature proliferating NK cells to MX-induced cytotoxicity. However, cytotoxicity alone may not entirely explain this rather late effect of the treatment, which manifested only after repeated applications of MX, at 12 months. MX treatment enhances the expression of Th2-related cytokines, including IL-4 (Vogelgesang et al. , 2010). IL-4 is known to induce NK cell maturation (Kitajima et al. , 2011). Therefore, we speculate that MX-induced IL-4 may contribute to the maturation of NK cells. Stratifying our data according to clinical response to MX revealed that the elevated frequency of mature NK cells occurred only in the responder patients, and was the only factor related to the beneficial effects of MX. The explanation for this relationship remains unclear. It could be that selective production of Th2-cytokines (Vogelgesang, Rosenberg, 2010) or selective alteration of the functionality of antigen-presenting cells (Neuhaus et al. , 2005) in responding patients may contribute directly or indirectly to NK cell maturation during MX treatment.

Thus, while increased circulation of immature CD56brigh NK cells have usually been associated with the beneficial effect of various MS treatment, such as IFN-beta or daclizumab (Bielekova et al. , 2006, Vandenbark et al. , 2009), we showed in our study with mitoxantrone that rather, it may be the generation of mature functional active subsets of NK cells that are beneficial during MS.

5 Summary

Research in the field of neuroinflammation has tended to focus exclusively on CNS-specific lymphocytes of the adaptive immune system, as these cells are clearly implicated in the pathogenic process. However, the implication of activated non-CNS specific lymphocytes, and the central role of innate lymphocytes, the NK cells remains under-explored.

Using a model of living organotypic brain slices, we demonstrated that non-CNS specific CD4-positive T cells are indeed able to interact with, and induce calcium influx in neurons. This interaction is antigen-independent, and seems to be mediated by perforin and glutamate. Moreover, we combined two imaging approaches, magnetic resonance imaging (MRI) and multiphoton microscopy (MPM), to monitor T cell infiltration and CNS alterations in a model of T cell transfer of OVA- and PLP-specific T cells *in vivo*. We showed that CNS-irrelevant T cells recognizing OVA have the capacity to not only enter the CNS, but also to promote alterations in the permeability of cerebral blood vessels without causing cellular neuropathology. Yet, in the inflamed brain, bystander perturbations of the BBB caused by non-specific effector cells may have further pathological consequences, and might contribute to infection-triggered relapses, as are observed in patients suffering from MS.

Regarding our work on NK cells, we showed that NK cells from MS patients had reduced CX3CR1 expression, and that the frequency of $CX3CR1^{+}$ NK cells correlated with disease activity. Regular CX3CR1 expression in patients clearly correlated with acute relapses and/or with the presence of gadolinium enhancing magnetic resonance lesions, which led to the question of whether this "normal" expression may account for disease remission after the attack. We further demonstrated that $CX3CR1^{-}$ NK cells display a rather immature phenotype and are distinct from fully differentiated CX3CR1-expressing cells in terms of cytolytic activity, cytokine profile, proliferative response and their impact on monocyte functionality. Based on the expression of CX3CR1 and CD56, we identified three stages of NK cell maturation: immature $CX3CR1^{neg}CD56^{bright}$, intermediary $CX3CR1^{neg}CD56^{dim}$ and fully mature $CX3CR1^{high}CD56^{dim}$ NK cells. Thus, in combination with other maturation markers, CX3CR1 can be used to delineate the sequential stages of human NK cell maturation.

To elucidate the implications of NK cells in neuroinflammation, we next characterized NK cells in blood and CSF samples from neurological patients. We demonstrated for the first time that in patients with inflammatory neurological diseases, CSF NK cells display an immature lymph node-like phenotype ($CX3CR1^{neg}CD56^{bright}$), which does not correspond to the phenotype of circulating blood NK cells, revealing a preferential recruitment of immature NK cells into the CSF of those patients. Immature NK cells may employ the same chemotactic tools as central memory T cells to traffic into the CSF. Thus, our data suggest

that the CSF may represent an intermediary compartment for NK cell trafficking and differentiation before entering the CNS parenchyma.

Finally, we demonstrated that NK cells are modulated during mitoxantrone (MX) therapy in patients with progressive MS. Sustained MX treatment promoted not only persistent NK cell enrichment but also NK cell maturation. Importantly, MX-induced NK cell maturation was the only parameter monitored in our study that was associated with the clinical response to MX treatment. To understand how drugs exert their beneficial effects is especially important for patients with progressive MS, since prolonged application of MX and the few other approved therapies cause severe side effects. We consider that our data may serve as a first step in the establishment of novel and safe treatments for this category of patients.

Altogether our studies point to potential collateral effects of non-CNS specific cells in neuronal injury and contribute to identify novel aspects of the biology of NK cells and their implications in neuroinflammation.

6 Conclusions and perspectives

Our data suggest that NK cells may have a protective, disease-limiting role in neuroinflammation. However, in both MS and its animal model, EAE, there are dissenting views that NK cells are rather pathogenic. A possible explanation for these discrepancies is that distinct subsets of NK cells may have divergent functions, or that NK cells exert different effects in distinct disease phases or pathological processes.

We previously demonstrated that expression of the fractalkine receptor CX3CR1 distinguishes between immature and mature NK cells and that the frequency of circulating immature NK cells is increased in stable MS patients, but decreased during relapses. While increased circulation of immature NK cells has usually been associated with the beneficial effect of different MS treatments such as IFN-beta or daclizumab, we showed in a study with mitoxantrone, that rather it is the generation of mature functional active subsets of NK cells that may be beneficial during MS. We also demonstrated that CSF NK cells display an immature phenotype that contrasts with the phenotype of circulating NK cells.

Moreover, our unpublished preliminary work on EAE demonstrated that transfer of competent NK cells prior EAE induction protects mice from developing disease. Moreover, it seems that CX3CR1-mediated migration of NK cells into the CNS may be necessary but not sufficient for controlling EAE progression, and that CX3CR1 may be crucial to maintain interactions of NK cells and dendritic cells during neuroinflammation. However, it remains elusive whether:

1. the CSF truly represents an intermediate compartment, or whether CSF NK cells reflect the phenotype of NK cells trafficking into the CNS parenchyma.
2. NK cells modulate inflammation locally inside the CNS, and/or modulate immune response inside the secondary lymphoid structures.
3. NK cells are in general protective, or whether a particular NK cell subset is responsible for protection and a different subset responsible for damage

Our current and future work aim to clarify these points by:

- Investigating how NK cells affect or are affected during the disease course; by means of NK cell transfer in EAE; and by monitoring NK cells not only in mice, but also during the natural course of MS and in response to MS treatments such as IFN-beta and fingolimod.
- Assessing the implications of CX3CR1 signalling in NK cell-mediated modulation of adaptive immune responses during neuroinflammation. Principally, by focusing on interactions between APC and NK cells using CX3CR1-defficient mice.
- Investigating NK cell trafficking into the CNS in mouse and human. We are presently establishing methods to investigate CSF NK cells in the mouse. In the future, we aim to characterize histologically NK cells inside the human CNS parenchyma on brain biopsy or autopsy material.

In summary, we aim to clarify whether and by which mechanisms NK cells modulate adaptive immune responses and traffic to the CNS. As mentioned above, we will work with

patient material and with the animal model of MS, EAE. While EAE does not recapitulate all aspects of MS, it is nevertheless a widely accepted model of neuroinflammation which is also useful for monitoring NK cell function. Interestingly, experimental depletion of NK cells in mice leads to a more severe form of EAE (Zhang et al. , 1997), and mice deficient for CX3CR1 experienced a more severe form of the disease, while showing reduced infiltration of NK cells into the CNS (Huang, Shi, 2006). Thus, these data suggest that NK cells are able to modulate CNS inflammation also in EAE.

In the mouse, expression levels of CD56 cannot be used to discriminate between different stages of NK cell maturation. However in recent years novel markers have emerged which facilitate the phenotyping of murine NK cells. Mature mouse NK cells express CD49b, CD122, CD161, NKG2D, NKp46 and Ly49 receptors, among others. Moreover, based on the expression of CD11b and CD27, mouse NK cells can be categorized into four different stages of maturation, starting at a $CD11b^{low}CD27^{low}$ stage, and proceeding through the following stages: $CD11b^{low}CD27^{high} \rightarrow CD11b^{high}CD27^{high} \rightarrow CD11b^{high}CD27^{low}$.

Although we must always exercise caution when extrapolating EAE data to the human situation, I hope that the investigation of the role of NK cells both in mouse and human will contribute to better understanding of the biology of NK cells in general and, in particular, their implications in neuroinflammation.

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Declaration / Erklärung

Gemäß § 4 Abs. 3 (k) der HabOMed der Charité

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