The importance of $CD8^+$ T cells and antigen-presenting cells in the immune reaction of primary inflammatory versus degenerative diseases

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Nicholas Schwab

Würzburg, April 2009

Für meine Eltern.

I could not prove the Years had feet —
Yet confident they run
Am I, from symptoms that are past
And Series that are done —

I find my feet have further Goals —
I smile upon the Aims
That felt so ample — Yesterday —
Today's — have vaster claims —

I do not doubt the self I was
Was competent to me —
But something awkward in the fit —
Proves that — outgrown — I see —

Emily Dickinson

#563

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1. Summary

The bidirectional influence of parenchymal cells and cells of the immune system, especially of antigen-presenting and $CD8^+$ T cells, in situations of putative auto-immune pathogenicity and degeneration was the main topic of this thesis.

In the first part, the influence of human muscle cells on antigen-presenting cells was investigated. In inflammatory myopathies prominent infiltrates of immune cells containing T cells and antigen-presenting cells like macrophages and dendritic cells are present. The hypothesis was that human myoblasts have an inhibiting influence on these antigen-presenting cells under homeostatic conditions. A dysfunction or impairment under inflammatory circumstances might contribute to the development of myopathic conditions. The surface analysis of dendritic cells cocultured with myoblasts showed that immature dendritic cells could be driven into a reversible semi-mature state with significantly elevated levels of CD80. These dendritic cells were additionally characterized by their inhibiting function on T-cell proliferation. It was also shown that the lysates of healthy myoblasts could strongly enhance the phagocytic ability of macrophages, which could help with muscle regeneration and which might be disturbed in myositis patients.

The second part of this thesis was about the clonal specificity of $CD8^+$ T cells in a mouse model with genetically induced over-expression of PLP in oligodendrocytes. Here, we could show that the cytotoxic T lymphocytes, which had previously been shown to be pathogenic, were clonally expanded in the CNS of the transgenic mice. The amino acid sequences of the corresponding receptor chains were not identical, yet showed some similarities, which could mean that these clones recognize similar antigens (or epitopes of the same antigen). The knockout of PD-1 in this setting allowed for an analysis of the importance of tissue immune regulation. It became evident that the absence of PD-1 induced a larger number of clonal expansions in the CNS, hinting towards a reduced threshold for clonal disturbance and activation in these T cells. The expansions were, however, not pathogenic by themselves. Only in the presence of tissue damage and an antigenic stimulus (in our case the overexpression of PLP), the PD-1 limitation exacerbated the immune pathogenicity. Therefore, only in the presence of a “tissue damage signal”, the dyshomeostasis of T cells lacking PD-1 achieved high pathogenetic relevance.

Finally, we investigated the pathogenetic role of $CD8^+$ T cells in Rasmussen encephalitis, a rare and chronic neurological disease mainly affecting children. The analysis of the T-cell receptor repertoire in Rasmussen encephalitis patients in the peripheral $CD4^+$ and $CD8^+$ T-cell compartments as well as the brain revealed the involvement of T cells in the pathogenicity of this disease. Many clonal expansions in the brain matched $CD8^+$ T-cell expansions in the periphery on the sequence level. These putatively pathogenic clones could be visualized by immunohistochemistry in the brain and were found in close proximity to astrocytes and neurons. Additionally, the expanded clones could be found in the periphery of patients for at least one year.

2. Zusammenfassung

Der Einfluss von Parenchymzellen auf Immunzellen und umgekehrt, im Besonderen von Antigen-präsentierenden Zellen und $CD8^+$ T-Zellen, im Zusammenhang von autoimmuner Pathogenese und Degeneration war das Hauptthema dieser Dissertation.

Im ersten Teil wurde der Einfluss menschlicher Muskelzellen auf Antigen-präsentierende Zellen untersucht. In entzündlichen Myopathien kommt es zu massiven Infiltraten von Immunzellen, die T-Zellen und auch Antigen-präsentierende Zellen wie Makrophagen und dendritische Zellen enthalten. Die Hypothese war, dass menschliche Myoblasten einen hemmenden Einfluss auf die Antigen-präsentierenden Zellen unter homöostatischen Bedingungen haben. Eine Störung dieses Einflusses oder eine Beeinträchtigung unter entzündlichen Rahmenbedingungen könnte eventuell zur Entwicklung eines myopathischen Zustands beitragen. In der Oberflächenanalyse der dendritischen Zellen, die mit Myoblasten kultiviert wurden, zeigte sich, dass unreife dendritische Zellen in einen halb-reifen Zustand versetzt werden konnten, der sich beispielsweise durch stark erhöhte CD80 Expression kennzeichnet. Diese dendritischen Zellen wurden weiterhin charakterisiert über ihre hemmende Funktion auf die T-Zell Proliferation. Außerdem wurde gezeigt, dass Zelllysate gesunder Myoblasten die Phagozytoserate von Makrophagen enorm verstärken, was die Regeneration des Muskelgewebes erhöhen und möglicherweise in Myositispatienten gestört sein könnte.

Im zweiten Teil der Dissertation ging es um die klonale Spezifität von $CD8^+$ T-Zellen in einem Mausmodell mit genetisch induzierter Überexpression von PLP in Oligodendrozyten. Hier konnte gezeigt werden, dass die zytotoxischen T-Zellen, deren Pathogenität Gegenstand früherer Arbeiten war, im ZNS der transgenen Mäuse klonal expandiert waren. Die Aminosäuresequenzen der TCRβ Kette der expandierten Klone waren nicht identisch, zeigten jedoch einige Ähnlichkeiten, die darauf hinweisen könnten, dass diese Klone ähnliche Antigene (oder Epitope des gleichen Antigens) erkennen. Die genetisch induzierte Abwesenheit von PD-1 ermöglichte es, in diesem Zusammenhang den Einfluss von spezifischer Immunregulation im Gewebe zu untersuchen. Es zeigte sich, dass die Deletion von PD-1 eine erhöhte Anzahl von klonalen Expansionen im ZNS der Mäuse erzeugte, was auf eine herabgesetzte Schwelle für klonale Störungen und Aktivierung schließen lässt. Diese Expansionen

waren jedoch für sich genommen nicht pathogen. Nur in der Anwesenheit eines Gewebeschadens und eines zusätzlicher Antigenstimulus (in unserem Fall in Form der PLP Überexpression) konnte man die erhöhte Pathogenität durch die PD-1 Deletion erkennen. Deswegen erreichten die PD-1 deletierten T-Zellen nur in der Gegenwart eines „Gewebeschaden-Signals" hohe pathogenetische Relevanz.

Schließlich untersuchten wir die pathogenetische Rolle von $CD8^+$ T-Zellen in der Rasmussen Enzephalitis, einer seltenen, chronischen Erkrankung des Gehirns, die hauptsächlich in Kindern vorkommt. Die Analyse des T-Zell-Rezeptor Repertoires in Rasmussen Enzephalitis Patienten in peripheren $CD4^+$ und $CD8^+$ T-Zell Populationen und im Gehirn zeigte die Beteiligung von T-Zellen in der Pathogenese dieser Krankheit auf. Viele klonale Expansionen waren zwischen Gehirn und der peripheren $CD8^+$ Population bis hin zur Aminosäuresequenz identisch. Diese vermutlich pathogenen Klone konnten in Gehirnbiopsien von Rasmussenpatienten histochemisch nachgewiesen werden und wurden in enger Nachbarschaft zu Astrozyten und Neuronen gefunden. Zusätzlich konnten diese expandierten Kone in der Peripherie von Patienten für die beobachteten Zeiträume (mindestens ein Jahr) nachgewiesen werden.

3. Introduction

3.1. Overview of the immune system

Although there are several mechanisms in plants as well as invertebrates to repel pathogens or parasites, the development of the vertebrate immune system was from an evolutionary point of view the first appearance of synergistic interaction of innate and especially adaptive immune components. The innate, antigen-unspecific immune system employs immune cells like mast cells, macrophages, neutrophils, basophils, and eosinophils, inflammatory chemical factors like histamine, bradykinin, and leukotrienes, and the complement system to deplete pathogens in a generic way, but fails to confer long-lasting immunity. The adaptive, antigen-specific immune system, however, has the ability to recognize and remember specific pathogens to confer immunity. The adaptive immune system relies on the somatic hypermutation of B cells and V(D)J recombination of B and T cells to react to virtually every possible antigen. These irreversible changes in the DNA of the respective cell are also key to lifelong immunological memory in the form of memory B and T cells (Murphy et al., 2007).

3.2. T-cell development

While B cells are the main mediators of the humoral arm of the adaptive immune system, T cells largely account for the cellular adaptive component and also integrate innate and adaptive immunity. T cells originate in the bone marrow from hematopoietic stem cells, which then populate the thymus and generate immature thymocytes. In the earliest stage, T cells express neither CD4 nor CD8 (double-negative, $CD4^-CD8^-$ T cells). Via the double-positive stage ($CD4^+CD8^+$), the T cells finally mature into single-positive ($CD4^+CD8^-$ / $CD4^-CD8^+$) cells and leave the thymus. An important part of the maturation process is the selection of T cells in the thymus (Zuniga-Pflucker, 2004).

The positive selection makes sure that the T-cell is able to interact with its respective MHC molecule (MHC class I for $CD8^+$ T cells, MHC class II for $CD4^+$ T cells). To achieve this, the double-positive T-cell migrates within the thymal cortex, where random self-antigens are presented on MHC molecules by epithelial cells. A cell which is able to

interact with an MHC class I molecule will later become a $CD8^+$ T-cell, while a T-cell with a working MHC class II / TCR interaction will later become a $CD4^+$ T-cell (positive selection for MHC interaction) (Singer et al., 2008).

The negative selection takes place in the medulla of the thymus, where antigen-presenting cells such as dendritic cells or macrophages present random self-antigens on their MHC molecules. If a T-cell shows a strong reaction to a self-antigen, it is negatively selected at this stage and goes into apoptosis. This is the most important step in T-cell development to prevent autoimmunity and promote immunological tolerance to self-antigens ("central tolerance") (Figure 1). Together, positive and negative selection sort out up to 98% of T lymphocytes to ensure functioning as well as tolerance against self-antigens (Ciofani and Zuniga-Pflucker, 2007).

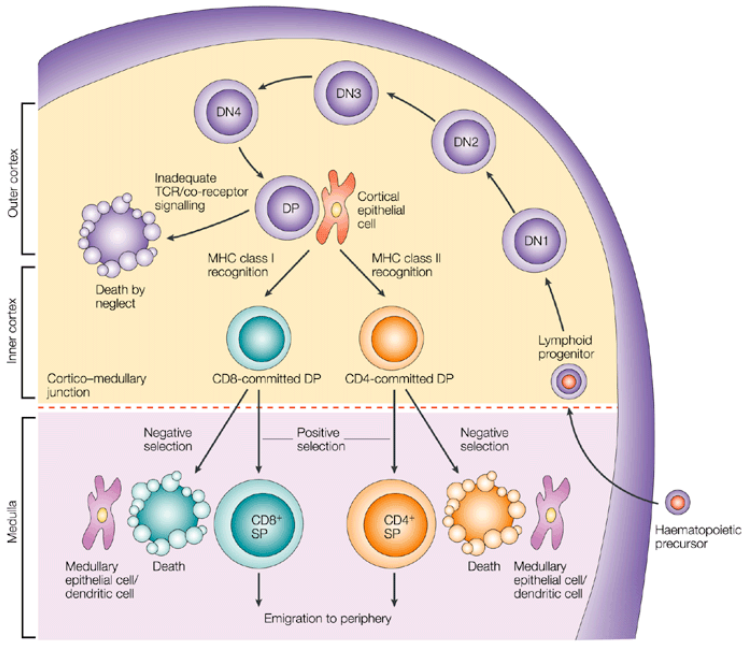

Figure 1: T-cell development in the thymus (Germain, 2002).

Although it has not been discovered yet, why these two mechanisms of selection do not eliminate all T cells, there are two hypotheses, which might explain this situation: The differential avidity hypothesis states that signal strength decides the outcome of the T-cell / MHC interactions (Girao et al., 1997) and the differential signaling hypothesis proposes that signal transduction differs in T cells, depending on their maturation stage.

3.3. The T-cell receptor

3.3.1. V(D)J recombination of the T-cell receptor

The V(D)J recombination occurs when segments of genes encoding for specific proteins in the immune system are randomly assembled by genetic recombination. It allows for a vast number of different antibodies (in B cells) and T-cell receptors (in T cells). Most T-cell receptors are composed of an α/β chain combination (there are also small numbers of γ/δ T cells). The genes of the T-cell receptor resemble the immunoglobulin genes in their organizational structure: there are multiple V (variant), D (diversity) and J (joining) genes (α chains are missing the D genes) rearranged during T-cell development to guarantee a unique antigen-specificity. During T-lymphocyte development, the genetic recombination starts with a D-to-J recombination in the β chain, followed by V-to-DJ rearrangements (Janeway, 2005). The genetic regions between the combined genes are permanently deleted to allow translation of a full length TCR β chain. The α chain rearrangement, which starts after a functional β chain has been produced, consists of a V-to-J rearrangement, followed by the formation of the functional αβ-TCR (Figure 2).

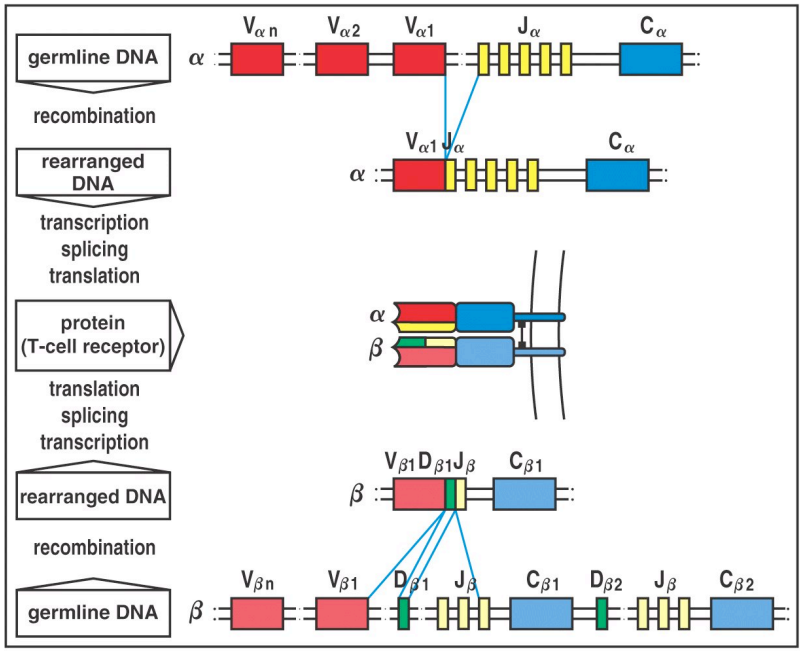

Figure 2: T-cell receptor α- and β-chain gene rearrangement and expression (Janeway, 2005).

3.3.2. The CDR3 of the TCR

The antigen-binding sites of the TCR, in contrast to those of immunoglobulins, have to conform to the principles of TCR / (MHC + antigen) binding ("trimolecular complex"). This implies limited fluctuation in the regions that connect with the MHC, whereas the TCR region linked to the bound antigen focuses on variability. The diversity of TCRs can be attributed mainly to combinatorial and junctional diversity, which is generated during gene rearrangement. In TCRs, the most variable regions are the CDRs (complementarity determining region). The CDR1 and CDR2 loops (encoded within the germline V gene segments) mainly contact the relatively less variable MHC section rather than the highly variable peptide component. The CDR3, to which the D and J segments contribute, form the center of the antigen-binding site (Jones et al., 2008) (Figure 3). The junctional region encoded by V, D, and J segments and modified by P- and N-nucleotides encodes the CDR3 loop and provides the TCR with variability, which surpasses the one of antibodies due to the very high number of J segments in the TCR chains (e.g. 61 J_α gene segments in humans). The theoretical diversity of an αβ-TCR is roughly 10^{18}, 10^4 times higher than the one of immunoglobulins (Davis and Bjorkman, 1988).

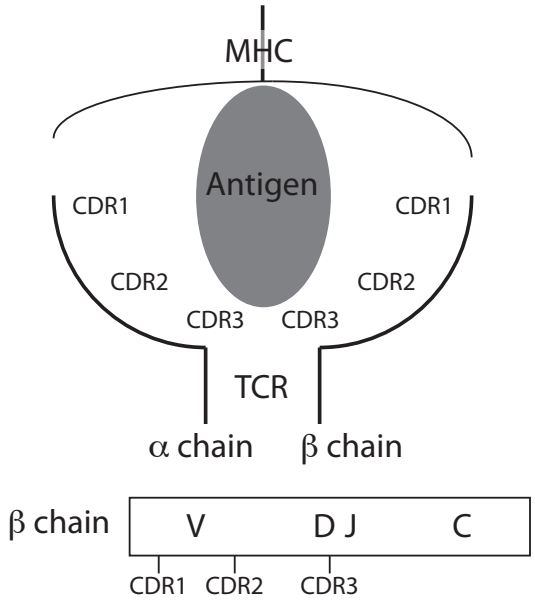

Figure 3: Position of the CDR loops within the TCR-MHC complex.

3.3.3. P- and N-nucleotides

The diversity of the CDR3 is increased immensely by the addition/deletion of nucleotides when forming the junction between the gene segments. The added nucleotides are called P- and N-nucleotides. P-nucleotides are named after palindromic nucleotide sequences added to the ends of gene segments during rearrangement, whereas N-nucleotides are nontemplate-encoded and are randomly added by the enzyme terminal deoxynucletidyl transferase (Gauss and Lieber, 1996).

3.4. T-cell activation and proliferation

Naive T cells need to be activated to be able to exert their specific functions. To be stimulated enough to undergo activation and clonal expansion, T cells mainly need two signals. The first signal (signal I) is the stimulation of the TCR by an antigen-MHC complex (which makes it antigen-specific), while the second signal (signal II) is provided by antigen-presenting cells and is usually called costimulatory (Grossman et al., 2004). The signal II in most cases is mediated through the B7:CD28 pathway, where B7-1 and B7-2 (CD80 and CD86) are expressed on the APC and CD28 is expressed on T cells. Other important points are that both signals have to be provided by the same APC and $CD8^+$ T cells are generally believed to need a stronger costimulatory signal to achieve sufficient activation (Emmrich et al., 1986). Sometimes, the induction of the rapamycin pathway by IL-2 is referred to as signal III (Vincenti, 2008).

After activation, a naive T-cell expresses several proteins contributing to sustaining and/or modifying the costimulatory signal, which is essential for clonal expansion and differentiation. For example, CD40 ligand is upregulated, which in turn binds to CD40 on the APC and thereby induces upregulation of the B7 molecules on the APC in a positive feedback loop (Kotowicz et al., 2000). Other CD28-related proteins involved in the activation of T cells are inducible co-stimulator (ICOS) on T cells (Kotowicz et al., 2000) or CTLA-4 (CD152) (Linsley et al., 1991), which resembles CD28, but binds to the B7 molecules about 20 times more avidly than CD28 and delivers an inhibitory signal to the activated T-cell. CTLA-4 is strongly expressed on naturally occurring regulatory T cells (Treg) and is involved in the mediation of Treg function. The inhibition by CTLA-4 leaves the T-cell less sensitive to stimulation by the APC and restricts the production of IL-2 (the most important autocrine T-cell growth factor). IL-2 production can be

regarded as the most prominent sign of successful T-cell activation, because only a two-signal activation increases the IL-2 production of the T-cell 100fold, while activation in the absence of co-stimulation through CD28 only induces little IL-2 production and fails to induce T-cell proliferation (Murphy et al., 2007).

Naive $CD8^+$ T cells differentiate into cytotoxic cells through antigen-specific activation, which can be provided by dendritic cells with high intrinsic costimulatory activity. DCs can directly induce IL-2 production, proliferation and differentiation in $CD8^+$ T cells, which has been previously used to generate cytotoxic T-cell responses against tumors (Nair et al., 2000). However, in some cases, $CD8^+$ T-cell responses require the presence of $CD4^+$ T cells, which have to recognize related antigens on the surface of the same APC. Presumably, these $CD4^+$ T cells are needed to compensate for inadequate costimulation by the APC, for example by expressing CD40 ligand (Janeway, 2005).

3.5. PD-1

In the recent years, more members of the B7:CD28 family and additional costimulatory pathways have been discovered. Interestingly, these pathways can act as positive and negative second signals, depending on the involved molecules.

Programmed death 1 (PD-1, CD279), which was discovered in 1992 (Ishida et al., 1992), can be expressed on T cells, B cells, NK-T cells, activated monocytes and DCs and exerts strong inhibitory signals through its ligands PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273). There are no data supporting a function for PD-1 in the absence of antigen receptor signaling (Keir et al., 2008). PD-1 interacts with its ligands to exert important and diverse immunoregulatory functions during T-cell activation and immune-mediated tissue damage (Figure 4). However, the most important role could be the control of potentially pathogenic effector T cells and the inhibition of early activation events taking place in lymph nodes, when naive T cells encounter antigens.

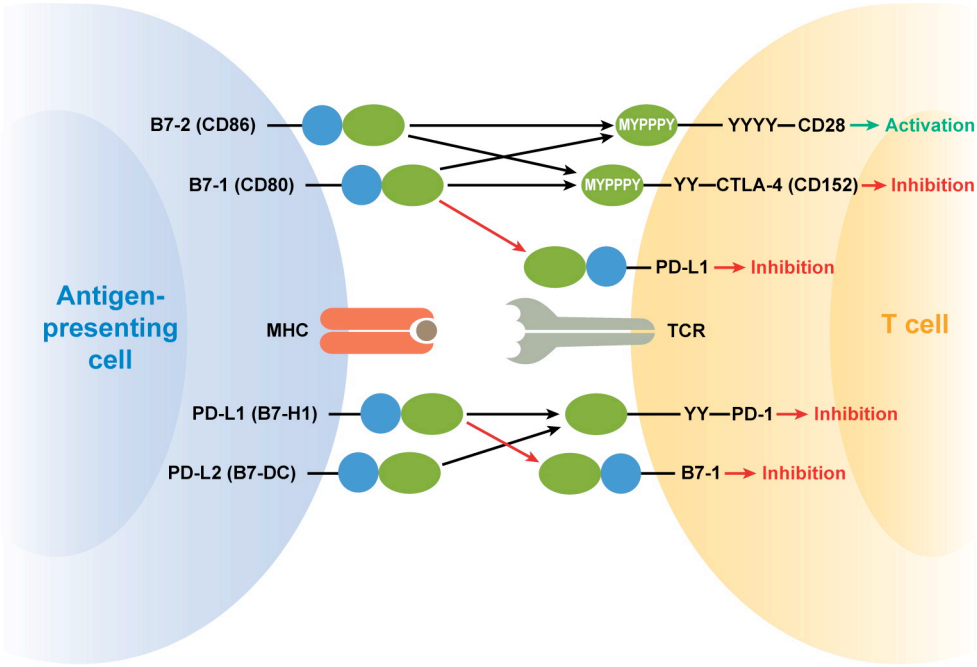

Figure 4: B7-1:PD-L1interaction pathways in the B7:CD28 family (Keir et al., 2008).

3.6. Proteolipid protein

The proteolipid protein (PLP) is the predominant myelin component of the CNS (about 50% of total CNS protein) (Milner et al., 1985). Although its function is not completely understood yet, it seems to have a structural role in the compaction of the extracellular surface. Its importance is underlined by its role in the pathogenesis of Pelizaeus-Merzbacher disease (PMD) (Woodward, 2008) and X-linked spastic paraplegia type 2 (Hodes et al., 1998), which can both be caused by PLP gene mutations. Additionally, overexpression of PLP can cause pathological changes (many PMD patients have PLP gene duplications). The molecular links between mutation and pathogenesis are still elusive.

3.7. Multiple sclerosis and gliopathy-induced inflammation

Muliple sclerosis (MS) is an inflammatory disorder of the CNS, mainly affecting young adults. The symptoms range from sensitivity dysfunctions to deficits of motor and cognitive functions. While there are several distinct forms of MS (relapsing-remitting, primary progressive, secondary progressive), the heterogeneity between patients is very high. Genetic as well as environmental factors are thought to play major roles in

the development and course of the disease (Sospedra and Martin, 2005). There are several aspects of MS still under discussion and heavy research, e.g. the involvement of the immune system as cause or epiphenomenon (Chaudhuri and Behan, 2004), the importance of $CD8^+$ T cells (Zozulya and Wiendl, 2008), and the relevance of animal models in an already complex human disease with many different patterns. However, the animal model of the experimental autoimmune encephalomyelitis (EAE) has been very useful in the past not only to investigate the pathogenic mechanisms putatively involved in the development of MS, but also in establishing pharmacological treatments.

The term "gliopathy" usually stands for the dysfunctional response of glial cells, specifically astrocytes, oligodendrocytes and microglia, to neural damage that is initiated by the sudden injury induced increase in extracellular concentrations of glutamate and concomitant production of several proinflammatory molecules (Hulsebosch, 2008). Gliopathy is thought to play a role in Multiple sclerosis as “dying-back gliopathy”, which is first noted in the most distal extension of the oligodendrocytes, meaning the myelin sheaths (Rodriguez, 1989). It is important to distinguish between immune-cell mediated gliotoxicity and primary gliopathy, both are discussed to be the cause of lesion development in MS.

3.8. The PLP-tg mouse as a model for gliopathy-induced inflammation

One of the mouse models used in this thesis overexpresses PLP in oligodendrocytes (PLP-tg mice), which causes a late-onset and slowly progressing demyelination associated with axonopathic changes (Anderson et al., 1998). The model has been established by Readhead et al. (Readhead et al., 1994) and was analyzed with respect to the relevance of secondary immune-mediated damage by Ip et al. (Ip et al., 2006). It was shown that $CD11b^+$ macrophage-like cells and $CD8^+$ T cells were pathologically involved in the primarily genetically caused myelinopathy. Also, bone marrow chimaera reconstitutions suggested a critical role of the $CD8^+$ T cells in the secondary immune reaction occuring after a primary glial damage. This process has previously been suggested as pathogenetic mechanism in some forms of Multiple sclerosis (oligodendropathy-induced inflammation, pattern III) (Lucchinetti et al., 2000). Therefore, this model was chosen to investigate the role of $CD8^+$ T cells, where the only possible trigger for an immune response is the overexpression of an (auto-)antigen.

3.9. The CNS - a site of immunological privilege?

At the end of the 19th century, experiments with specific dyes by Paul Ehrlich and colleagues showed that the CNS is separated from the remaining blood stream by a blood brain barrier. Later on in the 1920s, tissue graft experiments proved that the brain and the anterior chamber of the eye are sites in which tissues can be grafted without inducing rejection. These locations were termed "immunologically privileged" sites. Further studies showed that their extracellular fluid does not pass through conventional lymphatics. However, proteins placed artificially in these sites leave them and induce immunological effects. Also, the blood brain barrier (largely) excludes naive lymphocytes. Humoral factors (esp. cytokines as TGF-β) can be produced at privileged sites and leave them together with antigens (Pachter et al., 2003). As an additional point, Fas ligand is expressed by tissues of immunologically privileged sites and may provide protection by inducing apoptosis of Fas^+ lymphocytes (Ferguson and Griffith, 1997; Green and Ware, 1997).

Interestingly, antigens in such "privileged" sites as the brain are often the targets of autoimmune reactions (e.g. myelin protein in Multiple sclerosis). Obviously, these antigens do not induce tolerance due to clonal deletion of self-reactive T cells. They induce neither tolerance nor activation, but if activation is induced elsewhere they can become targets for autoimmune attacks. Probably, T cells specific for antigens of immunologically privileged sites are more likely to remain in a state of immunological ignorance. Effector T cells, in contrast to naive T cells, can enter these sites to fight off pathogens during infection.

In the last years, several aspects of immunological privilege have been reviewed and reevaluated (Galea et al., 2007). It has been shown that a certain level of privilege is indispensable for limiting damage during an immune response in an organ with inadequate regenerative abilities and crucial importance to the whole organism. However, this privilege does not comprise a complete absence of immunological reactions, but an extraordinary coordination and regulation of these potentially harmful immune responses. Therefore, privilege in the context of the brain has to be seen as relative, compartmentalized (Murphy and Sturm, 1923), and also active, meaning that lacking immune responses in the brain are not only due to the absence of immune cells, but also due to their active confinement by the brain itself. For example, neurons suppress the induction of MHC molecules by microglia (Neumann et

al., 1998) and microglial activation by expression of CD200 (Hoek et al., 2000). However, these confinemets are not always effective enough to prevent auto-immune damage in the CNS mediated by cytotoxic T lymphocytes.

3.10. $CD8^+$ T cells and $CD8^+$ T-cell-mediated cytotoxicity

Pathogens residing inside of cells (e.g. viruses, some bacteria) are not accessible to humoral immunity and can only be eradicated by destroying the host cell. In the adaptive immune system, this is the role of $CD8^+$ cytotoxic T lymphocytes (CTL). CTLs are also crucial in the defense of some protozoan infections (e.g. Toxoplasma gondii (Yamashita et al., 1998)). Fine-tuning the killing of target cells is the most accomplished achievement of this part of the adaptive immune system. CTLs kill their target cells by inducing apoptosis in as fast as 5 min after contact. In the course of apoptosis, not only the host cell is affected, but also the cytosolic pathogens. Nucleases activated in apoptosis to destroy cellular DNA can also degrade viral DNA. Thus, apoptosis is preferable to necrosis, because in the case of a necrotic cell, intact virions can be released to infect neighboring cells, whereas in apoptosis several mechanisms damage the pathogens in the process of cell death (Molloy et al., 1994).

CTL exert their killing through the calcium-mediated release of lytic granules to the surface of a target cell. These modified lysosomes contain perforin, which polymerizes to form pores in the target cell's membrane, and granzymes (serine proteases). These molecules work together in inducing apoptosis as the perforin forms cylindrical structures, lipophilic on the outside and hydrophilic in the center, to allow water and salts to be exchanged between cell and extracellular space. This alone could be shown *in vitro* to be enough to kill a target cell. However, *in vivo* the perforin effect is only the first step in inducing apoptosis. The granzymes can now enter the target cell and trigger an enzyme cascade. Granzyme B cleaves CPP-32, which activates a nuclease (caspase-activated deoxyribonuclease, CAD), leading to final DNA degradation (Rodrigues et al., 1992). It should be noted that a part of the cytotoxic ability of CTL is due to the expression of Fas ligand, which leads to the activation of caspases in target cells. It has been proposed that this mechanism is important in regulating peripheral immune responses and in terminating lymphocyte growth at the end of an adaptive response (Lenardo et al., 1999).

All CTL responses described up to this point were antigen-specific and dependent on a TCR / MHC I interaction. However, CTL can also act unspecifically through cytokines (IFN-γ (Berg et al., 2005), TNF-α (Chamberlain et al., 2006) and TNF-β (Adamthwaite and Cooley, 1994)) and thereby inhibit viral replication, induce expression of MHC I molecules, and activate macrophages.

3.11. Rasmussen encephalitis

Rasmussen encephalitis (RE) is a rare chronic progressive disorder of childhood associated with hemispheric atrophy, focal epilepsy, intellectual decline, and progressive neurological deficits (Bien et al., 2005; Dubeau et al., 2007; Rasmussen et al., 1958). Etiology and pathogenesis of this severely disabling inflammatory disease are still enigmatic. An intriguing feature of RE is the restriction of the inflammatory process to one brain hemisphere, setting it apart from any other inflammatory disease of the central nervous system (CNS). Histopathological findings in RE comprise lymphocytic infiltrates, microglial nodules, neuronal loss, and gliosis of the affected hemisphere. The hypothesis of RE as a primarily antibody-driven attack against neuronal structures (e.g. the glutamate receptor GluR3 (Rogers et al., 1994)) could not be confirmed in larger cohorts (e.g. (Watson et al., 2004; Wiendl et al., 2001)). Active brain inflammatory lesions contain large numbers of T lymphocytes, which are recruited early within the lesions suggesting that a T-cell-dependent immune response contributes to the onset and evolution of the disease (Farrell et al., 1995). Moreover, the histopathological observation of GranzymeB containing CTL in direct apposition to MHC class I positive neurons raised the hypothesis of a CTL mediated neuronal attack as a key pathogenetic mechanism underlying RE (Bien et al., 2002). Apart from neuronal cell death, CD8 cells may also be responsible for the degeneration of astrocytes found in RE lesions (Bauer et al., 2007). An antigen-driven, putatively CNS-directed adaptive T-cell response would, however, only be proven by demonstrating focusing of the T-cell receptor repertoire in CNS infiltrating immune cells and/or characterizing the antigen-specificity of the CNS infiltrating lymphocytes (Dornmair et al., 2003; Junker et al., 2007; Skulina et al., 2004). Thus far, only one study on the TCR repertoire in RE has been done (Li et al., 1997) and no information was available regarding the putatively pathogenic CD8 T cells.

3.12. Myositis

Idiopathic inflammatory myopathies are a heterogenous group of subacute, chronic, or acute acquired diseases of skeletal muscle characterized by moderate to severe muscle weakness and inflammation. The inflammatory myopathies comprise three major and distinct subsets: dermatomyositis (DM), polymyositis (PM) and inclusion body myositis (IBM) (Dalakas and Hohlfeld, 2003). Although each subset retains characteristic clinical, immunopathological, and morphological features, they all share the presence of varying amounts of infiltrating antigen-presenting cells (APC), B cells, macrophages, natural killer cells, and T cells within the muscle (Dalakas, 2004; Greenberg, 2007). So far, studies have mainly been analyzing the presence and relative frequencies of these specific cell types, immune system related changes in muscle fibres (e.g. the MHC class I expression on muscle fibres) and blood vessels (Wiendl et al., 2003a). The interactions between muscle cells and immune cells have mostly been described as an attack of the immune system on muscle cells, neglecting the influence of muscle cells on immune cells. In recent years, our group has focused on the influence of human myoblasts on the immune system and its implications in possible therapeutic options (Wiendl, 2008; Wiendl et al., 2005a, b; Wiendl et al., 2003b; Wiendl et al., 2003c), so the contribution of antigen-presenting cells in this setting, which is assessed in this thesis, appeared to be the next logical step.

3.13. Antigen-presenting cells

3.13.1. Dendritic cells

Dendritic cells arise from myeloid progenitors within the bone marrow, and emerge from the bone marrow to migrate in the blood to peripheral tissues. The main function of dendritic cells is the presentation of antigens to T cells, and mature DC, which have migrated to lymphoid organs (mainly lymphnodes), are by a large margin the most proficient stimulators of naive T cells. They express high levels of cell adhesion molecules, MHC molecules, CD80, CD86, and CD40 enabling them to interact with antigen-specific T cells (Steinman, 2007). Immature DC, which patrol the surface epithelia and internal organs like the heart and kidneys, are mainly responsible to internalize antigen fragments by phagocytosis and macropinocytosis, thereby creating

a snapshot memory of the antigen repertoire present in a certain microenvironment at a given time. In contrast to mature DC, immature DC are characterized by low MHC, CD80, CD83, and CD86 expression and high expression of DC-Sign (Ueno et al., 2007). It has previously been shown that immature DC can exert tolerogenic functions by secreting anti-inflammatory cytokines like IL-10 or inhibiting T-cell proliferation in a cell-cell contact dependent manner (Li et al., 2005). Additionally, immature DC that reach the end of their life span without ever having been activated by infection travel to local lymphoid tissue and present the gathered antigens. These DC do not express high amounts of costimulatory molecules and induce tolerance to the gathered self-antigens derived from peripheral tissues (Zanoni et al., 2007).

Immature dendritic cells persist in the peripheral tissues for variable lengths of time. When an infection occurs, they migrate via the lymphatics to the local lymphoid tissues, where they mature into a completely different phenotype. They are no longer able to engulf antigens. However, they now express very high levels of long-lived MHC class I and II molecules, enabling them to stably present peptides from proteins acquired from the infecting pathogens. They also express very high levels of adhesion molecules, as well as high levels of B7 molecules. These properties help dendritic cells to stimulate strong naive T-cell responses (Shreedhar et al., 1999).

The signals that activate tissue dendritic cells to migrate and mature after taking up antigen are clearly of key importance in determining whether an adaptive immune response will be initiated. The best-understood example is the response to gram-negative bacteria, whose cell walls contain lipopolysaccharides (LPS). Receptors recognizing LPS are found on dendritic cells and macrophages, and these associate with the Toll-like signaling receptor TLR-4, which then activates the transcription factor NFκB. Signaling through this pathway induces the expression of B7 molecules. Thus, an immature tissue dendritic cell that binds and internalizes a gram-negative bacterium is primed to migrate to local lymphoid tissue and present bacterium-derived peptide antigens to naive T cells (Anderson et al., 2008) (Figure 5).

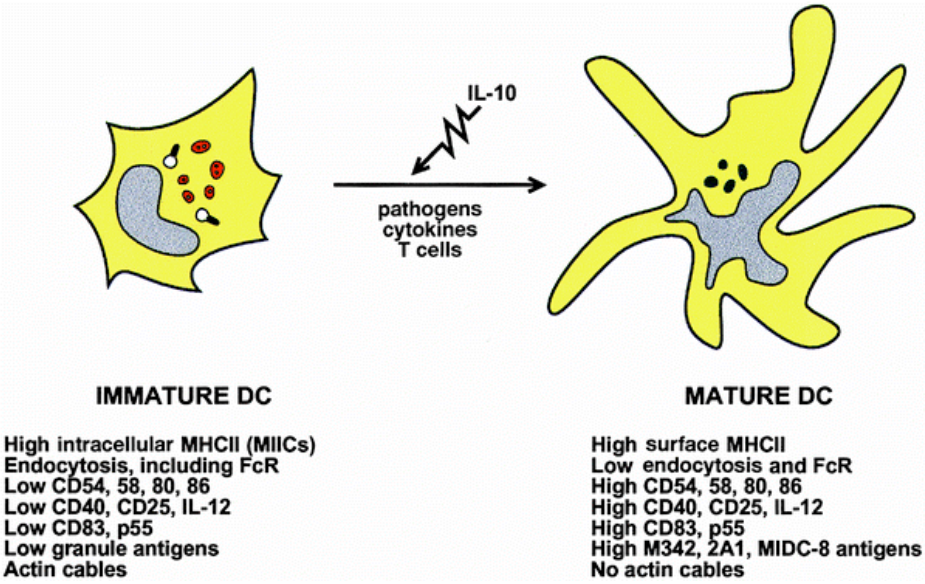

Figure 5: Features that change during DC maturation (Banchereau and Steinman, 1998).

Dendritic cells are also particularly important in stimulating T-cell responses to viruses, which fail to induce co-stimulatory activity in other types of antigen-presenting cell (Arico et al., 2005). Viral peptides will be presented on both MHC class I and II molecules as a result of viral particle uptake by phagocytic receptors such as the mannose receptor, which can recognize many viruses (Ezekowitz et al., 1990), or through macropinocytosis (de Baey and Lanzavecchia, 2000). The mechanism by which peptides generated by degradation of viral proteins in the endosomal pathway can be presented by MHC class I molecules is not known. Nevertheless, it is clear that extracellular proteins taken up by dendritic cells can give rise to peptides presented by MHC class I molecules. In this way, viruses that are not able to infect dendritic cells are still able to stimulate effective immune responses. Thus, any virus-infected cell is able to activate naïve $CD8^+$ T cells, generating cytotoxic $CD8^+$ effector T cells that can kill infected cells.

3.13.2. Macrophages

Many microorganisms entering the body are engulfed and destroyed by phagocytes, which provide an innate, antigen-nonspecific first line of defense against infection. Macrophages that have bound and ingested microorganisms, but have failed to destroy them, contribute to the adaptive immune response by acting as antigen-presenting cells. The adaptive immune response is in turn able to stimulate the microbicidal and phagocytic capacities of these cells.

Resting macrophages have few or no MHC class II molecules on their surface, and do not express B7 molecules. The expression of both MHC class II and B7 molecules is induced by the ingestion of microorganisms and recognition of their foreign molecular patterns (Chelen et al., 1995). Macrophages, like tissue dendritic cells, have a variety of receptors recognizing microbial surface components, including the mannose receptor, the scavenger receptor, complement receptors, and several Toll-like receptors. Once bound, microorganisms are engulfed and degraded, generating peptides that can be presented by MHC class II molecules. At the same time, the receptors recognizing these microorganisms transmit a signal that leads to expression of MHC class II molecules and B7 molecules (Gordon, 2003).

Thus, the induction of co-stimulatory activity by common microbial constituents occurs in both dendritic cells and macrophages. This is believed to allow the immune system to distinguish antigens borne by infectious agents from antigens associated with innocuous proteins, including self-proteins. Indeed, many foreign proteins do not induce an immune response when injected on their own, presumably because they fail to induce costimulatory activity in antigen-presenting cells.

As macrophages continuously scavenge dead or dying cells, which are rich sources of self-antigens, it is particularly important that they should not activate T cells in the absence of microbial infection (Schmitz et al., 1997).

3.14. Goal of this study

The interactions between cells of the immune system and parenchymal cells in the CNS as well as the muscle are complex. This thesis was designed to broadly study several aspects of these interactions and their involvement in diseases of presumed auto-immune origin and adequate animal models.

The first area of analysis was the bidirectional interaction between human myoblasts and antigen-presenting cells, which takes place in muscle tissue and is suspected to be disturbed in cases of inflammatory myopathies.

The next topic was the involvement of pathological $CD8^+$ T cells in the transgenic mouse model of PLP-overexpressing oligodendrocytes and the possibility of antigen specificity of these cells. Additionally, the introduction of the PD-1 knock down allowed us to analyze the influence of specific co-stimulation/inhibition in this system and its influence on $CD8^+$ T-cell expansions and clonality.

The most extensive part of this thesis, however, was the analysis of the TCR repertoire in Rasmussen encephalitis patients in the CNS as well as the periphery. The separate analysis of $CD4^+$ and $CD8^+$ T cells in the periphery in comparison with concomitant brain biopsy specimens allowed us to assess the pathological relevance of $CD8^+$ T-cell clones in these patients with respect to long-term disease course and their putative pathogenic relevance.

4. Materials

4.1. Buffers and media

RPMI-Medium: RPMI basic medium
FCS (10%)
Glutamate (1%)
Penicillin/Streptomycin (1%)
Sterile filtration through 0.2µm filter

Skeletal Muscle Growth Medium (SMGM) from Promocell (Heidelberg, Germany)

10x PBS: NaCl 80g
$Na_2HPO_4 * 2H_2O$ 14.2g
KCl 2g
KH_2PO_4 2g
Aqua dest ad 1l
Adjustion to pH=6.8

1x PBS: 1:10 dilution of 10x PBS in Aqua dest

10x TBE: Tris 108g
Boric acid 55g
0.5 M EDTA 40ml
Aqua dest ad 1l

1x TBE: 1:10 dilution of 10x TBE in Aqua dest

Erythrocyte lysis buffer: NH_4Cl 0.15M
$KHCO_3$ 1mM
Na_2EDTA 0.1mM
In Aqua dest
Adjustion to pH=7.2-7.4
Sterile filtration through 0.2µm filter

FACS buffer:	1x PBS
	BSA (0.1%)
	Sodium azide (0.1%)
MACS buffer:	1x PBS
	BSA (0.5%)
	EDTA (1mM)

Buffers, media, and solutions were stored at 4°C.

4.2. Antibodies

The following primary antibodies were used for flow cytometry: phyco-erythrin (PE) conjugated anti-human B7-H1 (clone MIH1, eBioscience, NatuTec GmbH, Frankfurt, Germany), allophycocyanin (APC) conjugated anti-HLA-ABC (clone W6/32), anti-HLA-DR-APC (clone L243, both Leinco Technologies, St. Louis, MO, USA); anti-CD14-PE and -APC (clone MOP9), fluorescein isothiocyanate (FITC) conjugated anti- neural cell adhesion molecule (NCAM) (clone NCAM16.2), anti-CD80-PE (clone L307.4), anti-CD86-PE (clone 2331(FUN1)), anti-DC-Sign-PE and -APC (clone DCN46) and the respective isotype controls were all purchased from BD, Heidelberg, Germany.

4.3. Cytokines

Interferon (IFN)-γ and tumor necrosis factor (TNF)-α were from Peprotech EC Ltd (London, England). Macrophage colony stimulating factor (M-CSF), and granulocyte macrophage stimulating factor (GM-CSF) and Interleukin-4 (IL-4) were from R&D Systems (Minneapolis, MN, USA).

4.4. Primers

4.4.1. Spectratyping primers (human samples)

Name	Fluorescence	Sequence 5'-3'
Vβ1		CAA CAG TTC CCT GAC TTG CAC
Vβ2		TCA ACC ATG CAA GCC TGA CCT
Vβ3		TCT AGA GAG AAG AAG GAG CGC
Vβ4		CAT ATG AGA GTG GAT TTG TCA TT
Vβ5.1		TTC AGT GAG ACA CAG AGA AAG
Vβ5.2		CCT AAC TAT AGC TCT GAG CTG
Vβ6		AGG CCT GAG GGA TCC GTC TC
Vβ7		CTG AAT GCC CCA ACA GCT CTC
Vβ8		TAC TTT AAC AAC AAC GTT CCG
Vβ9		AAA TCT CCA GAC AAA GCT CAC
Vβ11		ACA GTC TCC AGA ATA AGG ACG
Vβ12		GAC AAA GGA GAA GTC TCA GAT
Vβ13.1		GAC CAA GGA GAA GTC CCC AAT
Vβ13.2		GTT GGT GAG GGT ACA ACT GCC
Vβ14		TCT CGA AAA GAG AAG AGG AAT
Vβ15		GTC TCT CGA CAG GCA CAG GCT
Vβ16		GAG TCT AAA CAG GAT GAG TCC
Vβ17		CAC AGA TAG TAA ATG ACT TTC AG
Vβ18		GAG TCA GGA ATG CCA AAG GAA
Vβ19		CCC CAA GAA CGC ACC CTG C
Vβ20		TCT GAG GTG CCC CAG AAT CTC
Vβ21		GAT ATG AGA ATG AGG AAG CAG
Vβ23		TCA TTT CGT TTT ATG AAA AGA TGC
Vβ24		AAA GAT TTT AAC AAT GAA GCA GAC
Jβ1.1	HEX	ACT GTG AGT CTG GTG CCT TG

Jβ1.2	6-FAM	ACA ACG GTT AAC TTG GTC CCC GAA
Jβ1.3	NED	GGT CCT CTA CAA CAG TGA GCC AAC
Jβ1.4	HEX	AAG AGA GAG AGC TGG GTT CCA CT
Jβ1.5	6-FAM	GGA GAG TCG AGT TCC ATC A
Jβ1.6	NED	TGT CAC AGT GAG CCT GGT CCC ATT
Jβ2.1	HEX	CCT GGC CCG AAG AAC TGC TCA
Jβ2.2	6-FAM	GTC CTC CAG TAC GGT CAG CCT AGA
Jβ2.3	NED	TGC CTG GGC CAA AAT ACT GCG
Jβ2.4	HEX	TCC CCG CGC CGA AGT ACT GA
Jβ2.5	6-FAM	TCG AGC ACC AGG AGC CGC
Jβ2.6	NED	CTG CTG CCG GCC CCG AAA GTC
Jβ2.7	HEX	TGA CCG TGA GCC TGG TGC CCG
Cβ-R	HEX	CTT CTG ATG GCT CAA ACA C
SpTy-β-out		ACA CCA GTG TGG CCT TTT GG
Cβ-RT (cDNA)		GAA GAA GCC TGT GGC C

4.4.2. Spectratyping primers (murine samples)

Name	Fluorescence	Sequence 5'-3'
Vβ1		CTG AAT GCC CAG ACA GCT CCA AGC
Vβ2		TCA CTG ATA CGG AGC TGA GGC
Vβ3.1		CCT TGC AGC CTA GAA ATT CAG T
Vβ4		GCC TCA AGT CGC TTC CAA CCT C
Vβ5.1		CAT TAT GAT AAA ATG GAG AGA GAT
Vβ5.2		AAG GTG GAG AGA GAC AAA GGA TTC
Vβ5.3		AGA AAG GAA ACC TGC CTG GTT
Vβ6		CTC TCA CTG TGA CAT CTG CCC

Vβ7		TAC AGG GTC TCA CGG AAG AAG C
Vβ8.1		CAT TAC TCA TAT GTC GCT GAC
Vβ8.2		CAT TAT TCA TAT GGT GCT GGC
Vβ8.3		TGC TGG CAA CCT TCG AAT AGC A
Vβ9		TCT CTC TAC ATT GGC TCT GCA GGC
Vβ10		ATC AAG TCT GTA GAG CCG GAG GA
Vβ11		GCA CTC AAC TCT GAA GAT CCA GAG C
Vβ12		GAT GGT GGG GCT TTC AAG GAT C
Vβ13		AGG CCT AAA GGA ACT AAC TCC CAC
Vβ14		ACG ACC AAT TCA TCC TAA GCA C
Vβ15		CCC ATC AGT CAT CCC AAC TTA TCC
Vβ16		CAC TCT GAA AAT CCA ACC CAC
Vβ17		AGT GTT CCT CGA ACT CAC AG
Vβ18		CAG CCG GCC AAA CCT AAC ATT CTC
Vβ19		CTG CTA AGA AAC CAT GTA CCA
Vβ20		TCT GCA GCC TGG GAA TCA GAA
Jβ1.1	HEX	AGT GTG AGT CTG GTT CCT TTA CC
Jβ1.2	6-FAM	AAA GCC TGG TCC CTG AGC CGA AG
Jβ1.3	NED	CTT CCT TCT CCA AAA TAG AGC
Jβ1.4	HEX	GAC AGC TTG GTT CCA TGA CCG
Jβ1.5	6-FAM	GAG TCC CCT CTC CAA AAA GCG
Jβ1.6	NED	TCA CAG TGA GCC GGG TGC CTG C
Jβ2.1	HEX	GTG AGT CGT GTT CCT GGT CCG AAG
Jβ2.2	6-FAM	CCA GCA CTG TCA GCT TTG AGC
Jβ2.3	NED	GTT CCT GAG CCA AAA TAC AGC G
Jβ2.4	HEX	GTG CCC GCA CCA AAG TAC AAG
Jβ2.5	6-FAM	GTG CCT GGC CCA AAG TAC TGG
Jβ2.7	HEX	CTA AAA CCG TGA GCC TGG TGC
Cβ-R	NED	CAC TGA TGT TCT GTG TGA CA

4.5. Mice

4.5.1. Proteolipid transgenic (PLP-tg) mice

Experiments involving the transgenic mice have been performed in very close collaboration with the group of R. Martini (A. Kroner-Milsch). The generation of PLP-tg mice expressing autosomal copies of the entire wild-type PLP gene has been described previously (Readhead et al., 1994). These mutant mice have been bred and genotyped as reported in a recent study (Ip et al., 2006). PLP-tg mice and wild-type littermates were kept in our animal facility under specific pathogen-free conditions. Presence of the transgene was surveyed by PCR with the primer set 5'-CAGGTGTTGAGTCTGATCTACACAAG-3' and 5'-GCATAATACGACTCACTATAGGGATC-3' following a standard protocol with an annealing temperature of 55°C and an elongation time of 45 seconds at 72°C.

4.5.2. PD-1 deficient mice

Absence of PD-1 in PD-1-/- mice (Nishimura et al., 1998) was also confirmed by PCR. For this, the wildtype allele was amplified using the primer pair 5´-CCGCCTTCTGTAATGGTTTG-3´ and 5´-TGTTGAGCAGAAGACAGCTAGG-3´ with an annealing temperature of 54°C and an elongation time of 45 seconds. Additionally, the Neo Cassette inside the knockout allele was detected using the primer pair 5´-GCCCGGTTCTTTTTGTCAAGACCGA-3´ and 5´-ATCCTCGCCGTCGGGCATGCGCGCC-3´ with an annealing temperature of 60°C and an elongation time of 45 seconds.

4.5.3. Double transgenic mice

Double mutants (PLP-tg/PD-1-/-) were obtained by cross breeding PLP-tg with PD-1-/- mice. The offspring in the F1 generation was tested for the presence of the PLP transgene, all being heterozygously deficient for PD-1. Offspring from the first generation (F1) were crossbred with PD-1-/- mice resulting (among others) in PLP-tg/PD-1-/- mice in the F2 generation.

4.5.4. Bone marrow chimerization

PD-1-/- or wildtype bone marrow was transplanted into PLPtg mice with PLPwt mice as controls, which were irradiated with 5 Gy. Transplantation was performed as described before (Ip et al., 2006; Maurer et al., 2001). Briefly, bone marrow from donor mice was isolated by flushing out femoral bone marrow with sterile PBS, washed and filtered through a mesh with 70 µm pore size to remove debris. Afterwards, cells were resuspended in 500 µl PBS and approximately 2 x 10^7 cells were injected intravenously into recipient mice. Animals were kept under frequent controls until the age of 10 months and then transcardially perfused.

4.6. Reagents

Ammonium chloride (NH_4Cl) Merck (Darmstadt, Germany)
Agarose Sigma (Munich, Germany)
Ampicillin Roth (Karlsruhe, Germany)
Biotherm DNA Polymerase Genecraft (Cologne, Germany)
Boric acid Merck (Darmstadt, Germany)
Bovine Serum albumine (BSA) 96% Sigma (Munich, Germany)
CD3/CD28 T-cell expander Dynabeads Dynal Biotech ASA (Oslo, Norway)
CFSE Molecular Probes (Eugene, OR, USA)
Di-sodium hydrogen phosphate (Na_2HPO_4) Merck (Darmstadt, Germany)
Ethanol J.T.Baker (Deventer, Netherlands)
Ethidium bromide Sigma (Munich, Germany)
Ethylendiaminetetraacetic acid (EDTA) Merck (Darmstadt, Germany)
Fetal Calf Serum (FCS) Gibco Invitrogen (Karlsruhe, Germany)
Fluoresbrite 2.0 micron Microspheres Polysciences, Inc (Warrington, PA, USA).
Glutamate Gibco Invitrogen (Karlsruhe, Germany)
Hydrochloric acid (HCl) Merck (Darmstadt, Germany)
Gel (Agarose) 100bp DNA ladder Peqlab (Erlangen, Germany)
Lipopolysaccharides (LPS) (S. thyphi (L-7261)) Sigma-Aldrich (Munich, Germany)
6x Loading Dye Fermentas (St. Leon-Rot, Germany)
Phosphate-buffered saline (PBS) Biochrom AG (Berlin, Germany)
Penicillin / Streptomycin Biochrom AG (Berlin, Germany)

Percoll GE Healthcare (Munich, Germany)

Potassium di-hydrogen phosphate (KH_2PO_4) Merck (Darmstadt, Germany)

Potassium chloride (KCl) Merck (Darmstadt, Germany)

Potassium hydrogen carbonate ($KHCO_3$) Merck (Darmstadt, Germany)

Propidium iodide (PI) Sigma-Aldrich (Munich, Germany)

ROX-500 Applied Biosystems (Foster City, CA, USA)

RPMI PAA (Coelbe, Germany)

Sodium azide (NaN_3) Merck (Darmstadt, Germany)

Sodium chloride solution (NaCl) Merck (Darmstadt, Germany)

4.7. Consumables

Cell strainer 40 and 70 µm BD Biosciences Pharmingen (San Jose, CA , USA)

Cell culture flasks Sarstedt (Nuembrecht, Germany)

Cell culture dishes Nunc (Roskilde, Denmark)

FACS Tubes BD Biosciences Pharmingen (San Jose, CA , USA)

PCR tubes Sarstedt (Nuembrecht, Germany)

PCR plates Sarstedt (Nuembrecht, Germany)

Reaction tubes (15 ml, 50 ml) Greiner Bio-one (Frickenhausen, Germany)

Sequencer plates Applied Biosystems (Foster City, CA, USA)

4.8. Equipment

BioPhotometer 6131 Eppendorf (Hamburg, Germany)

Centrifuges Biofuge 15R Heraeus (Hanau, Germany)

Centrifuge 5810R Eppendorf (Hamburg, Germany)

Centrifuge 5415C Eppendorf (Hamburg, Germany)

FACSCalibur BD Biosciences Pharmingen (San Jose, CA, USA)

Heater Eppendorf (Hamburg, Germany)

Incubators HeraCell150 Heraeus (Hanau, Germany)

LabCycler (96well) Sensoquest (Göttingen, Germany)

Microscopes IX70 Olympus (Hamburg, Germany)

Pipettes Abimed (Berlin, Germany)

Eppendorf (Hamburg, Germany)

Gilson (Bad Camberg, Germany)

Sequencer ABI3130 Applied Biosystems (Foster City, CA, USA)

4.9. Software

4peaks Mek&Tosj Software (Amsterdam, Netherlands)

Adobe Photoshop CS3 Adobe Systems (Dublin, Republic of Ireland)

Adobe Illustrator CS3 Adobe Systems (Dublin, Republic of Ireland)

CellQuest Pro BD Biosciences Pharmingen (San Jose, CA, USA)

FlowJo7 software Tree Star Inc. (Ashland, OR, USA)

Genemarker SoftGenetics (State College, PA, USA)

Excel Microsoft (Seattle, WA, USA)

Prism GraphPad Software (La Jolla, CA, USA)

5. Methods

5.1. Isolation of peripheral blood mononuclear cells (PBMC)

Human peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient isolation from blood samples. Peripheral blood mixed 1:1 with phosphate buffered saline (PBS) was applied on a Lymphoprep (Fresenius Kabi Norge AS, Oslo, Norway) layer and centrifuged (20 min, 300 g, 20 °C). The PBMC in the interphase of the gradient were collected and washed twice in PBS (12 min, 300 g, 4 °C) prior to use.

5.2. Isolation of splenocytes

Splenocytes were isolated by homogenizing a spleen through a 70 µm pore filter, followed by an erythrocyte lysis step using ACK hypoosmotic buffer on the pelleted cells (5 min, room temperature). The splenocytes were washed with PBS, filtered, and counted prior to use.

5.3. Lymphocyte purification from murine CNS

Brains and spinal cords from PBS-perfused mice were isolated as published previously (Ortler et al., 2008). In brief, CNS material was cut into pieces and CNS cells were isolated from the interface of 30-50% Percoll (Amersham Biosciences, Freiburg, Germany) centrifuged for 30 min at 2500 rpm. Mononuclear cells were washed and pelleted for RNA isolation.

5.4. Magnetic activated cell separation (MACS®)

Cells were magnetically isolated following the manufacturer's instructions. Briefly, PBMC were suspended in sterile, de-gased MACS buffer (PBS, 0.5% bovine serum albumine, 2 mM EDTA) at a concentration of 10^7 cells per 40 µl. 10 µl Biotin-Antibody cocktail (from the negative isolation MACS kits, Miltenyi Biotec, Bergisch Gladbach, Germany) were added to the cells (for the $CD4^+$ or $CD8^+$ T-cell selection, respectively) and incubated for 10 min at 4-8 °C. 30 µl of buffer and 20 µl of Anti-Biotin MicroBeads

were then added, followed by an additional incubation of 15 min at 4-8 °C. The cells were washed with 5 ml of buffer and centrifuged at 300 g for 10 min at 4 °C. The supernatant was discarded and the cells resuspended in 500 µl of buffer. The magnetically labeled cells were purified using the appropriate MACS® columns (Miltenyi Biotec).

5.5. Fluorescence staining for flow cytometry

Cells were harvested using Accutase (PAA, Cölbe, Germany), blocked with mouse IgG (Sigma, Munich, Germany) and washed with FACS-buffer (PBS 0.1% Sodiumazide 0.1% BSA). After a 30 min incubation period with the respective antibody, cells were washed once and resuspended in 200 µl FACS-buffer. Antibody concentrations were carefully titrated prior to experiments. In some experiments, one minute prior to the flow cytometry analysis 50 µl PI (2µg/ml) were added to a sample to allow exclusion of dead cells (PI+).

5.6. Cell culture

Myoblasts (MB) were isolated from biopsies of healthy donors as described previously (Wiendl et al., 2003c). MB cultures and muscle biopsy specimens were obtained from the Muscle Tissue Culture Collection at the Friedrich-Baur-Institute in Munich. Proliferating MB were further purified by NCAM (clone 5.1H11, hybridoma supernatant) magnetic bead separation and cultured in SMGM. After purification, MB cultures stained >95% positive for the neural cell adhesion molecule (NCAM/CD56) by flow cytometry.

Monocytes were selected by adhesion of PBMC to plastic flasks for 1 h. Dendritic cells were generated as previously described (Schreiner et al., 2004). In brief, monocytes (>90% pure, as assessed by flow cytometry) were cultured in RPMI 10% FCS supplemented with GM-CSF (100 ng/ml) and IL-4 (40 ng/ml). After 5 days, the cells exhibited an immature DC phenotype (DC-Sign++ CD14- CD1a+ MHC-II low CD86 low CD80 low/- CD83-). Maturation was induced by 48h-incubation of the immature DCs (DC-i) with LPS (5 µg/ml). High levels of surface MHC class II and costimulatory molecules (CD86, CD80), as well as low levels of DC-Sign identified mature DCs (DC-m).

To generate macrophages (MPh), monocytes were cultured in RPMI 10% FCS supplemented with GM-CSF (100 ng/ml) and M-CSF (10 ng/ml) for 7 days. MB and APC were cocultured in a 1 : 1 mixture of RPMI 10% FCS and SMGM in 6-well (1x 10^6 /well) or 12-well plates (1x 10^5 /well). After 48 h cells were harvested and subjected to flow cytometry or tested in functional assays.

5.7. Assessment of cell proliferation by CFSE labeling

PBMC were isolated and washed twice with PBS. 1x 10^6 PMBC were incubated in 1 ml CFSE-staining-solution (2,5 µmol CFSE in PBS) for 1 minute. Adding 1 ml FCS stopped the reaction. Cells were washed twice in PBS and cultured in 96-well plates (5x 10^4 /well). CD3/CD28 beads were added to each well at a 1:1 ratio to induce T-cell proliferation. APC were pipetted carefully from the coculture to avoid contamination with adherent MB. Pure APC were added to the proliferating PBMC at a 1:1 ratio.
After 60h, the PBMC were harvested. Proliferation of T cells was assessed by analyzing CFSE staining intensity/dilution in the subsequent FACS analysis (FACSCalibur™, Becton/Dickinson, Heidelberg, Germany) and the positive control (stimulated with beads, but no DC) was set to the value of 1 in each corresponding experiment.

5.8. Macrophages phagocytosis assay

Macrophages were grown in 12-well plates (1-5x 10^5 /well) and incubated for 48h with different cell lysates, which were generated by performing 5 freeze/thaw-cycles with liquid nitrogen. 1x 10^6 fluorescent beads (Polysciences, Warrington, PA, USA) per 1x 10^5 MPh were coated in 100 µl PBS 1% BSA for 30 min at room temperature (RT). The coated beads were added to 300 µl RPMI 10% FCS and the mixture was added to the macrophage culture. MPh were incubated with beads for 30 min at RT. Extensive washing in cold PBS (5 times) was performed to eliminate non-ingested particles. Afterwards the cells were harvested by adding EDTA/Trypsin to remove beads that adhered to the cell-membrane. Fluorescence was displayed in a FL1-histogram and phagocytosis was determined as the percentage of cells which incorporated one or more particles in the 30 min incubation period. The percentage of phagocytic MPh in the control was set to the value of 1 in each corresponding experiment.

5.9. RNA isolation

5.9.1. RNA isolation from tissue

Tissue samples were homogenized in 1 ml of Trizol® reagent before subjecting the samples to the protocol for RNA isolation from cells.

5.9.2. RNA isolation from cells

5.9.2.1. TriZol®

The cells were pelleted by centrifugation and lysated in Trizol® (Invitrogen, Karlsruhe, Germany) by repetitive pipetting (1 ml of Trizol per 5-10x 10^6 cells). The samples were incubated for 5 min at room temperature to permit the complete dissociation of nucleoprotein complexes before adding 0.2 ml chloroform per 1 ml of Trizol®. After vortexing for 15 s, the samples were incubated for 3 min at room temperature and centrifuged at 12000 g for 15 min at 4 °C. The aqueous phase was transfered to a fresh tube and the RNA was precipitated by mixing with isopropyl alcohol (0.5 ml of isopropyl alcohol per 1 ml of initial Trizol®). The samples were incubated at room temperature for 10 min and centrifuged at 12000 g for 10 min at 4 °C. The supernatant was removed and the RNA pellet was washed once with 75% ethanol (1 ml of 75% ethanol per 1 ml of Trizol®). The samples were vortexed and centrifuged at 7500 g for 5 min at 4 °C. Finally, the RNA was dried briefly, dissolved in RNAse-free water by pipetting and incubating for 10 min at 60 °C, and stored at -80 °C.

5.9.2.2. Column-based spin kit

The cells were pelleted and lysated in the provided RLT buffer (all reagents Qiagen, Hilden, Germany). The RNA was precipitated with 70% ethanol and subsequently purified using the provided spin columns. After precipitation, the RNA was washed with a high-salt washing buffer and a low-salt washing buffer, before eluating it in DEPC H_2O.

5.10. cDNA synthesis

5.10.1. Unspecific

cDNA synthesis with random hexamer primers was performed using standard methods as previously described (Wiendl et al., 2002). Reagents were obtained from Applied Biosystems (Foster City, CA, USA). For each sample, 250 ng RNA were transcribed using random hexamer primers and M-MLV reverse transcriptase. In short, 250 ng RNA (in 17.5 µl) were transcribed using 5 µl RT-buffer, 10 µl dNTPs, 2.5 µl random hexamer primers, 1 µl RNAse inhibitor, 3 µl reverse transcriptase, and 11 µl $MgCl_2$ (25 mM). PCR conditions: 25 °C (10 min), 37 °C (60 min), 95 °C (15 min).

5.10.2. TCR specific (human samples)

For human cDNA from tissue samples, a previously published TCR β-chain specific cDNA primer 'Cβ-RT' (100 pmol/µl) (Seitz et al., 2006), as well as SuperScriptIII™ reverse transcriptase (Invitrogen, Karlsruhe, Germany) was used, following the manufacturer's instructions.

5.11. CDR3 spectratyping PCRs

5.11.1. Human system

For the human CDR3 spectratyping, I used the Vβ forward primers as described in Monteiro et al. (Monteiro et al., 1996), the Jβ reverse primers as described previously (Puisieux et al., 1994), and two different Cβ reverse primers: 'SpTy-β-out' (Junker et al., 2007) and 'Cβ-R' (Monteiro et al., 1996). A complete list of primers can be found in the materials section. The Vβ nomenclature according to Arden et al. (Arden et al., 1995a) is used throughout the thesis.

For the peripheral blood derived samples, the following protocol was applied: First, the cDNA was used in 24 Vβ-Cβ reactions: 1.25 µl Vβ primer (10 pmol/µl), 1.25 µl Cβ-R primer (10 pmol/µl), 0.5 µl cDNA, 0.25 µl dNTPs (2.5 mM, Promega, Mannheim, Germany), 2.5 µl 10xbuffer (Applied Biosystems, Foster City, CA, USA), 0.1 µl Taq Polymerase (Applied Biosystems), 19.15 µl DEPC-H_2O. PCR conditions: 94 °C (6 min); 94 °C (1 min), 59 °C (1 min), 72 °C (1 min) for 40 cycles; 72 °C (7 min). After those first-round PCRs, every PCR product

was subjected to 13 individual Vβ-Jβ 'run-off' reactions (modified from (Batliwalla et al., 1996; Pannetier et al., 1993)) with 13 5'-fluorescence-tagged Jβ primers to differentiate between individual TCR Jβ regions and also with a 5'-fluorescence-tagged Cβ-R primer. In the case of tissue samples (e.g. CNS samples), I used a more sensitive protocol (Junker et al., 2007) to compensate for the low T-cell numbers in tissue specimens. Briefly, I introduced a semi-nested pre-amplification PCR step before the PCR reaction described above. I used the same forward primers as above, but employed the Cβ-specific reverse primer 'SpTy-β-out' (10 pmol/µl) that hybridizes downstream of Cβ-R, but upstream of the RT-primer Cβ-RT. Samples were incubated for 5 min at 94 °C. The PCR was run for 30 cycles of: 94 °C (1 min), 56 °C (1 min), 72 °C (1 min), followed by an incubation of 72 °C (10 min). From this PCR, 1 µl/reaction was used as template in the protocol mentioned before.

5.11.2. Murine system

For the murine CDR3 spectratyping, the Vβ forward and Jβ reverse primers as described in (Pannetier et al., 1993) and the common Cβ-R reverse primer published previously in (Menezes et al., 2007) were used. The PCR composition was identical to the human protocol. However, the thermal cycler conditions of the first round PCRs were as follows: 94 °C (1 min); 94 °C (1 min 10 s), 60 °C (1 min), 72 °C (4 min) for 40 cycles; 72 °C (10 min). In the murine system, there was no need for a modified protocol for CNS samples. The nomenclature is according to Arden et al. (Arden et al., 1995b).

5.12. Fragment analysis

The length of the fluorescence-labeled PCR products was analyzed on an ABI3130 genetic analyzer (Applied Biosystems), applying a module for fragment analysis. 500-ROX (Applied Biosystems) was the internal standard in each sample.

5.13. Data normalization and analysis

Data were processed by GeneMarker® software (SoftGenetics, State College, PA, USA). For all PCR products of Vβ- and Cβ-primers, or Vβ- and Jβ-primers, respectively, I

plotted the peak intensities versus the fragment lengths. Unskewed repertoires yield Gaussian length distribution, whereas skewed repertoires show distortions (Pannetier et al., 1995). To evaluate the relative skew of the repertoires in the human system, the data obtained from blood of the healthy control persons were used as a standard: For each Vβ-Cβ-combination, the average fragment lengths were measured and the positions of the maxima of the Gauss distribution were determined. At these length positions, the peak intensity of the corresponding Vβ-Cβ product from the patients was defined as "1.0". A Vβ-Cβ product from a patient was considered skewed, if a peak exceeded the intensity on this semi-quantitative scale for a factor of greater than two. If the factor was between 1.0 and 2.0, the repertoire was considered slightly disturbed. Very high peaks were reamplified with unlabeled Vβ and Jβ primer and the PCR products were sequenced directly, after isolating the PCR product from an agarose gel (3 %).

5.14. Statistics

The two-sided, unpaired Student's t-test was used to assess statistical significance. Significance was indicated as * $p < 0.05$, ** $p < 0.01$. For non-parametrical situations, the Kruskal-Wallis and Mann-Whitney-U tests were used.

6. Results and Discussion

For Results and Discussion, I would like to refer to these publications in the Appendix of the thesis.

1. Leder, C.*, **Schwab, N.***, Ip, C.*, Kroner, A.*, Nave, K., Dornmair, K., Martini, R.*, and Wiendl, H.* (2007). Clonal expansions of pathogenic CD8+ effector cells in the CNS of myelin mutant mice. Mol Cell Neurosci *36*, 416-424.

2. **Schwab, N.***, Waschbisch, A.*, Wrobel, B., Lochmuller, H., Sommer, C., and Wiendl, H. (2008). Human myoblasts modulate the function of antigen-presenting cells. J Neuroimmunol *200*, 62-70.

3. Kroner, A., **Schwab, N.**, Ip, C.-W., Leder, C., Nave, K.-A., Mäurer, M., Wiendl, H., and Martini, R. (2009). PD-1 Regulates Neural Damage in Oligodendroglia-Induced Inflammation. PLoS ONE *4*, e4405. DOI: 10.1371/journal.pone.0004405

4. **Schwab, N.***, Bien, C.G.*, Waschbisch, A., Becker, A., Vince, G.H., Dornmair, K.*, and Wiendl, H.* (2009). CD8+ T-cell clones dominate brain infiltrates in Rasmussen encephalitis and persist in the periphery. Brain. DOI: 10.1093/brain/awp003

5. Kroner, A., **Schwab, N.**, Ip, C.-W., Ortler, S., Göbel, K., Nave, K.-A., Mäurer, M., Martini, R. and Wiendl, H. (2009). Accelerated course of experimental autoimmune encephalomyelitis in PD1-deficient CNS myelin mutants. Am. J. Pathol. *In press.*

6. Kroner, A., **Schwab, N.**, Ip, C., Sommer, C., Wessig, C., Wiendl, H., and Martini, R. (2009). The co-inhibitory molecule PD-1 modulates disease severity in a model for an inherited, demyelinating neuropathy. Neurobiol Dis *33*, 96-103.

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7. Final assessment

The studies in this thesis were performed under the overriding question how the interactions between parenchymal cells and immune cells contribute to pathogenic situations in primary inflammatory versus primary degenerative disorders. As disease models we used mouse mutants in addition to paradigmatic human diseases such as myositis, Rasmussen encephalitis and Multiple sclerosis. These connections between cell types might have been underrated in the past. Immune reactions have often been patronized as being either a clear-cut beneficial response in the case of fighting infections or detrimental in the case of autoimmune disorders. In recent years, the complexity of these cells' cross-reactions has been found to be important in an increasing number of pathogenic situations (reviewed for the CNS by (Russell, 2005)). The development of immune cell-based therapies (e.g. dendritic cells (El Marsafy et al., 2009) and regulatory T cells (Edinger, 2008)) or therapeutic antibodies (Tol et al., 2009) shows that the immune system can be a powerful instrument in the treatment of (auto)immune disorders, but also of diseases of non-immune origin (e.g. cancer).

The muscle microenvironment had been termed immunologically inactive and passive for a long time before studies in recent years showed that many processes of physiological and pathophysiological importance take place in muscle tissue (Wiendl et al., 2005a). We could show the induction of inhibitory functions in dendritic cells by human myoblasts as an example where a parenchyma uses the immune system as a way of protection. Additionally, we could prove the significant upregulation of macrophage phagocytosis by muscle cell fragments. We hypothesize that this putatively facilitates the regeneration of damaged muscle tissue. From an evolutionary point of view, functional skeletal muscle tissue is vital to the immediate survival of the organism. Therefore, it makes sense that mechanisms exist to enhance or maintain tissue integrity or to enhance its regenerative abilities. In the case of inflammatory myopathies, APC are already considered as cell-based therapy (Sonnet et al., 2006)

In our studies of the immune interaction between immune cells and CNS tissue cells (specifically oligodendrocytes), we further investigated the concept of gliopathy-induced inflammation. Having proven the pathogenic relevance of macrophages and $CD8^+$ T cells in a model of primary myelinopathy (PLP-tg mice), we looked here for the specificity of the adaptive immune reaction in the case of the PLP overexpressing

oligodendrocytes. It became clear that it is possible to induce an adaptive immune reaction based solely on the transgenic tissue overexpression of a self-protein, which was not known before. This finding of paradigmatic importance has important implications for Multiple sclerosis. Among other links, PLP mutations have been proposed to play a role in the development of the disease (Warshawsky et al., 2005). Also, in X-linked adrenoleukodystrophy, a very serious neurological disorder of childhood, which is caused by a PLP mutation as well, $CD8^+$ T lymphocytes have been proposed as being pathogenic (Moser et al., 2004).

Additionally, the induction of the PD-1 knockout in the context of our transgenic mouse model illustrated the importance of tissue regulators in the immune reaction of the central nervous system. Mice lacking PD-1 showed much more prominent clonal expansions of $CD8^+$ T cells in their CNS. The effect of the PD-1 knockout alone, however, induced no pathogenicity. It seemed to lower the threshold for clonal expansions and when added to the overexpression of a self-antigen (PLP), it turned out to significantly exacerbate the immune response against the PLP overexpression. Therefore, a chronic disease course of the gliopathy-induced inflammation was induced, again with no foreign antigen or immunization. We have subsequently analyzed this effect in more detail in the context of actively induced experimental autoimmune encephalomyelitis (Kroner et al., 2009) to assess the interplay of CNS vulnerability and immune regulation under conditions of an additional inflammatory hit.

In the third part of my thesis, the focus lay on the specificity of the immune interaction between T cells and CNS parenchymal cells in Rasmussen encephalitis. RE is a disorder where the interaction between immune cells and the parenchyma is evidently detrimental. We could show that $CD8^+$ T cells attack structures in the CNS (neurons and astrocytes) and thereby putatively induce severe brain damage leading to symptoms like paresis, seizures, and intellectual decline. Interestingly, in the situation of RE these interactions are once again not random attacks, but turned out to be finely tuned, antigen-specific killings of target cells. We thereby confirmed and expanded previous observations (Bien et al., 2002; Li et al., 1997) and provided strong evidence for the pathogenicity of the $CD8^+$ T cells. Knowing that, the next question is the antigen of these pathogenic cytotoxic T cells. The identification of the antigen would help us not only to understand the cause of the inflammatory, putative auto-immune attack, but also to even begin to look for a treatment of the disease cause and not the symptoms.

A virus infection still seems a very distinct possibility as a trigger for this immune response and, therefore, some way of treatment could be feasible. The search for the antigen will be the topic of further studies and we are hopeful that the results of this thesis will be the starting point of promising projects focusing on the pathogenic $CD8^+$ T-cell clones in RE patients.

8. Abbreviations

APC	antigen-presenting cell
APC	allophycocyanin
BSA	bovine serum albumine
CAD	caspase-activated deoxyribonuclease
CD	cluster of differentiation
cDNA	complementary desoxyribo nucleic acid
CDR	complementarity determining region
CFSE	carboxyfluorescein succinimidyl ester
CNS	central nervous system
CTL	cytotoxic T lymphocyte
CTLA	cytotoxic T-lymphocyte antigen
D chain	diversity chain
DC	dendritic cell
DEPC	diethylpyrocarbonate
DM	dermatomyositis
DNA	deoxyribonucleic acid
EAE	experimental autoimmune encephalomyelitis
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescence-activated cell sorting
FAM	fluorescein amidite
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GluR	glutamate receptor
GM-CSF	granulocyte macrophage colony stimulating factor
HLA	human leukocyte antigen

IBM	inclusion body myositis
ICOS	inducible T-cell co-stimulator
IFN	interferon
IL	interleukin
J chain	joining chain
LPS	lipopolysaccharides
M-CSF	macrophage colony stimulating factor
MACS	magnetically activated cell sorting
MB	myoblasts
MHC	major histocompatibility complex
MPh	macrophages
MS	Multiple sclerosis
NCAM	neural cell adhesion molecule
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	programmed death
PE	phyco-erythrin
PI	propidium iodide
PLP	proteolipid protein
PM	polymyositis
PMD	Pelizaeus-Merzbacher disease
RE	Rasmussen encephalitis
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute medium
RT	room temperature
s/min/h	second/minute/hour

SMGM	skeletal muscle growth medium
TBE	tris/borate/EDTA
TCR	T-cell receptor
TGF	transforming growth factor
TLR	toll-like receptor
TNF	tumor necrosis factor
Treg	regulatory T-cell
V chain	variable chain

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11. Curriculum Vitae

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2000-2005	Studies of Biology, Julius-Maximilians-University, Würzburg, Germany
September 2002	Pre-Diploma of Biology General biology Physics Chemistry (organic, inorganic, physical)
May 2005	University Diploma of Biology Main Subject: Neurobiology Secondary Subjects: Immunology Pharmaceutical Biology Thesis: The role of PKB/Akt in T-cell activation
2005-2009	PhD thesis at the Clinical Research-Group for Multiple Sclerosis and Neuroimmunology (Head: Prof. H.Wiendl), neurological university clinic, Würzburg, Germany

12. Publications

12.1. Peer-reviewed publications

Leder, C.*, **Schwab, N.***, Ip, C.*, Kroner, A.*, Nave, K., Dornmair, K., Martini, R.*, and Wiendl, H.* (2007). Clonal expansions of pathogenic CD8+ effector cells in the CNS of myelin mutant mice. Mol Cell Neurosci *36*, 416-424.

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12.2. Poster presentations

Schwab, N., Leder, C., Kroner, A., Ip, C.-W., Nave, K.-A., Dornmair, K., Martini, R. and H. Wiendl. Clonal expansions of pathogenic CD8+ effector cells in the CNS of myelin mutant mice. 23rd Congress of the European Committee for Treatment and Research in Multiple Sclerosis/ 12th Annual Conference of Rehabilitation in MS, Prague, Czech Republic, October 11th-14th, 2007.

Schwab, N., Leder, C., Kroner, A., Ip, C.-W., Nave, K.-A., Dornmair, K., Martini, R. and H. Wiendl. Clonal expansions of pathogenic CD8+ effector cells in the CNS of myelin mutant mice. 37th Annual Meeting of the German Society for Immunology, Heidelberg, Germany, September 5th-8th, 2007.

Schwab, N., Bien, C.G., Waschbisch, A., Becker, A., Vince, G.H., Dornmair, K. and H. Wiendl. CD8+ T-cell clones dominate brain infiltrates in Rasmussen encephalitis and persist in the periphery. 9th International Congress of NeuroImmunology, Fort Worth, Texas, October 26th-30th, 2008.

Schwab, N., Bien, C.G., Waschbisch, A., Becker, A., Vince, G.H., Dornmair, K. and H. Wiendl. CD8+ T-cell clones dominate brain infiltrates in Rasmussen encephalitis and persist in the periphery. France – German Meeting on Multiple Sclerosis, Paris, France, March 6th, 2009.

12.3. Oral presentations

Schwab, N., Waschbisch, A., Wrobel, B., Lochmüller, H., Sommer, C. and H. Wiendl. Humane Myoblasten modulieren die Funktion Antigen-präsentierender Zellen. 18.

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Schwab, N., Bien, C.G., Waschbisch, A., Becker, A., Vince, G.H., Dornmair, K. and H. Wiendl. CD8+ T-cell clones dominate brain infiltrates in Rasmussen encephalitis and persist in the periphery. Treffen der AG Neuroimmunologie – Kloster Seeon, Seeon, Germany, February 6th-8th, 2009.

13. Appendix

13.1. Manuscripts for the cumulative dissertation

1. Leder, C.*, **Schwab, N.***, Ip, C.*, Kroner, A.*, Nave, K., Dornmair, K., Martini, R.*, and Wiendl, H.* (2007). Clonal expansions of pathogenic CD8+ effector cells in the CNS of myelin mutant mice. Mol Cell Neurosci *36*, 416-424.

2. **Schwab, N.***, Waschbisch, A.*, Wrobel, B., Lochmuller, H., Sommer, C., and Wiendl, H. (2008). Human myoblasts modulate the function of antigen-presenting cells. J Neuroimmunol *200*, 62-70.

3. Kroner, A., **Schwab, N.**, Ip, C.-W., Leder, C., Nave, K.-A., Mäurer, M., Wiendl, H., and Martini, R. (2009). PD-1 Regulates Neural Damage in Oligodendroglia-Induced Inflammation. PLoS ONE *4*, e4405. DOI: 10.1371/journal.pone.0004405

4. **Schwab, N.***, Bien, C.G.*, Waschbisch, A., Becker, A., Vince, G.H., Dornmair, K.*, and Wiendl, H.* (2009). CD8+ T-cell clones dominate brain infiltrates in Rasmussen encephalitis and persist in the periphery. Brain. DOI: 10.1093/brain/awp003

5. Kroner, A., **Schwab, N.**, Ip, C.-W., Ortler, S., Göbel, K., Nave, K.-A., Mäurer, M., Martini, R. and Wiendl, H. (2009). Accelerated course of experimental autoimmune encephalomyelitis in PD1-deficient CNS myelin mutants. Am. J. Pathol. *In press.*

6. Kroner, A., **Schwab, N.**, Ip, C., Sommer, C., Wessig, C., Wiendl, H., and Martini, R. (2009). The co-inhibitory molecule PD-1 modulates disease severity in a model for an inherited, demyelinating neuropathy. Neurobiol Dis *33*, 96-103.

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Clonal expansions of pathogenic CD8+ effector cells in the CNS of myelin mutant mice

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Tissue damage in the CNS is critically influenced by the adaptive immune system. Primary oligodendrocyte damage (by overexpression of PLP) leads to low-grade inflammation of high pathological impact, which is mediated by CD8+ T cells. To yield further insight into pathogenesis and nature of immune responses in myelin mutated mice, we here apply a detailed immunological characterization of CD8+ T cells in PLP-transgenic and aged wild type mice.

We provide evidence that T effector cells accumulate in the CNS of PLP-transgenic and wild-type mice and show a higher level of activation in mutant mice, indicated by surface markers and clonal expansions, as demonstrated by T cell receptor CDR3-spectratype analysis. Vβ–Jβ similarities suggest specificity against a common antigen, albeit we could not find specific responses against myelin-antigen-derived peptides. The association of primary oligodendrocyte damage with secondary expansions of pathogenic cells underlines the role of adaptive immune reactions in neurodegenerative and neuroinflammatory diseases.

Keywords: PLP; Oligodendrocyte damage; Inflammation; CD8+ T-cells; Clonal expansion; Multiple sclerosis

Introduction

The interplay between adaptive as well as innate immune reactions and the immune privileged central nervous system (CNS) has been known to play a critical role in the development and course of many neuroinflammatory and neurodegenerative diseases. Multiple sclerosis (MS) for example is an acquired CNS disorder of young adults and characterized by inflammatory demyelination as well as axonal degeneration. A variety of findings support the hypothesis that in some subtypes of MS the immune system may not be the initial trigger but suggest that inflammation is a secondary response to a primary degenerative stimulus in the CNS. Recent functional studies elucidating the interactions of the immune system with CNS structures have provided novel insights into the molecular mechanisms of inflammatory damage. The detailed histopathological classification of MS lesions demonstrated their remarkable heterogeneity. In general, MS lesions can be divided into four different patterns (patterns I–IV) (Lucchinetti et al., 2000). While cellular and humoral immune components are the prevailing elements found in pattern I and II, a "primary oligodendropathy" with less inflammation dominates in pattern III and IV lesions. On the basis of histopathological analysis of a small set of MS cases, a recently published study postulates a primarily oligodendroglial damage as initiator of MS lesions in general (Barnett and Prineas, 2004). Whereas the concept(s) of CNS lesion development are

Abbreviations: CD, cluster of differentiation; CDR3, complementarity determining region 3; CM, complete medium; CNS, central nervous system; CTL, cytotoxic T lymphocyte; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; FACS, fluorescence activated cell sorting; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; IFNγ, interferon-γ; IL-2, interleukin-2; MBP, myelin basic protein; MS, multiple sclerosis; MHC, major histocompatibility complex; MOG, myelin oligodendrocyte glycoprotein; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PE, phycoerythrin; PerCP, peridinium chlorophyllic protein; PLP, proteolipid protein; RAG, recombination activating gene; TCR, T cell receptor; Tg, transgenic; TNFα, tumor necrosis factor α; Wt, wild type.

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currently under controversial discussion (Lassmann, 2005), there is consent that molecular epitopes related to the myelin sheath play important roles as immunological targets. This is of particular pathological and clinical relevance since the myelin sheath is important not only for mediating the saltatory conduction of axonal action potentials but also for the maintenance of axonal integrity and survival (Bjartmar et al., 1999; Edgar et al., 2004; Frei et al., 1999; Griffiths et al., 1998; Lappe-Siefke et al., 2003; Martini, 2001; Samsam et al., 2003; Wrabetz et al., 2000; Yin et al., 1998; Yin et al., 2000).

We have recently investigated a mouse myelin mutant over-expressing proteolipid protein (PLP) in oligodendrocytes leading to myelin degeneration and late onset axonal degeneration that was accompanied by an elevation of CD11b+ macrophages and CD8+ T lymphocytes in the central nervous system (Ip et al., 2006). By crossbreeding these mutants with RAG-1 deficient

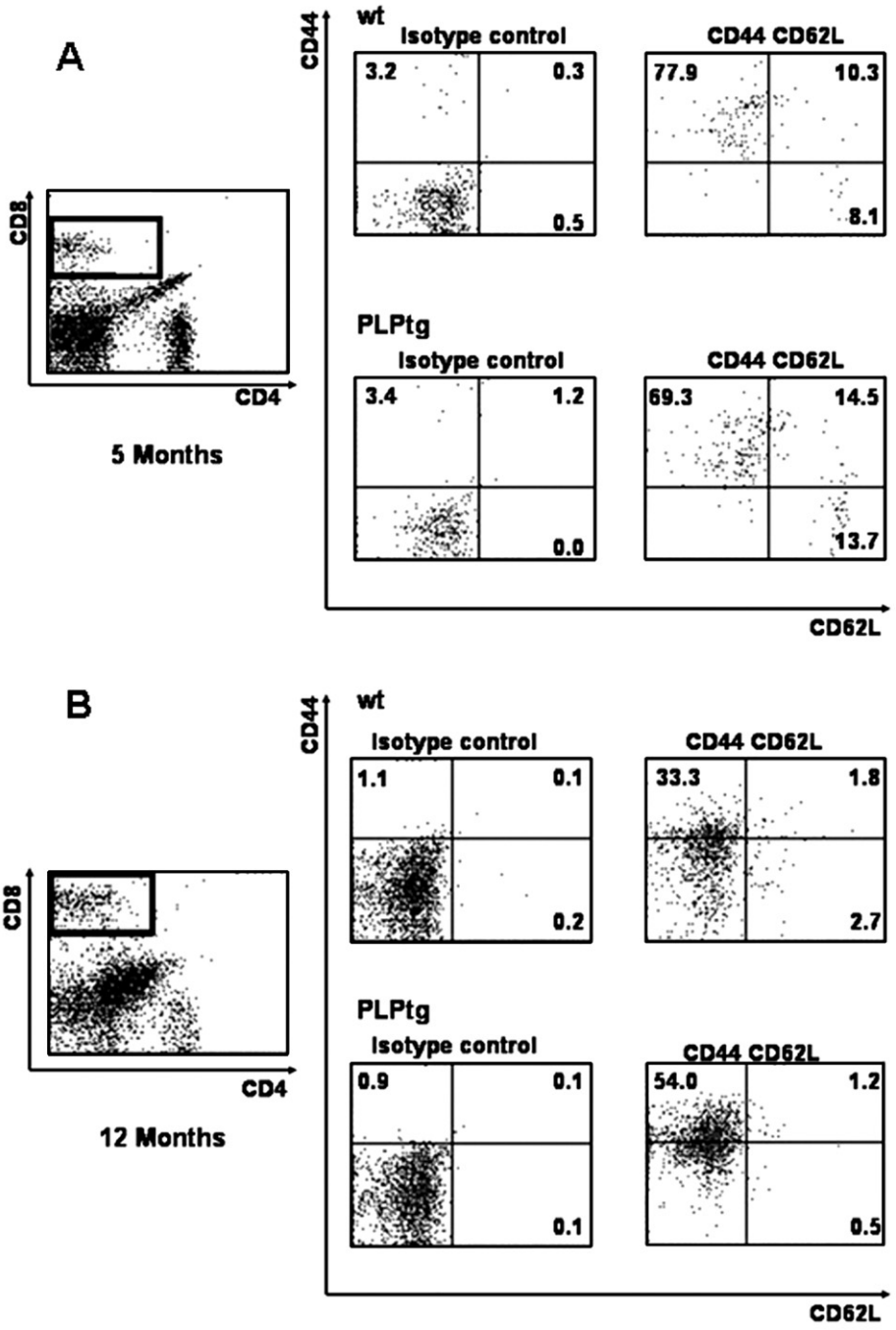

Fig. 1. Flow cytometry-based phenotyping of CD8+ T cells derived from CNS of wild-type and PLP-transgenic mice (5 months of age (A), 12 months of age (B)). Isolated lymphocytes were stained for CD8, CD44 and CD62L. After collecting all events of the stainings, the data were analyzed by gating on CD8+ cells and subsequently displaying their activation status by showing CD44 and CD62L expression.

mice that lack mature lymphocytes and by transplantating bone marrow of either CD8+/CD4− or CD8−/CD4+ mutants, we could clearly identify CD8+ T lymphocytes as pathogenic effector cells (Ip et al., 2006). Therefore, this model can serve as a paradigm to challenge the question how components of the adaptive immune system (CD8+ T cells) are interrelated or linked to a primary myelinopathy.

Results

CD8+ T effector cells appear in aging wild-type and PLP-transgenic mice

In addition to previous published results about the phenotype of CNS-specific T cells in 12-month-old PLP-transgenic mice (Ip et al., 2006), we compared the T cell phenotype in young (5 month) and old (12 month) wild-type and PLP-transgenic mice (Fig. 1). Strikingly, we observed T lymphocytes with effector cell phenotype (CD44+ CD62L low) in 12-month-old PLP-transgenic and wild-type mice. The CD8+ T cells in PLP-transgenic mice showed higher CD44 expression, suggesting a stronger activation.

In younger mice (5 month) fewer T cells were detectable in the CNS of PLP-transgenic and wild-type mice. A fraction of approximately 10% of CD8+ T cells displayed a naive phenotype (CD62L high, CD44-), while the majority of CD8+ T cells already had an effector phenotype (CD62L low, CD44+). In contrast to 12-month-old individuals, the CD44 expression level did not differ between T cells from PLP-transgenic mice and wild-type mice at the age of 5 months.

Clonal T cell expansions in CNS of aging PLP-transgenic mice

We screened CD8+ and CD4+ T cells from spleens of 12-month-old PLP-transgenic or wild-type mice with a Vβ region specific antibody panel covering approximately 85% of the T cell compartment. We found no evidence for systemic changes in the Vβ repertoire in PLP-transgenic or in wild-type mice (Fig. 2).

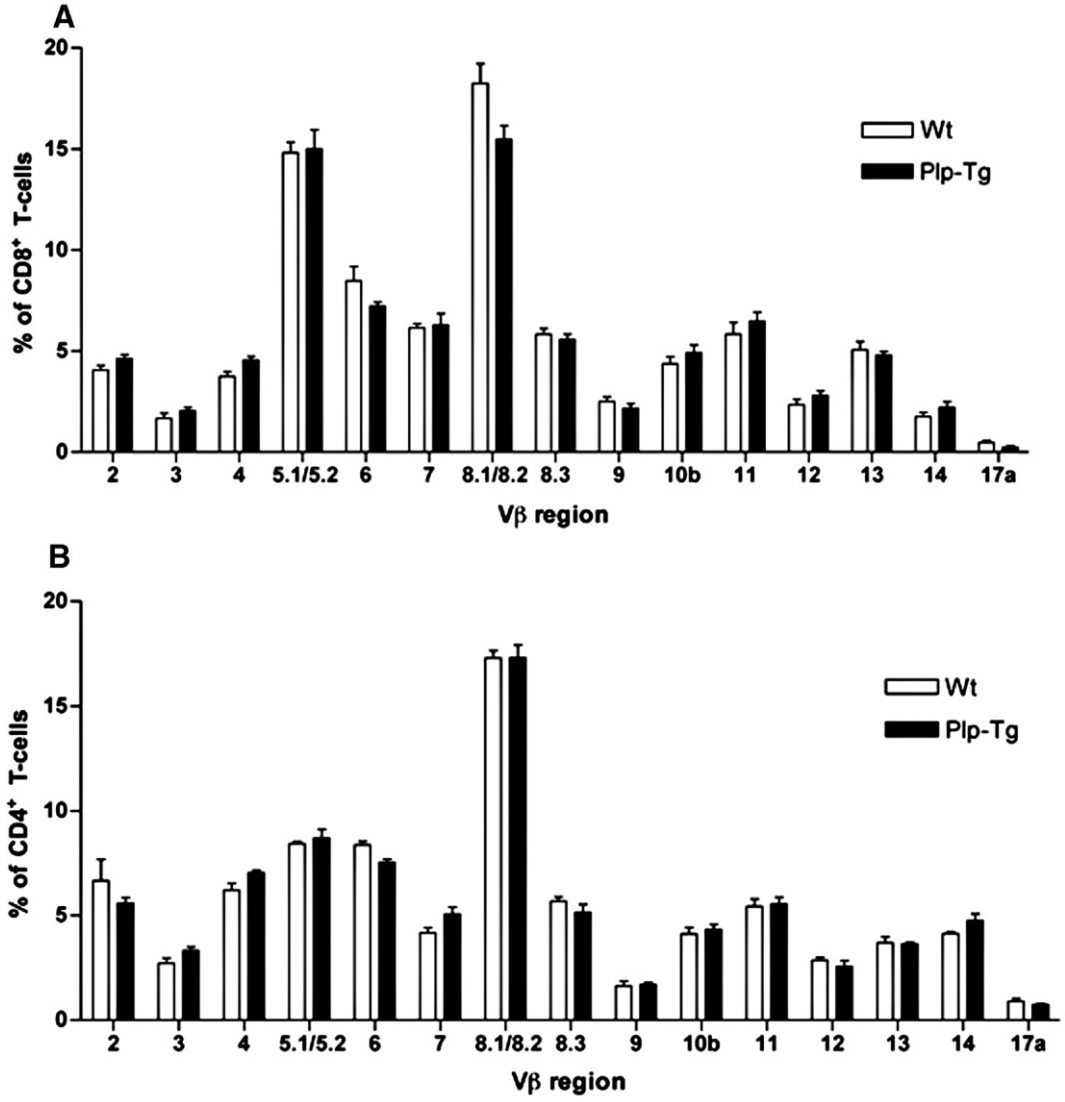

Fig. 2. Vβ analysis of splenic CD8+ (A) and CD4+ (B) T cells derived from wild-type and PLP-transgenic mice. 5×10^5 splenocytes were stained for CD8 and a panel of 15 Vβ specific antibodies. PLP-Tg: PLP-transgenic mice; wt: wild-type mice.

In order to further characterize the recently discovered CD8+ T cells involved in demyelination and axonopathy (Ip et al., 2006), we chose the sensitive PCR-based spectratyping method. This method detects the lengths of Vβ–Jβ regions of T cell receptor β chains (Pannetier et al., 1993) and reliably detects repertoire skewings and clonal TCR expansions.

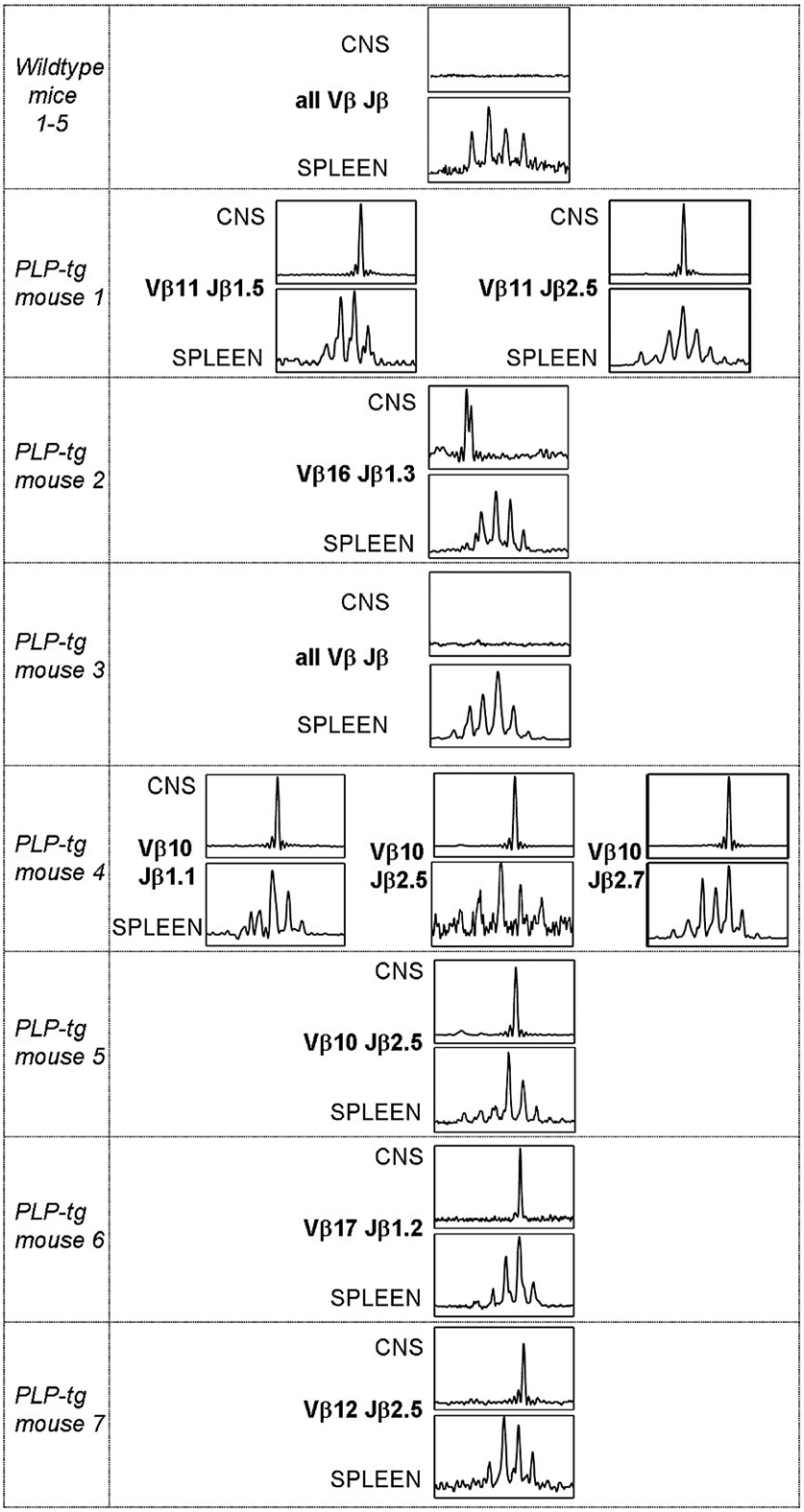

Fig. 3. CDR3 spectratyping of T cells derived from PLP-transgenic mice. Isolated CNS-specific lymphocytes and corresponding splenocytes were collected, RNA was isolated and cDNA was analyzed by PCR-based CDR3 spectratyping to determine Vβ–Jβ combinations of the T cell receptor composition.

Interestingly, we found that CD8+ T cells were clonally expanded in the CNS of 12-month-old PLP-transgenic mice, while no similar changes were found in the peripheral repertoire (splenocytes) (Fig. 3). No clonal expansions could be found in wild-type mice of the same age. The majority of transgenic mice showed 1 to 3 alternative Vβ–Jβ combinations. Interestingly, in each mouse the expanded T cells only use one Vβ segment, suggesting related TCR specificity. In two mice we found the same Vβ–Jβ combination and CDR3 peak position, possibly indicating a very similar antigen specificity of these expanded TCR. We were also able to amplify and sequence the CDR3 region of 5 of these expanded T cell populations.

By sequencing the Vβ–Jβ peaks that were clonally expanded in three transgenic mice (mouse 4, 5 and 7), we determined the amino acids of the CDR3 region (Fig. 4). Interestingly, there were negatively charged amino acids (e.g. D and E) in the end region of all sequences. However, these charges vary between position 6 and 10 after the conserved cystein. In 3 of the sequences (1, 2 and 5), there were positive charges between the C and the mentioned negative charge, but this positive charge is missing in sequences 3 and 4. Also, all sequences end with two aromatic amino acids (sequence 4 only one). A difference between the sequences is that number 3 and 4 show hydrophobic amino acids (F and L) instead of the positive charge observed in the others. All in all, these data suggest a common antigen underlying the expansion of the CD8+ T cells.

PLP-transgenic do not show signs of a systemic myelin antigen specific CD8+ T cell response

We next addressed the question if we can detect myelin antigen specific CD8+ T cell responses in PLP-transgenic mice. Therefore, we predicted possible MHC-I-restricted peptides for PLP, MOG and MBP by the bioinformatical tool www.syfpeithi.de. Predicted binders with consensus sequences for binding to H2-Kb and -Db were chosen and synthesized (Fig. 5). In a control experiment we immunized C57Bl6 mice with PLP-Db1 and 2 or PLP-Kb1 and 2 and detected the CD8+ T cell response against these peptides in spleens on day 21 post immunization after in vitro restimulation. Although these control experiments demonstrated the principle relevance of the predicted peptides (Fig. 6), we did not find evidence for systemic myelin antigen specific CD8+ T cell responses in PLP-transgenic or wild-type mice with any of the predicted epitopes by performing intracellular cytokine staining after in vitro restimulation (Supplementary Fig. 1).

Peptide	Amino acids
PLP-Db1	VCGSNLLSI
PLP-Db2	AATYNFAVL
PLP-Kb1	ATYNFAVL
PLP-Kb2	NYQDYEYL
MBP-Db1	ADPGNRPHL
MOG-Kb1	LIICYNWL
MOG-Kb2	VGLVFLFL
MOG-Db1	SPGKNATGM
MOG-Db2	FYWVNPGVL

Fig. 5. Myelin antigen peptides.

Discussion

We have previously shown that a transgenic mouse mutant overexpressing the major CNS myelin component PLP develops a low-grade inflammatory reaction comprising CD8+ effector cells that showed substantial pathogenic relevance with regard of myelin and axonal damage (Ip et al., 2006). By contrast, CD4+ cells were neither upregulated in the mutant CNS nor played a pathogenic role as revealed by bone marrow chimeric myelin mutants (Ip et al., 2006).

In order to yield further insights in the pathogenic relevance and nature of the adaptive immune response in myelin mutated mice, we here provide a detailed immunological characterization of systemic and CNS CD8 T cell responses. We provide data showing that T effector cells are surveying the CNS of PLP-transgenic and wild-type mice. These cells accumulate with age and show a more activated phenotype in PLP-transgenic mice. Exclusively in PLP-transgenic mice these T cells are clonally expanded suggesting an antigen-specific pathomechanism. Also, the same Vβ–Jβ combination occurred in two individual mice and supports the assumption that the corresponding T cells recognize a single epitope of a specific antigen.

By sequencing the Vβ–Jβ peaks that were clonally expanded in three transgenic mice (mouse 4, 5 and 7), we determined the amino acids of the CDR3 region (Fig. 4). Taken together, the analyses of the amino acids show a number of similarities between the

	PLP-Tg mouse	TCRVβ region	TCRVβ sequence	NDN region	TCRJβ sequence	TCRJβ region
1	4	10	CAS	SYRGA	NTEVFFGKG	1.1
2	4	10	CAS	RRTE	DTQYFGPG	2.5
3	4	10	CAS	LGLGI	EQYFGPG	2.7
4	5	10	CAS	SFGL	QDTAQFGPG	2.5
5	7	12	CAS	KT	QDTQYFGPG	2.5

Fig. 4. Vβ–Jβ amino acid sequences of clonally expanded TCR.

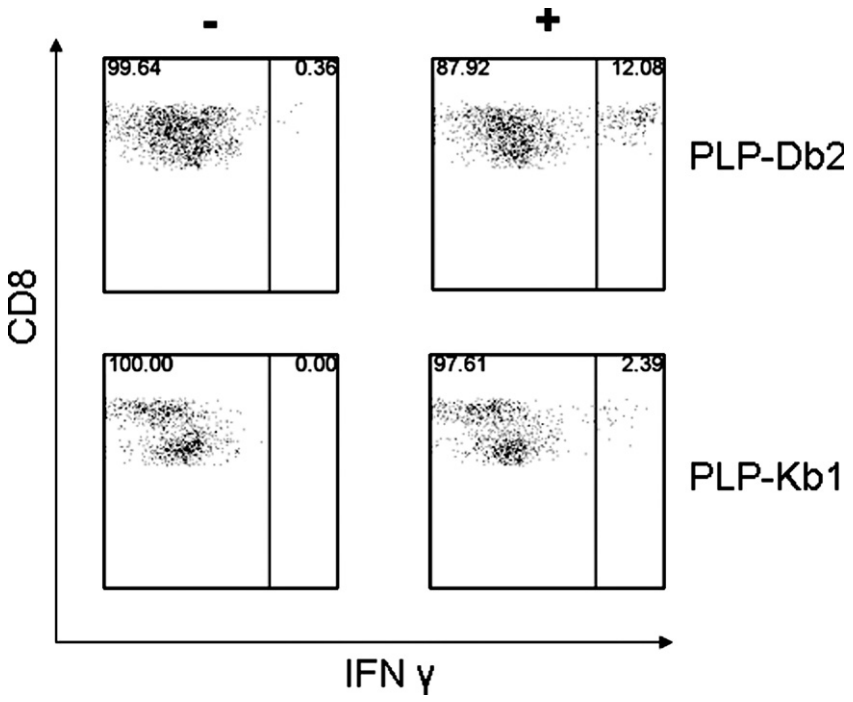

Fig. 6. Validation of predicted PLP-specific MHC-I-restricted peptides. C57Bl6 mice were immunized with peptides derived from bioinformatically predicted epitopes of PLP on H2-b background. 21 days post immunization spleens were removed and splenocytes were restimulated twice in the presence of PLP peptide and interleukin-2. Peptide-specific IFNγ responses were detected by intracellular cytokine staining. − Control peptide (for Db: GP33, for Kb: SIY), + relevant peptide.

expanded clones. Although one could have expected an even higher similarity in the sequences 2 and 4 due to the use of the same Vβ and Jβ regions, we consider the similarities as not being coincidental but pointing to a common antigen that triggers these expansions since the immense amount of possible CDR3 variations has to be taken into account.

Additionally, by performing flow cytometry-based Vβ screening and PCR-based spectratyping with spleen-derived cells, we did not find evidence for systemic clonal expansions of CD8+ or CD4+ T cells (Figs. 2 and 3). This suggests that the expansions are limited to the CNS of the transgenic mice.

Clonal restrictions of the T cell repertoire, as found in the CNS of myelin mutant mice, indicate a focused adaptive cellular immune reaction. Mutant myelin herein represents a putative local trigger of such an immune reaction since wild-type mice of similar age, strain and breeding condition do not show this phenomenon. Since no exogenous triggers are identifiable in our model system, these clonal expansions of the CD8+ T cell repertoire could be interpreted as a secondary "autoimmune" response or loss of tolerance of T cells that occurs in aging mutant mice. It is tempting to speculate that CD8+ cells in the CNS, that have been proven to be of pathogenic relevance in this model previously (Ip et al., 2006), putatively recognize self-antigen(s) that are most likely under the control of central tolerance (Kyewski and Klein, 2006). This concept implies that high-avidity clones against self-antigens are deleted during T cell development in the thymus while low-avidity clones can escape. Therefore, the pathogenic CD8+ T cells would most likely represent low avidity T cell clones, the only clones that sustain the process known as negative selection (Liu et al., 1995). Low avidity T cells are known to have a selective loss of function compared to high avidity clones. Most notably, the capacity to achieve optimal clonal expansion and the production of IL-2 and the IL-2-responsiveness are decreased in low avidity clones (de Visser et al., 2001). Therefore, it is consistent that only low numbers of CD8+ T cells were detected in the mutant CNS compared to the robust elevation of lymphocytes which is seen during an immune response with high avidity clones (e.g. in acute viral infections). However, this should not imply that the CD8+ effector cells in the mutants have a low pathogenic impact; by contrast, only relatively few cells cause a substantial neurological damage reflecting that individual cells act highly effective (Ip et al., 2006).

After providing strong first evidence for clonal expansions of CD8+ T lymphocytes in the CNS of our myelin mutants, we addressed the question about antigen specificity. We tried to detect CD8+ T cell responses against bioinformatically predicted MHC class I restricted epitopes of various myelin antigens including PLP in PLP-transgenic mice. The epitopes were predicted with the SYFPEITHI algorithm (Rammensee et al., 1999) and only peptides of epitopes with the appropriate consensus sequences for binding to H2-Kb and-Db were chosen and synthesized (Fig. 5). In order to prove the relevance of the predicted epitopes and the applied readout, we performed a peptide immunization using the predicted PLP peptides. We were able to show that these peptides are H2-Kb or -Db binders and that the readout for detecting CD8+ T cell responses was appropriate (Fig. 6). Screening for CD8+ T cell responses against the predicted epitopes of myelin antigens provided no evidence for a systemic response against the chosen myelin antigens in 12-month-old (or younger) PLP-transgenic mice (Supplementary Fig. 1). Nevertheless, we cannot exclude the possibility that these epitopes might be detected by the T cells in the target organ under *in vivo* conditions.

After observing that the CD8+ effector cells in the CNS of the myelin mutants show clonal expansions, it is tempting to hypothesize how this "autoreactivity" is generated. Intriguingly, T effector

cells are surveying the CNS both in wild-type and PLP-transgenic mice. We found CD8 effector T cells in both mice (Fig. 1). In wild-type mice, however, these cells remain ineffective because the physiological antigen densities on possible target cells may be too low. Low avidity T cells are known to need higher antigen densities to become responsive (Walter et al., 2003) and both antigen density on target cells and T cell avidity are important criteria for the constitution of CTL effector functions (Shimonkevitz et al., 1985). PLP is approximately 2-fold overexpressed in oligodendrocytes of the investigated PLP-transgenic mice (Readhead et al., 1994), probably resulting in an elevated antigen density. Autoreactive low avidity T effector cells surveying the CNS and particularly the oligodendrocytes (Ip et al., 2006) might be triggered by these supraphysiological antigen densities. This might lead to direct cytolysis of oligodendrocytes by lymphocytic FasL or the perforin/granzyme system and/or the release of TNFα and IFNγ, which subsequently activate macrophages in the CNS. Indeed, our previous results provided evidence that, in some CNS areas, particularly in the corpus callosum, the T cells tend to appear earlier than the macrophages (Ip et al., 2006). Consistent with this possibility is that we can show that the CD8+ T effector cells are stronger activated in the CNS of PLP-transgenic mice compared to age-matched wild-type mice documented by a higher CD44 expression (Fig. 1).

As an alternative possibility, one could hypothesize that the overexpression of PLP induces intracellular stress which could occur due to an accumulation of the protein and cholesterol in intracellular compartments of the oligodendrocytes (Simons et al., 2002). This intracellular stress could cause several immune-relevant glial reactions, such as expression of cytokines and, as seen in one of our previous studies, upregulation of MHC-I molecules and sialoadhesin on the surface of the diseased myelinating glial cells and macrophage-like cells, respectively (Ip et al., 2007).

In any case, our studies in PLP overexpressing mice as a model for primary glial damage and accompanying immunological reactions might have substantial clinical implications. For instance, it is well known that, in X-linked adrenoleukodystrophy, a serious neurological disorder in childhood, the most detrimental and rapidly progressing forms are usually associated with inflammation (Berger et al., 2001). In this disorder, CD8+ cytotoxic T lymphocytes displaying CD44 are often tightly attached to oligodendrocytes, as we have observed in the PLP mutants, suggesting cellular toxicity (Moser, 2004). In Leber's hereditary optic neuropathy, in which mitochondrial DNA mutations are the primary causes, inflammatory reactions can lead to a close clinical and histopathological relationship to MS (Kovacs et al., 2005). Most strikingly, it has been shown recently that PLP mutations in humans can be linked to a primary progressive or relapsing-remitting MS exhibiting steroid-responsive relapses (Gorman et al., 2007; Warshawsky et al., 2005).

These examples are highly related to the recent view that the primarily immune-independent damage or dystrophy of oligodendroglial cells may underlie some forms of MS (pattern III and IV lesions, see Introduction (Lucchinetti et al., 2000)) or – even more provocatively – might be a major pathological pathway for this disorder (Barnett and Prineas, 2004). It is therefore highly relevant to disclose the pathogenic pathways from primary oligodendroglial injury to secondary immune reactions of significant pathogenic relevance, implicating antigen presentation, T lymphocyte activation, mono- or oligoclonal expansion and cytotoxic cell damage. Our present study provides evidence that a primary degenerative CNS disorder is associated with secondary changes of the T cell repertoire, which is of significant pathogenic relevance.

Experimental methods

Mice

The generation of PLP-transgenic mice expressing autosomal copies of the entire wild-type PLP gene has been described previously (Readhead et al., 1994). These mutants have been bred and genotyped as reported in a recent study (Ip et al., 2006). PLP-transgenic mice and wild-type littermates were kept in our animal facility under specific pathogen-free conditions.

Antibodies and peptides

The myelin-antigen-derived peptides (Fig. 5) and control peptides for H2-Kb (SIYRYYGL) and H2-Db (KAVYNFATC) were purchased from Genscript Corp. (Piscataway, NJ, USA). The peptides were dissolved in DMSO at 1 mM and stored at −20 °C. For immunization the PLP-derived peptides were dissolved in DMSO at a concentration of 20 mg/ml.

The following primary antibodies were used: anti-murine-CD8-FITC (clone 53-6.7), anti-murine CD4-PerCP (clone RM4-5) and anti-murine-IFNγ-PE (clone XMG1.2) all purchased from BD Pharmingen (Heidelberg, Germany). Additionally, the TCR Vβ screening panel (557004) from BD Pharmingen (Heidelberg, Germany) was used. Isotype control rat-IgG2b-FITC (clone A95-1) was purchased from BD Pharmingen (Heidelberg, Germany).

Isolation of splenocytes and CNS leukocytes

Splenocytes were passed through a 70 μm cell strainer (Becton Dickinson) and red blood cells were lysed with the hypotonic ACK buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA, pH 7.2–7.4). Splenocytes were incubated in complete medium (CM) consisting of DMEM with 5% FCS, 10 mM HEPES, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 1% non essential amino acids and 25 μg/ml gentamycin.

The CNS-lymphocytes were collected as described previously (Becher et al., 2001). Briefly, the spinal cords were removed by flushing the spinal column with sterile PBS and the brains were collected. Both tissues were homogenized and strained through a 40 μm nylon filter. After centrifugation, the cell pellet was resuspended in 9 ml Percoll. PBS was added up to 30 ml. After gentle vortexing, the gradient was centrifuged at 2900×*g* for 60 min at 4 °C (brake was disabled). The interphase cells were collected and extensively washed before RNA isolation.

Stimulation of leukocytes

Splenocytes (8×10^6/well) were stimulated in 12-well plates in complete medium supplemented with 1 nM of myelin-antigen-derived peptide and 20 IU/ml of human recombinant IL-2 (PROLEUKIN, Chiron Corporation, Emeryville, CA, USA). After 5 days of in vitro culture, myelin-antigen-specific CD8+ T cells were analyzed by intracellular cytokine staining for IFNγ secretion.

For detecting the CD8+ T cell responses after immunization with PLP-derived peptides a second restimulation was pursued in order to achieve a more pronounced IFNγ response.

Vβ screening and flow cytometry-based phenotyping

For flow cytometry-based Vβ screening, splenocytes of PLP-transgenic and wild-type mice (5×10^5/staining) were stained with the Vβ screening panel (BD Pharmingen) according to the manufacturer's instructions, co-stained with anti-CD4 and anti-CD8 antibodies and subsequently analyzed by flow cytometry.

The CNS-specific lymphocytes were phenotyped by staining isolated leukocytes from brain and spinal cord for CD8 and the activation markers CD62L and CD44. In order to achieve meaningful analyses we collected all

events of the stainings, subsequently gated on CD8+ T cells and displayed their activation status.

Intracellular cytokine staining

Intracellular cytokine staining for IFNγ was performed after stimulation with 100 nM of the myelin-antigen-derived peptide or control peptide as described previously (Pavlenko et al., 2005). The samples were analyzed on a FACSCalibur (Becton Dickinson) and 1×10^4 to 5×10^4 events were collected. Acquisition and analysis of samples were performed using the CELLQuestPro software (Becton Dickinson).

RNA isolation and cDNA synthesis

RNA isolation was performed using the Absolutely RNA Microprep Kit (Stratagene, Edinburgh, UK) for up to 5×10^5 cells and the RNeasy Mini Kit (Qiagen, Hilden, Germany) for 5×10^5 to 1×10^7 cells according to the manufacturer's instructions. Five hundred nanograms of RNA (in 35 µl) was then transcribed into cDNA, using 10 µl RT-Buffer, 20 µl dNTP, 5 µl Random Hexamer Primers, 2 µl RNAse Inhibitor and 6 µl Reverse Transcriptase (all purchased from Applied Biosystems) and 22 µl 25 mM $MgCl_2$ (Roche, Mannheim, Germany). PCR program: 25 °C, 10 min; 37 °C, 60 min; 95 °C, 15 min.

Spectratyping

The CDR3 spectratyping with the corresponding primers was performed as described previously (Pannetier et al., 1993). The Wilson Vβ nomenclature has been used. Briefly, 500 ng of leukocyte mRNA was transcribed into cDNA and then used in PCRs with a Cβ specific reverse primer and 24 Vβ specific forward primers. The PCR steps were as follows: 94 °C, 1 min; 94 °C, 1 min 10 s, 60 °C, 1 min, 72 °C, 4 min (40 steps); 72 °C, 10 min. Each Vβ–Cβ PCR product was labeled during a PCR, using an NED-labeled Cβ reverse primer. The PCR steps were: 94 °C, 2 min, 60 °C, 1 min, 72 °C, 15 min (5 steps). The NED-labeled Vβ–Cβ PCR products were analyzed on an ABI Prism 3130 capillary sequencer (Applied Biosystems) to determine their length and distribution, using a module for fragment analysis. As an internal length standard, 500-ROX (Applied Biosystems) was used in every sample. If the analysis rendered an expansion in the CNS, the corresponding (non-labeled) Vβ–Cβ product was used in PCRs with 12 different (fluorescence-labeled) Jβ reverse primers, after which the products were again analyzed on the capillary sequencer, to determine the Vβ–Jβ specification of the expanded T cells. In case of splenocyte cDNA, all Vβ–Jβ PCRs were performed to assess the status of clonal T cell expansions in blood.

Sequencing of specific Vβ–Jβ peaks

If clonal expansions were visible, the corresponding PCRs were performed with unlabeled Vβ, Cβ and Jβ primers as described in the spectratyping section. The resulting PCR products were cloned into the pGEM®-T vector (Promega, Madison, Wisconsin, USA) following the manufacturer's instructions. In short, the PCR product was ligated with the vector overnight, transformed into competent *E. coli* bacteria and then subjected to X-Gal blue/white screening on LB-plates with ampicillin, IPTG and X-Gal (all from Sigma, Munich, Germany). The white clones were selected and after incubation overnight, the DNA of the bacteria was extracted by plasmid preparation (QIAprep Spin Miniprep Kit, Qiagen, Hilden, Germany) and sequenced by Seqlab sequence laboratories, Goettingen.

Immunization with PLP peptides

C57Bl/6 mice were subcutaneously immunized with 20 µg of each PLP-derived peptide (Genscript, New Jersey, USA) in 100 µl complete Freunds Adjuvants (Sigma, Steinheim, Germany). Four hundred nanograms of Pertussistoxin (Listlabs, Campbell, CA, USA) in 200 µl PBS was applied at days 0 and 2 post immunization intraperitoneally. The peptides were divided into two groups (PLP-Db1+PLP-Db2 and PLP-Kb1+PLP-Kb2). Peptides of one group were immunized simultaneously. Twenty-one days later the animals were sacrificed and splenocytes were restimulated and analyzed by peptide-based intracellular cytokine staining.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mcn.2007.08.002.

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Human myoblasts modulate the function of antigen-presenting cells

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Abstract

Muscle biopsy specimens of myositis patients were analyzed for the presence of dendritic cells (DC) and macrophages (MPh) by immunohistochemistry. The interaction of DC and myoblasts (MB) was studied by coculture and effects on DC phenotype and function were assessed by flow cytometry and T-cell proliferation assays. Effects of MB-lysates on the phagocytic capacity of MPh were analyzed in bead-incorporation assays.

Myositis specimens revealed a tendency towards more immature DC. MB modulated the maturation state of DC and DC recovered from MB-coculture had an inhibitory effect on T-cell proliferation. MB-lysates strongly stimulated MPh phagocytosis. Hypothetically, MB might modulate APC, counterbalancing immune-mediated damage.

Keywords: Myositis; Antigen-presenting cells; Dendritic cells; Macrophages; Myoblasts

1. Introduction

Idiopathic inflammatory myopathies are a heterogenous group of autoimmune disorders characterized by progressive muscle weakness. The inflammatory myopathies comprise three major and distinct subsets: dermatomyositis (DM), polymyositis (PM) and inclusion body myositis (IBM). Although each subset retains characteristic clinical, immunopathological and morphological features, they all share the presence of infiltrating antigen-presenting cells (APC) and T cells within the muscle (Dalakas, 2004).

In addition to macrophages (MPh), dendritic cells (DC) have been identified in cellular infiltrates within muscle biopsies giving rise to the hypothesis that DC, the most potent APC, might play a key role in the initiation of a self-directed T-cell response in inflammatory muscle disorders (Greenberg et al., 2007; Page et al., 2004). Skeletal muscle is considered a specialized immunological microenvironment, which adds to the incentive of analyzing immune reactions in inflammatory muscle disorders. Recent findings suggest that MB act as facultative antigen-presenting cells through the expression of

Abbreviations: APC, antigen-presenting cells; BSA, bovine serum albumine; CCL, CC chemokine ligand; CCR, CC chemokine receptor; CFSE, 5(6)-Carboxyfluorescein diacetate *N*-succinimidyl ester; DC, dendritic cells; DC-i, immature dendritic cells; DC-Lamp, DC lysosome-associated membrane glycoprotein; DC-m, mature dendritic cells; DC-Sign, DC-specific ICAM grabbing non-integrin; DM, dermatomyositis; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; Glioma, human glioblastoma; GM-CSF, granunocyte macrophage colony stimulating factor; IBM, inclusion body myositis; IFNγ, interferon gamma; IL, interleukin; LPS, lipopolysaccharides; MB, myoblasts; MPh, macrophages; M-CSF, macrophages colony stimulating factor; MHC, major histocompatibility complex; Mono, monocytes; NCAM, neural cell adhesion molecule; PBMC, peripheral blood mononuclear cells; PE, phyco-erythrin; PI, propidium iodide; PM, polymyositis; SMC, skeletal muscle cells; TGFβ, transforming growth factor beta; TNFα, tumor necrosis factor alpha.

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major histocompatibility complex (MHC) and costimulatory molecules (Wiendl et al., 2003a,b). Moreover, cultured human MB secrete a variety of cytokines and chemokines and express cell adhesion molecules favouring muscle-immune cell interactions (Wiendl et al., 2005a). Thus, muscle cells can be considered as active participants rather than passive targets of immune reactions in inflammatory muscle diseases (Wiendl et al., 2005b).

Previous studies have demonstrated the accumulation of specific DC populations in myositis specimens, such as immature DC expressing the chemokine ligand 20/chemokine receptor 6 (CCL20/CCR6) complex or plasmacytoid DC (pDC) (Greenberg et al., 2007; Page et al., 2004). Therefore, it has been hypothesized that APC play a key role in the modulation of muscular immune reactions.

The aim of this study was to challenge if human skeletal muscle cells are able to modulate the function of APC, thereby dictating the outcome of APC-T cell interactions in inflamed muscle tissue. We were interested if human MB influence the maturation of monocyte-derived DC. Moreover, we addressed the question whether MB modulate the expression of immunologically relevant cells surface molecules (MHC and costimulatory molecules) or cytokines by APC. *In vitro* proliferation and phagocytosis assay were performed to analyze the influence of MB on APC function.

2. Materials and methods

2.1. Antibodies and reagents

The following primary antibodies were used for flow cytometry: phyco-erythrin (PE) conjugated anti-human B7-H1 (clone MIH1, eBioscience, NatuTec GmbH, Frankfurt, Germany), allophycocyanin (APC) conjugated anti-HLA-ABC (clone W6/32), anti-HLA-DR-APC (clone L243, both Leinco Technologies, St. Louis, MO, USA); anti-CD14-PE and -APC (clone MOP9), fluorescein isothiocyanate (FITC) conjugated anti-neural cell adhesion molecule (NCAM) (clone NCAM16.2), anti-CD80-PE (clone L307.4), anti-CD86-PE (clone 2331(FUN1)), anti-DC-Sign-PE and -APC (clone DCN46) and the respective isotype controls were all purchased from BD, Heidelberg, Germany. Interferon (IFN)-γ and tumor necrosis factor (TNF)-α were from Peprotech EC Ltd (London, England). CFSE (5(6)-Carboxyfluorescein diacetate *N*-succinimidyl ester) was purchased from Molecular Probes (Eugene, OR, USA). Macrophage colony stimulating factor (M-CSF), and granulocyte macrophage stimulating factor (GM-CSF) and Interleukin-4 (IL-4) were from R&D Systems (Minneapolis, MN, USA). CD3/CD28 T-cell expander Dynabeads were purchased from Dynal Biotech ASA (Oslo, Norway), Fluoresbrite Carboxy YG 2.0 micron Microspheres from Polysciences, Inc (Warrington, PA, USA). Propidium iodide (PI) and Lipopolysaccharides (LPS) (S. thyphi (L-7261)) were bought from Sigma-Aldrich (Munich, Germany). RPMI1640 was from Gibco Invitrogen GmbH (Karlsruhe, Germany), Fetal Calf Serum (FCS) from PAA (Cölbe, Germany) and Skeletal Muscle Growth Medium (SMGM) from Promocell (Heidelberg, Germany).

DC were histochemically charaterized by staining with DC-Sign (clone 120612, R&D Systems, Minneapolis, MN, USA), DC-Lamp (clone 104.G4, Immunotech), CD1a (clone 010, Immunotech, Krefeld, Germany) and CD83 (HB15e, BD, Heidelberg, Germany). Monocyte and macrophage populations were visualized by staining for CD68 (clone KP1, DAKO, Hamburg, Germany) or MRP 8/14 (clone 27E10, BMA Biomedicals, Augst, Switzerland).

2.2. Immunohistochemical studies

Diagnostic muscle biopsy specimens were obtained from patients with inflammatory myopathies [polymyositis (PM, $n=5$), dermatomyositis (DM, $n=5$), inclusion body myositis (IBM, $n=5$)]. Written consent was obtained from all donors and tissue sampling was approved by the local ethics committee. Samples analyzed in this study were chosen according to the presence of typical clinical features in the patient's history and the presence of inflammatory lesions in the biopsy meeting the diagnostic criteria for each myositis entity.

Flash-frozen muscle biopsy specimens were cut into 8–10 μm cryostat sections and analyzed by immunohistochemistry. Acetone-fixed air-dried sections were blocked with 10% BSA and human IgG followed by incubation with primary antibodies or corresponding nonimmune IgG isotype controls for 45 min. The reaction product was visualized with the streptavidin-biotin method (reagents from DAKO, Hamburg, Germany) using diaminobenzidine (Serva, Heidelberg, Germany) as a substrate.

2.3. Cell isolation and culture

Myoblasts (MB) were isolated from biopsies of healthy donors as described previously (Wiendl et al., 2003a). MB cultures and muscle biopsy specimens were obtained from the Muscle Tissue Culture Collection at the Friedrich-Baur-Institute in Munich. Proliferating MB were further purified by NCAM (clone 5.1H11, hybridoma supernatant) magnetic bead separation and cultured in SMGM. After purification, MB cultures stained >95% positive for the neural cell adhesion molecule (NCAM/CD56) by flow cytometry.

Peripheral blood mononuclear cells (PBMC) were isolated from healthy volunteers by centrifugation on a Lymphoprep™ (Fresenius Kabi Norge AS, Oslo, Norway) density gradient. Monocytes were selected by adhesion to plastic flasks for 1 h. Dendritic cells were generated as previously described (Schreiner et al., 2004). In brief, monocytes (>90% pure, as assessed by flow cytometry) were cultured in RPMI 10% FCS supplemented with GM-CSF (100 ng/ml) and IL-4 (40 ng/ml). After 5 days, the cells exhibited an immature DC phenotype (DC-Sign++ CD14− CD1a+ MHC II low CD86 low CD80 low/− CD83−). Maturation was induced by 48 h-incubation of the immature DCs (DC-i) with LPS (5 μg/ml). High levels of surface MHC class II and costimulatory molecules (CD86, CD80), as well as low levels of DC-Sign identified mature DCs (DC-m).

To generate macrophages (MPh), monocytes were cultured in RPMI 10% FCS supplemented with GM-CSF (100 ng/ml) and M-CSF (10 ng/ml) for 7 days. MB and APC were cocultured in a

1:1 mixture of RPMI 10% FCS and SMGM in 6-well (1×10^6/well) or 12-well plates (1×10^5/well). After 48 h cells were harvested and subjected to flow cytometry or tested in functional assays.

2.4. CFSE-proliferation assay

PBMC from healthy donors were isolated and washed twice with PBS. 1×10^6 PMBC were incubated in 1 ml CFSE-staining-solution (2.5 μmol CFSE in PBS) for 1 min. The reaction was stopped by adding 1 ml FCS. Cells were washed twice in PBS and cultured in 96-well plates (5×10^4/well). CD3/CD28 beads were added to each well at a 1:1 ratio to induce T-cell proliferation. APC were pipetted carefully from the coculture to avoid contamination with adherent MB. Pure APC were added to the proliferating PBMC at a 1:1 ratio.

After 60 h, the PBMC were harvested. Proliferation of T cells was assessed by analyzing CFSE-staining intensity/dilution in the subsequent FACS analysis (FACSCalibur™, Becton/Dickinson, Heidelberg, Germany) and the positive control (stimulated with beads, but no DC) was set to the value of 1 in each corresponding experiment.

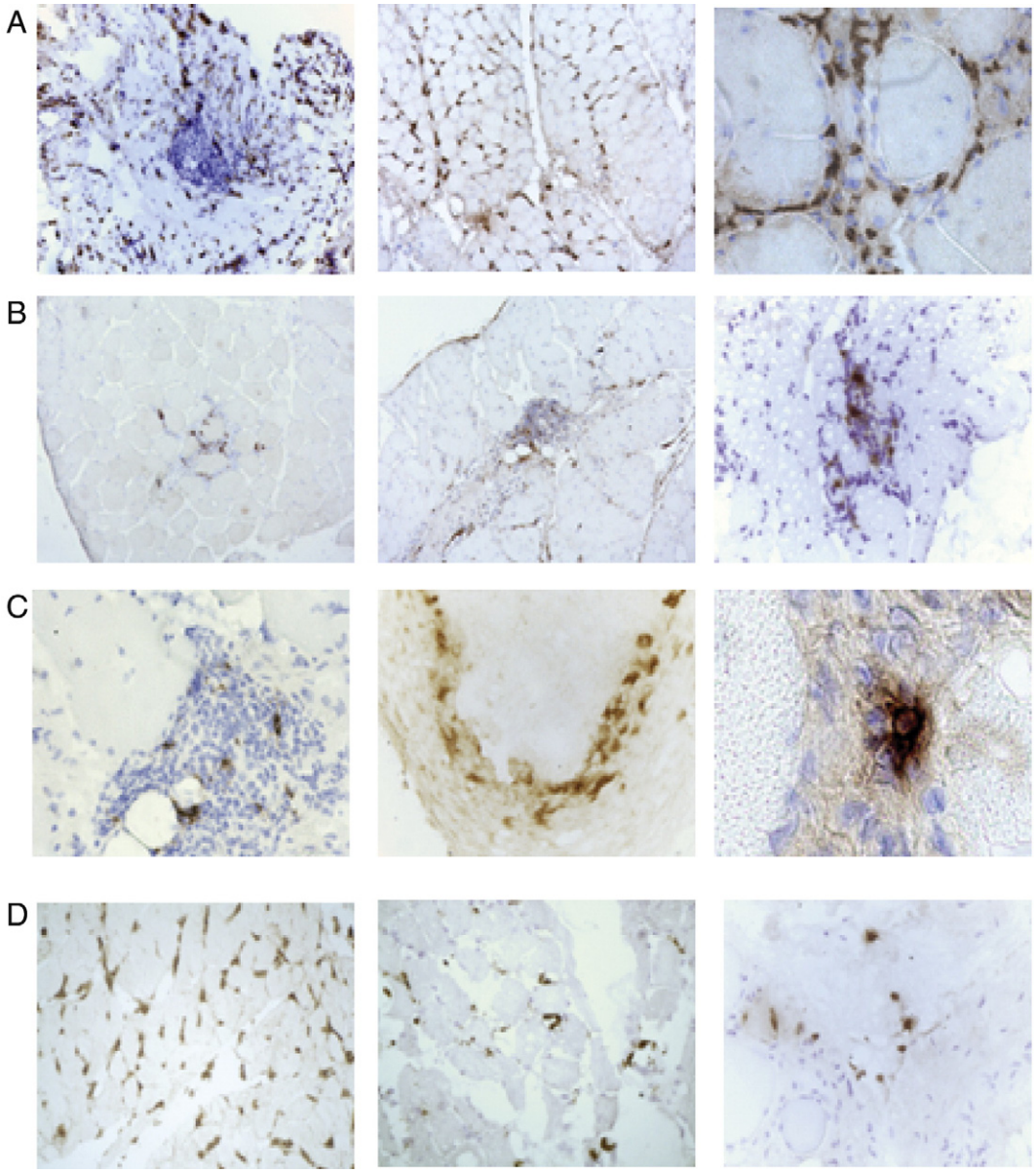

Fig. 1. Different types of antigen-presenting cells (APC) are present in inflammatory myopathies. A) Abundant *DC-SIGN* immunoreactive cells are detected in a muscle biopsy of a patient with polymyositis (PM, left panel; 100×), dermatomyosits (DM, middle; 100*x*100×) and inclusion body myositis (IBM, right panel; 400×). B) Mature *DC-LAMP* positive DC in muscle biopsies of differents patients with polymyositis (PM, left panel; 100×), dermatomyositis (DM, middle; 100×) and polymyositis (PM, right panel; 200×). C) *CD83* positive mature DC in a muscle biopsy of a dermatomyositis patient (DM, left; 400×). *CD1a* identifies immature DC in human tonsil (positive control, middle; 100×) and in a polymyositis specimen (PM, right panel; 1000×) D) Abundant expression of *CD68* by tissue macrophages in a dermatomyositis specimen (DM, left; 100×). Infiltrating macrophages characterized by expression of MRP8/14 in dermatomyositis (DM, middle; 100×) and in inclusion body myositis (IBM, right; 200×).

2.5. Macrophage bead-incorporation assay

Macrophages were grown in 12-well plates ($1–5 \times 10^5$/well) and incubated for 48 h with different cell lysates, which were generated by performing 5 freeze/thaw-cycles with liquid nitrogen. 1×10^6 fluorescent beads per 1×10^5 MPh were coated in 100 µl PBS 1% BSA for 30 min at room temperature (RT). The coated beads were added to 300 µl RPMI 10% FCS and the mixture was added to the macrophage culture. MPh were incubated with beads for 30 min at RT. Extensive washing in cold PBS (5 times) was performed to eliminate non-ingested particles. Afterwards the cells were harvested by adding EDTA/Trypsin to remove beads that adhered to the cell-membrane. Fluorescence was displayed in a FL1-histogram and phagocytosis was determined as the percentage of cells which incorporated one or more particles in the 30 min incubation period. The percentage of phagocytic MPh in the control was set to the value of 1 in each corresponding experiment.

2.6. Flow cytometry

Cells were harvested using Accutase (PAA, Cölbe, Germany), blocked with human IgG (Sandoglobulin, ZLB Behring, Marburg, Germany) and washed with FACS-buffer (PBS 0.1% Sodiumazide 0.1% BSA). After a 30 min incubation period with the respective antibody, cells were washed once and resuspended in 200 µl FACS-buffer. Antibody concentrations were carefully titrated prior to experiments. 1 min prior to the flow cytometry analysis, 50 µl PI (2 µg/ml) were added to a sample to allow exclusion of dead cells (PI+).

2.7. Statistical analysis

The two-sided, unpaired Student *T*-test was used to assess statistical significance. Significance was indicated as $*p<0.05$, $**p<0.01$.

3. Results

3.1. Dendritic cells and macrophages are found in close contact to muscle fibers in myositis

Immunohistochemical analysis of DC subpopulations was performed on muscle biopsy specimens from patients diagnosed with dermatomyositis (DM, $n=5$), polymyositis (PM, $n=5$) and inclusion body myositis (IBM, $n=5$). Staining for DC-Sign (DC-specific ICAM grabbing non-integrin, CD209), a C-type lectin receptor expressed by both immature and mature DCs (Page et al., 2004) revealed large numbers of DCs surrounding muscle fibers (Fig. 1A). In contrast to the abundant and widespread expression of DC-Sign, the expression of DC-Lamp (DC lysosome-associated membrane glycoprotein, CD208) characterizing mature DC (Page et al., 2004) was restricted to a small subgroup of dendritiform cells located in the centers of larger cellular infiltrates (Fig. 1B). Cells characterized by the expression of CD83, another marker for mature DC (Serafini et al., 2006) were even less numerous, however, the staining pattern closely resembled that of DC-Lamp (Fig. 1C, left panel). With respect to the large numbers of DC-Sign positive cells and the relatively small fraction of DC characterized by the expression of the maturation markers CD83 or DC-Lamp, we presumed that most of the DC should have an immature phenotype. However, we failed to detect CD1a immunoreactivity – a known marker for immature DC (Page et al., 2004) – in all but 1 PM and 1 IBM specimens. In those biopsies, CD1a positive cells had a ramified morphology and were found sporadically in between the muscle fibres (Fig. 1C, right panel). Overall, there was no significant difference with respect to the distribution or estimated numbers of DC populations in between the different entities of inflammatory myopathies.

The presence of MPh in myositis specimens ($n=2$ PM, 2 DM and 3 IBM) was demonstrated using the marker CD68. CD68 immunoreactive cells showed a widespread and abundant

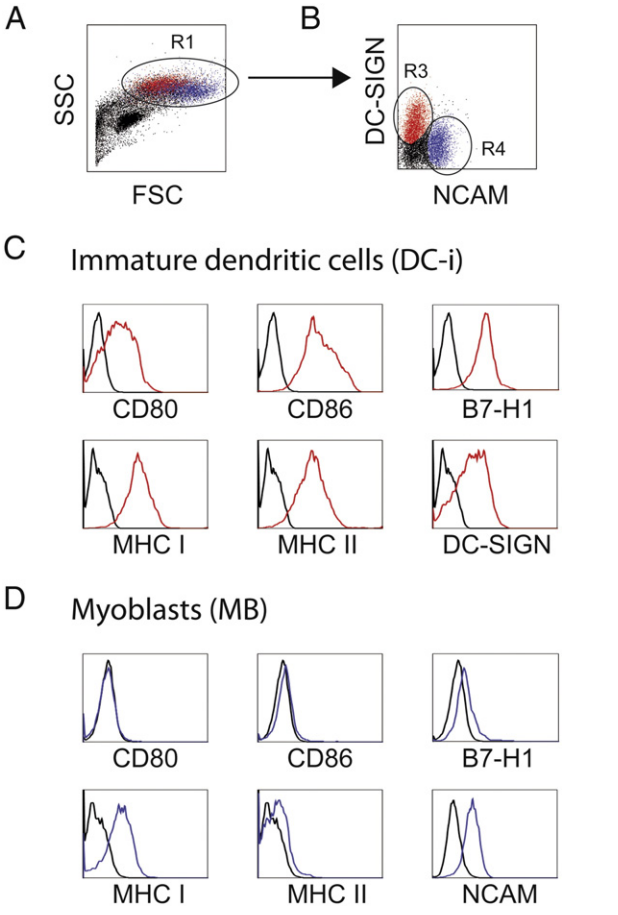

Fig. 2. Flow cytometry assessment and gating procedure of muscle cell — dendritic cell cocultures. Human myoblasts (MB) and dendritic cells (DC) were cocultured and subsequently subjected to flow cytometric assessment. Gating procedure for MB-DC cocultures is shown here. Region R1 was defined based on forward and side scatter properties (FSC/SSC) to exclude debris, platelets and T-lymphocytes (A). Region R2 was defined based on propidium iodide (PI) to exclude PI+ cells (not shown). Cells contained in R1 and R2 were displayed on a dot blot showing anti-DC-Sign-APC (DC marker) vs anti-NCAM-FITC (MB marker). Region R3 describes DC-Sign+ cells (DC) and region R4 NCAM+ cells (MB) (B). Cells contained either in R1+R2+R3 (DC) (C) or R1+R2+R4 (MB) (D) were further analysed concerning the expression of CD80, CD86, B7-H1, MHC I and MHC II (histograms of one representative example are shown; black line: isotype control).

distribution in all myositis specimens under investigation (Fig. 1D, left panel). Immature MPh were identified by staining for the heterodimer of myeloid-related protein 8 and 14 (MRP8/14), which are calcium-binding proteins belonging to the S100 protein family (Seeliger et al., 2003). The presence of MRP8/14 positive MPh reflects an acute inflammatory stage and MRP8/14 positive MPh have been shown to directly inhibit MB proliferation and to induce apoptosis in MB (10). In our samples, MRP8/14 immunoreactive MPh were found within the inflammatory infiltrates, especially in close vicinity of necrotic muscle fibers (Fig. 1D, middle and right panel).

3.2. Influence of muscle-APC interactions on phenotype and maturation of DC

To study the potential effects of skeletal muscle cells on APC phenotype and function, we established a coculture model of human primary MB and PBMC-derived APC (immature dendritic cells, mature dendritic cells, macrophages). We were interested if MB affect the maturation of DC and modulate the outcome of APC-T cell interactions by influencing the tightly regulated expression of costimulatory and coinhibitory molecules on the cell surface.

Flow cytometry confirmed the constitutive expression of MHC class I molecules, the inducible expression of MHC class II molecules (HLA-DR) and B7-H1 (data not shown) as well as the absence of the classical costimulatory molecules CD80, CD86 (Wiendl et al., 2003a,b) on neuronal cell adhesion molecule (NCAM) positive MB. MB were cocultured with DC for 48 h. Subsequently, cells were subjected to flow cytometry and analysed for the immunological phenotype by assessing expression levels of costimulatory molecules (CD80, CD86, B7-H1) and MHC molecules (MHC I and II) (Fig. 2). Coculture had no significant effect on the expression of these molecules on NCAM+ MB (data not shown). Since immunohistochemical studies in inflammatory myopathies have previously demonstrated the

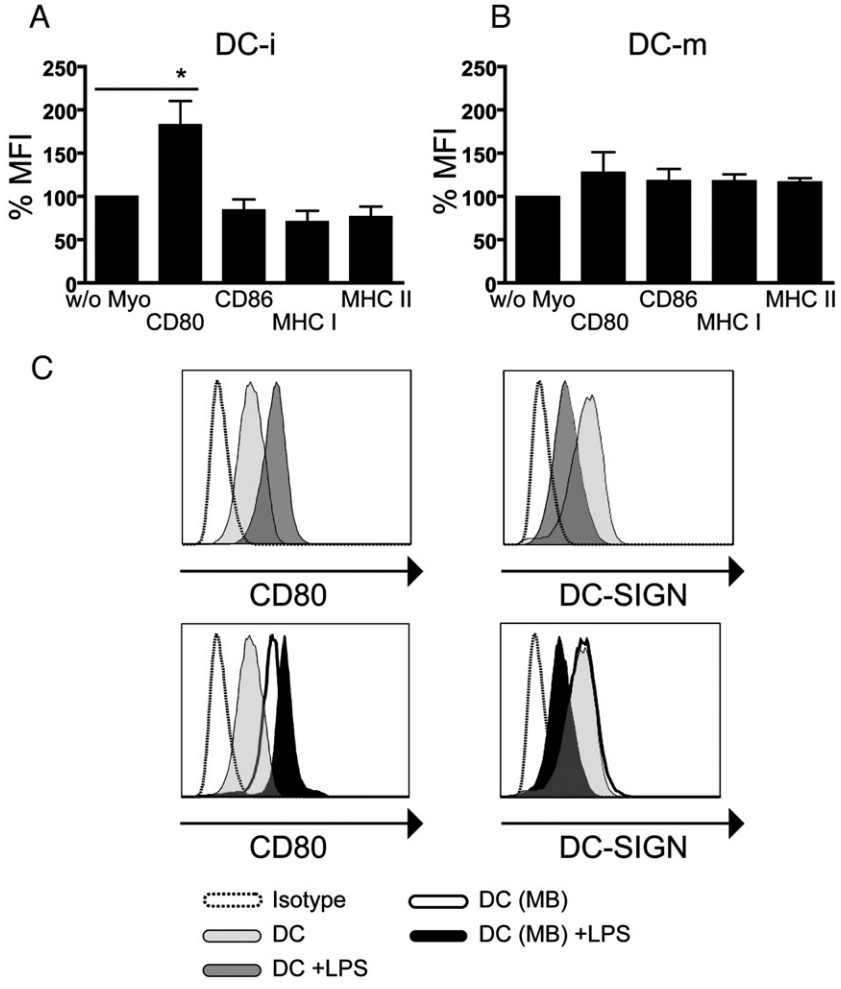

Fig. 3. Human myoblasts modulate the maturation status of DC. Different APC were either cultured in the presence or absence of MB for 48 h. Cell surface expression of CD80, CD86, MHC I (HLA-ABC) and MHC II (HLA-DR) was analysed by flow cytometry on immature dendritic cells (DC-i) (A) and mature dendritic cells (DC-m) (B). The change in the mean fluorescence index (MFI) as a consequence of DC-MB coculture is shown in percent. 3 independent experiments were conducted for each APC population. Bars represent the mean change in MFI, compared to DC without MB. DC cocultured in the presence or absence of MB were induced for maturation and analyzed by flow cytometry. Previous contact with MB did not affect LPS-induced changes of CD80 and DC-Sign expression as an indicator of DC maturation. One representative experiment is shown for the maturation markers CD80 and DC-Sign (C). The histograms represent: Dotted line — isotype control, light grey — DC, dark grey — DC maturated with LPS, black line — DC cultured with MB, filled out black — DC cultured with MB and maturated with LPS.

accumulation of immature DC in myositic lesions (Page et al., 2004), we were interested if MB might modulate the maturation process of DC. While levels of the maturation markers CD86, MHC II and DC-Sign were unaffected by coculture, CD 80 was significantly upregulated on immature DC after the 48 h MB coculture (Fig. 3A). Coculture with MB did not modulate the expression of maturation markers on mature DC (Fig. 3B). However, DC recovered from 48 h MB cocultures, which already showed an upregulation of CD80, could still be matured by appropriate antigenic stimuli (bacterial lipopolysaccharides, LPS), resulting in further upregulation of CD80 and downregulation of DC-Sign, thereby demonstrating that maturation was not impaired by the influence of MB (Fig. 3C). Since classical and non-classical MHC I molecules have been discussed in the pathogenesis of skeletal muscle inflammation we studied whether blocking of MHC I molecules by specific antibodies (clone TP25.99 reactive with the α3 domain of HLA-A, HLA-B, HLA-C, but not HLA-G molecules, clone W6/32 reactive with classical and non-classical MHC molecules) would effect the upregulation of CD80 expression levels by MB. However, blocking MHC I did not abrogate the impact of MB on DC maturation (Supplementary Fig. 1A).

3.3. Influence of myoblasts on dendritic cell function

Next, we were interested if MB modulate the costimulatory properties of DC. DC in different maturation status were recovered from MB-APC cocultures and analyzed for their stimulatory potency as a 3rd party in allogeneic proliferation assays. In these assays, no MB were present, as the non-adherent APC were separated from the strongly adherent MB mechanically (purity was higher than 98%, assessed by microscopy and flow cytometry). This was especially important, because it has been shown that MB can exert APC functions (Goebels et al., 1992) and we had to make sure that observed effects could be attributed to the DC in our culture. Both immature and mature DC stimulated the proliferation of allogeneic PBMC. When separated by transwell inserts, MB did not affect the capacity of DC-i to stimulate PBMC proliferation (Fig. 4A). Surprisingly, once the immature dendritic cells (DC-i) had been in contact with the MB, they had an inhibitory effect on the proliferation of PBMC (DC-i: −17% (+/−9%)), which was highly significant ($p=0.001$) compared to the proliferative effect of DC-i that had not been in contact with MB (DC-i: +13.8% (+/−4.6%)). In contrast to DC-i, mature DC seemed to be less prone to the influence of MB (Fig. 4B). Since the outcome of a T cell-APC interaction is largely determined by the balance between costimulatory and coinhibitory molecules on the cell surface, the differential effect of DC on T-cell proliferation might be secondary to an upregulation of the expression of coinhibitory members of the B7 family (Subudhi et al., 2005). As the exposure to MB resulted in an upregulation of the costimulatory B7 molecule CD80, we studied if this was paralleled by upregulation of coinhibitory B7 family members such as B7-H1, B7-DC and B7-H4. Although a trend towards a higher B7-H1 protein expression on DC-i, cultured in the presence of MB, was noted (Fig. 4C), overall changes were not significant. Also, the flow cytometry did not reveal a significant effect of MB coculture on B7-DC or B7-H4 (data not shown). Regarding the secretion levels of IFN-γ and IL-10 as paradigms for pro-inflammatory and anti-inflammatory cytokines, we did not observe any differences in the secretion pattern of DC and DC cultured in the presence of MB (data not shown). MHC I (antibodies TP25.99 and W6/32) and II (antibody L243) blocking experiments demonstrated that the constitutive MHC expression on MB was not involved in the modulation of DC function by MB (Supplementary Fig. 1B). Of note, the size and auto-fluorescence of cocultured DC change characteristically. This effect could also not be altered by blocking MHC I and II (Supplementary Fig. 1C).

3.4. Influence of myoblasts on the phagocytic capacity of macrophages

The second prominent group of APC in inflammatory muscle infiltrates are MPh. Muscle damage is known to induce

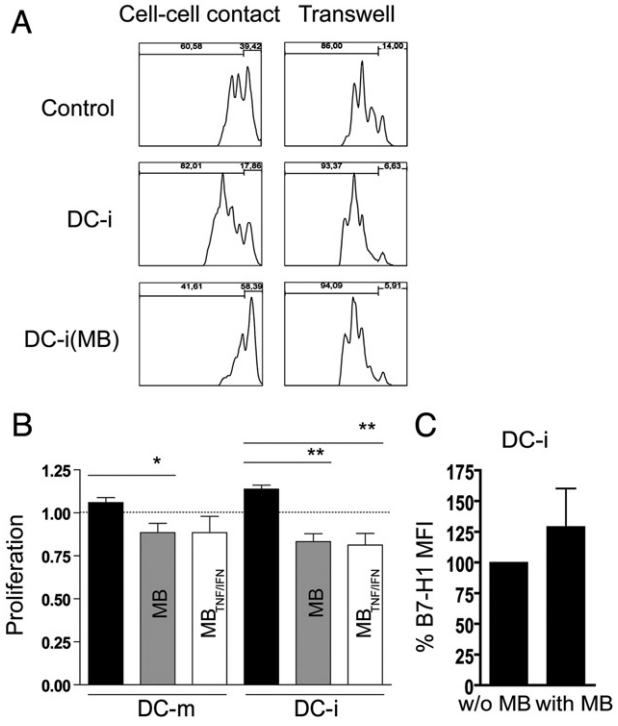

Fig. 4. Human myoblasts modulate DC function. DC function was analyzed by measuring capacity to induce PBMC proliferation in CFSE-proliferation assays. Shown are CFSE-histograms of one representative experiment: Positive control (only CD3/CD28 beads, no DC), DC-i (beads and DC, cultured alone) and DC (MB) (beads and DC, cultured with MB). In the right column, transwells were used to segregate DC-i and MB during the coculture period (A). DC were cultured alone (black), with MB (grey) or with TNFα/IFNγ treated MB (white) for 48 h. Thereafter, 5×10^4 human PBMC were used in a CFSE-proliferation assay in the presence of those DC. DC-i that have been cultured alone stimulate the proliferation of PBMC. After coculture with MB, both DC-i and DC-m significantly inhibit the proliferation of PBMC. At least 3 independent experiments have been performed for each DC population (B). Immature dendritic cells were either cultured alone or with myoblasts and then analyzed by flow cytometry, concerning the expression of the coinhibitory molecule B7-H1. 4 independent experiments have been conducted. Bars represent the mean change in MFI, compared to DC without MB (100%) (C).

massive MPh infiltration and MB-lysates have been shown to induce chemotaxis of MPh in mice (Pimorady-Esfahani et al., 1997). We were interested if MB-lysates affect the phagocytic capacity of MPh. To distinguish a specific influence of muscle cell lysates on MPh function from an overall effect of necrotic cells, we used lysates derived from a glioblastoma cell line (U373-MG) as a control. LPS (5 μg/ml) stimulation of monocyte-derived MPh for 48 h was used as a positive control. Lysates of MB, were added to MPh, which were afterwards used in a bead-incorporation assay (Fig. 5).

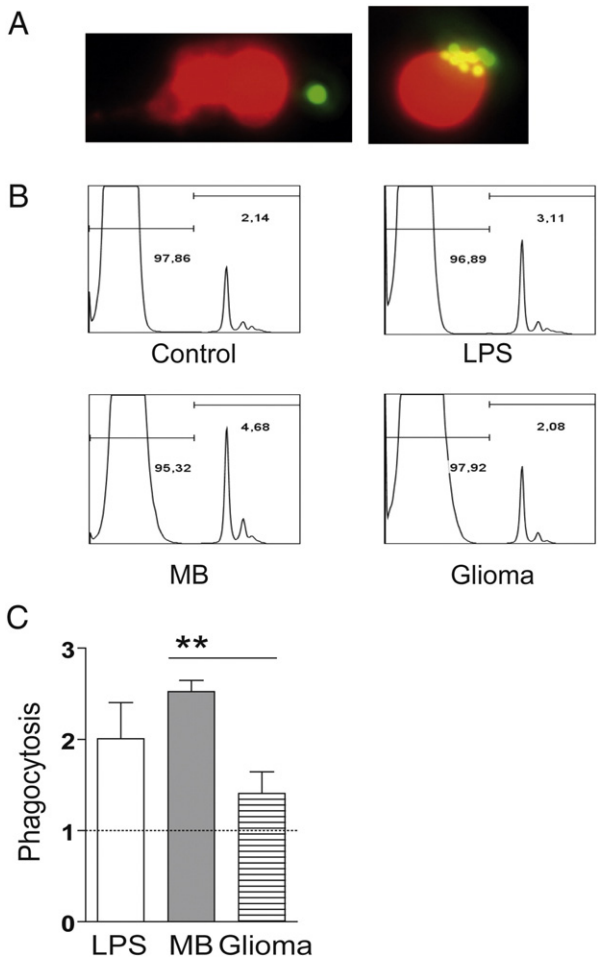

Fig. 5. Muscle cell lysates stimulate the phagocytotic capacity of macrophages. Macrophages were cultured in the presence of myoblast lysates (MB), glioma cell lysates (Glioma), lipopolysaccharides (LPS) (used as positive control) or medium (Control) and subsequently analyzed for their capability to incorporate polystyrene beads as a measure of phagocytosis. A shows PKH-26 stained macrophages (red) with incorporated latex beads (green): live image of the phagocytosis process. Left picture: macrophage without incorporated beads, right picture: macrophage during phagocytosis. B shows flow cytometry histograms of one representative bead-incorporation experiment. The ability of macrophages to incorporate fluorescent beads was assessed after 48 h coculture with lipopolysaccharides (LPS/white), lysates of MB (MB/grey) or lysates of glioma cells (Glioma/streaked). The lysates of MB stimulate the phagocytic properties of macrophages more efficiently than the lysates of glioma cells. 4 independent experiments were conducted (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

MB-lysates stimulated the phagocytic ability of MPh. Interestingly, the stimulatory effect of MB-lysates (mean+152% compared to control) on phagocytosis was comparable or higher than a high dose of LPS (mean+100%). In contrast, glioma cell lysates were very weak stimulators of MPh phagocytosis (mean+41% (+/−24%) (Fig. 5C).

4. Discussion

This study demonstrates that human skeletal muscle derived-MB are capable of modulating function of antigen-presenting cells, whereas the phenotype is only slightly changed. We were able to show that upon encounter with a MB, DC acquire a “semi-mature” phenotype, which has reduced capacity to boost subsequent lymphocyte proliferation, suggesting the induction of inhibitory DC by MB. In turn, MPh that have been exposed to MB-lysates were found to gain increased phagocytic capacity.

Previously published data suggested the accumulation of immature DC in myositic lesions in polymyositis and dermatomyositis (Chevrel et al., 2006; Page et al., 2004). In line with these findings we found that most DC recruited to myositic lesions do not express markers of mature DC such as CD83 or DC-Lamp. However, only a small minority of cells identified in 2 out of 15 myositis specimens expressed the marker CD1a described to identify immature DC. Since CD1a expression is an early event in DC differentiation, the lack of CD1a on the majority of infiltrating DC in myositis might reflect an impairment in the local DC maturation process within the skeletal muscle microenvironment and supports the hypothesis that infiltrating DC are held at an immature or rather “semi-mature” stage in myositis.

To study the interaction of APC and MB, we chose a well-characterized and widely accepted *in vitro* model for analyzing the immunobiological properties of skeletal muscle cells under inflammatory conditions (Wiendl et al., 2005a). MB derived from skeletal muscle satellite cells were selected by expression of the neuronal cell adhesion molecule (NCAM), which characterizes proliferating and differentiating MB as part of the ongoing regenerative process in inflammatory myopathies (Charlton et al., 2000; Winter and Bornemann, 1999); and cocultured with different APC populations in the presence or absence of inflammatory stimuli. Cultured MB share a lot of properties with skeletal muscle cells, however, while muscle fibers are large syncytial cells specialized for generating mechanical force (Wiendl et al., 2005b), MB derived from muscle satellite cells represent the cell population within the muscle which seems to be crucial in orchestrating muscle repair processes (Ehrhardt and Morgan, 2005) and is assumed to play an important role in the autoimmunity of myositits (Casciola-Rosen et al., 2005). In line with our observations on DC maturation in myositis lesions, we found that exposure of DC to MB modulated the maturation process leading to the acquisition of a “semi-mature” state by DC, which was characterized by the expression of CD80 and the absence of classical maturation indicators such as DC-Sign downregulation or MHC II upregulation. A similar phenotype has previously been described for

tumor-infiltrating DC in different types of malignancies (Gabrilovich, 2004; Liu, 2001; Perrot et al., 2007). Interestingly, these tumor-infiltrating "semi-mature" DC were found to be refractory to classical maturation stimuli (Perrot et al., 2007) suggesting that tumor-exposed DC may be arrested at an immature stage. Therefore we studied, whether MB-induced "semi-mature" DC could still react to classical maturation stimuli, proving that MB "semi-mature" DC are not permanently held in this intermediate state but can regain complete DC function if required. In this property, MB-induced "semi-mature" DC closely resemble TNF-alpha induced tolerogenic DC that can undergo two subsequent maturation steps (Voigtlander et al., 2006).

The acquisition of a "semi-mature" phenotype has been repeatedly shown to come along with a tolerogenic DC function resulting in the inhibition of T-cell proliferation (Lutz and Schuler, 2002), which holds true for MB-exposed DC. The balance between costimulatory molecules such as CD80 and CD86 and coinhibitory members of the B7 family determines whether an immunogenic or tolerogenic T-cell response will develop (Nurieva et al., 2006). However, in our setting, these molecules did not seem to be responsible for the tolerogenic properties of the cultured DC, as other B7 family members (B7-DC, B7-H4 or B7-H1) were not significantly modulated by MB. Soluble factors such as IL-10 and TNF-alpha have been implicated in the generation of tolerogenic DC (Buelens et al., 1997; Corinti et al., 2001). Since transwells abrogated the modulation of DC phenotype and function by MB, the induction of tolerogenic properties in DC by MB seemed to involve a cell–cell contact dependend mechanism. This point was further strengthened, when there were no differences in IFN-γ or IL-10 production by coculture. In contrast to normal skeletal muscle fibers, cultured MB and muscle fibers within myositic lesions do express high levels of MHC I molecules. Histochemical studies in inflammatory myopathies have suggested that the upregulation of MHC class I antigens in muscle fibers is an early event and precedes the inflammatory cell infiltration in myositis, implying a potential role in the pathogenesis of myositis. Using MHC blocking antibodies we evaluated the impact of MB MHC I and II expression on DC maturation. However, MHC I did not seem to be involved in the induction of a "semi-mature" DC phenotype and seemed unlikely to mediate the tolerizing effects on DC function since DC do not express cognate receptors for MHC I molecules. Albeit the molecular mechanisms underlying the immunoinhibition by MB-exposed DC remain elusive, our findings do provide important insights into DC maturation and function under the influence of MB. It is tempting to speculate that rendering DC "immunosuppressive" is a mechanism of immunoprotection employed by MB: these might protect themselves by inhibiting T-cell proliferation and thus effectively counterbalance immune-mediated damage. However, it seems questionable that pathogenic alterations in the small albeit significant modulation of DC function could be solely responsible for the damage observed in inflammatory myopathies; they are surely only one part of a more complex pathogenic process.

MPh are abundant in myositis and it has previously been shown that muscle damage induces massive MPh infiltration of the injury site (McLennan, 1996; Pimorady-Esfahani et al., 1997). MPh phagocytose necrotic muscle cells and orchestrate muscle repair processes. We were interested if MB-lysates modulate the phagocytic capacity of MPh. As a control to exclude an overall and unspecific effect of necrotic cells we chose glioblastoma cell lysates. Surprisingly, the tumor cell lysates proved to be very poor stimulators of MPh phagocytosis. Previous studies have shown that glioblastoma cells potently inhibit the development of an effective anti-tumor immune response by the expression of immunotolerogenic molecules (e.g. HLA-G, B7-H1) (Wintterle et al., 2003). It is conceivable that the poor effect on MPh phagocytosis is secondary to the high expression of these immunomodulatory molecules conferring the tumor immunparalytic properties. In contrast to glioblastoma cell lysates, which proved to constitute a negative control, MB-lysates strongly amplified MPh phagocytosis in an extent comparable to the positive control (LPS). It is assumed that stimulation of phagocytosis is beneficial for muscle regeneration (Sonnet et al., 2006). Recently it was reported that phagocytosis of necrotic muscle cells enhances TGF-beta 1 secretion by MPh and promotes a switch towards an anti-inflammatory MPh phenotype (Arnold et al., 2007). If this is taken into account, it is tempting to speculate about the existence of a negative-feedback inhibition loop, in which muscle cell destruction drives MPh to phagocytose cell debris and secrete anti-inflammatory cytokines in order to attenuate aggressive immune reactions within the muscle and protect from further damage. The question whether this effect on MPh is defective in myositis patients will be addressed in future research.

How could our *in vitro* experiments translate to the situation *in vivo*? The conclusions we drew from our experiments were within given limitations. Our peripheral cells as well as our MB came from healthy donors, which leads to a somewhat artificial situation compared to the one in myositis, meaning that we cannot say for sure, how much the observed effect really applies to the situation *in vivo*. However, our study sheds new light on the role of professional APC and MPh in the (patho)physiology of muscular immune reactions. Professional APC, which are infiltrating skeletal muscle, have recently been taken into consideration to trigger the development of autoimmune responses and perpetuate ongoing inflammation. This conception unfortunately neglects a potential impact of MB on immune cell function. APC-muscle cross-talk might modulate APC function *in vivo,* enabling APC to exert "immunosuppressive" functions. Understanding if and how immune cells interact with MB constitutes a major goal for developing effective therapies for muscle diseases, mainly because APC are already considered as possible cell-based tools for the treatment of myopathies (Sonnet et al., 2006). Strategies are in development to modulate myeloid DC function for treatment of autoimmune diseases, such as inflammatory myopathies (Figdor et al., 2004). These strategies include approaches like vaccination with genetically or cellularly engineered DC, designed to induce tolerance to specific antigens (Matsue et al., 2002) and the

development of drugs, promoting the immunosuppressive properties of DC-i (Greenberg, 2007). Our findings could contribute to the understanding of basic immunological characteristics of muscle, which is necessary for these clinical applications of cell- or drug-based immunotherapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jneuroim.2008.06.012.

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PD-1 Regulates Neural Damage in Oligodendroglia-Induced Inflammation

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Abstract

We investigated the impact of immune regulatory mechanisms involved in the modulation of the recently presented, CD8+ lymphocyte mediated immune response in a mouse model of oligodendropathy-induced inflammation (PLPtg-mutants). The focus was on the role of the co-inhibitory molecule PD-1, a CD28-related receptor expressed on activated T- and B-lymphocytes associated with immune homeostasis and autoimmunity. PLPtg/PD-1-deficient double mutants and the corresponding bone marrow chimeras were generated and analysed using immunohistochemistry, light- and electron microscopy, with particular emphasis on immune-cell number and neural damage. In addition, the immune cells in both the CNS and the peripheral immune system were investigated by IFN-gamma elispot assays and spectratype analysis. We found that mice with combined pathology exhibited significantly increased numbers of CD4+ and CD8+ T-lymphocytes in the CNS. Lack of PD-1 substantially aggravated the pathological phenotype of the PLPtg mutants compared to genuine PLPtg mutants, whereas the PD-1 deletion alone did not cause alterations in the CNS. CNS T-lymphocytes in PLPtg/PD-1-/- double mutants exhibited massive clonal expansions. Furthermore, PD-1 deficiency was associated with a significantly higher propensity of CNS but not peripheral CD8+ T-cells to secrete proinflammatory cytokines. PD-1 could be identified as a crucial player of tissue homeostasis and immune-mediated damage in a model of oligodendropathy-induced inflammation. Alterations of this regulatory pathway lead to overt neuroinflammation of high pathogenetic impact. Our finding may have implications for understanding the mechanisms leading to the high clinical variability of polygenic or even monogenic disorders of the nervous system.

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Introduction

We have recently investigated a mouse myelin mutant overexpressing the proteolipid protein (PLP) in oligodendrocytes leading to myelin degeneration and late onset axonal degeneration. Although being primarily caused by the glial mutation, the neuropathological phenotype was accompanied by an elevation of CD11b+ macrophages and CD8+ T-lymphocytes in the central nervous system [1]. Reconstitution experiments with RAG-1 deficient myelin mutants, receiving bone-marrow from either CD8+/CD4− or CD8−/CD4+ mutants, clearly identified CD8+ T-lymphocytes cells as pathogenic mediators. Lack of the macrophage-restricted molecule sialoadhesin in the PLP mutants, that mediates interactions of macrophage-like cells and T-lymphocytes [2], abrogates the elevation of CD8+ T-lymphocytes and substantially ameliorates the myelin-phenotype of the PLP mutants, further supporting the pathogenetic role of CD8+ cells in PLP transgenic mice [3]. In this model, CD8+ lymphocytes show clonal expansions in the diseased CNS but not in peripheral lymphatic organs. This serves as a strong hint for a pathogenetic, antigen-specific role of these cells [4]. The link between oligodendrocyte damage and components of the adaptive immune system is particularly relevant for inflammatory disorders of the nervous system. It has been recently hypothesized that subtypes of multiple sclerosis (MS) may be caused by a primary oligodendropathy [5,6]. This hypothesis is strongly supported by recent clinical reports, showing that PLP mutations in humans can be linked to primary progressive or relapsing-remitting MS [7,8]. Thus, our recent work identifying a primary oligodendropathy as a ''trigger'' for immune-driven pathological changes is important for our understanding of pathomechanisms occurring in some forms of MS.

It is well known, that genetic and environmental factors control disease onset and disease course of CNS inflammatory autoimmune disorders [9]. The co-inhibitory molecule ''programmed death'' (PD)-1 (CD279) is a CD28-related receptor expressed on activated T- and B-lymphocytes and associated with immune

homeostasis and autoimmunity [10,11]. Accordingly, we recently demonstrated that a polymorphism of the PD-1 gene is associated with a progressive disease course in MS [12], therefore corroborating the importance of PD-1 as a disease modifying gene. Moreover, inactivation of PD-1 in an animal model for inherited demyelination in the peripheral nervous system implicating T-lymphocytes [13] leads to a substantial aggravation of the primarily genetically-caused neuropathy [14].

In the present study, we investigate the impact of immune-regulatory mechanisms involved in the modulation of the immune response in PLP transgenic mice, a model of oligodendropathy-induced inflammation. Our present study identifies PD-1 as a crucial factor regulating tissue homeostasis of T-lymphocytes and indicates that a primary oligodendropathy combined with alterations in this regulatory pathway can lead to accelerated neuroinflammatory reactions of high pathogenetic impact.

Materials and Methods

Animals and Determination of Genotypes

PLP transgenic mice [15] were bred and genotyped as described previously [1]. PD-1-/- mice [16] were kindly provided by T. Honjo and C. Blank. Absence of PD-1 was verified by PCR genotyping as described [14]. To generate double mutants, PLPtg and PD-1-/- mice were crossbred. All resulting genotypes were investigated at 2, 6 and 12 months of age.

To exclude organ autoimmunity in the PD-1-/- mice [16], urine samples were investigated for protein and glucose with CombiScreen® urine tests (BioconDiagnostik, Voehl-Marienhagen, Germany). For bone marrow chimerization, PLPtg/RAG-1-/- mice were used as recipients. RAG-1 deficiency was identified as previously described [1,14,17].

All mice were bred and kept in our animal facility under barrier conditions (University of Wuerzburg, Department of Neurology). All animal experiments were approved by the local authorities (Regierung von Unterfranken).

Bone marrow chimerization

Two strategies to generate bone marrow chimeras were performed: In one group, the recipients (PLPtg mice with PLPwt mice as controls) were sublethally irradiated (5 Gy), while mice in the other group were deficient for the recombination activating gene (RAG)-1 (PLPtg/RAG-1-/- recipients with PLPwt/RAG-1-/- as controls). The bone marrow chimeras were transplanted with PD-1-/- or wildtype bone marrow at the age of 6–8 weeks and investigated at the age of 10 months (n = 8–9). Transplantation and control of successful transplantation was performed as described before [1,18,19].

Purification of splenocytes

Spleens were passed through a cell strainer (BD Biosciences Pharmingen, San Jose, CA USA), erythrocytes were lysed with a lysis buffer (150mM NH_4Cl_2, 10 mM $KHCO_3$, 0.1 mM EDTA in distilled water at pH 7.3) and cells were washed and processed for the respective experiments.

Preparation of CNS mononuclear cells and flow cytometry of splenocytes and CNS lymphocytes

Mice were killed with CO_2 and transcardially perfused with cold 0.1 M PBS. The CNS was prepared, tissues were homogenized and cells were gradient isolated as described [1]. Flow cytometry was performed using standard methods as described [4,20].

Tissue preparation and immunohistochemistry

For identification of macrophage-like cells, mice were transcardially perfused with 4% paraformaldehyde in 0.1 M cacodylate buffer. Tissue was dissected, postfixed for 2 hours and cryoprotected in 30% sucrose overnight. For T- lymphocyte and MBP staining, mice were perfused with 0.1 M phosphate buffered saline (PBS) only. After snap freezing, 10 μm thick transverse sections of the spinal cord and longitudinal or transverse sections of the optic nerve were cut.

Immunohistochemistry for CD11b, Sialoadhesin, CD4, CD8 and MBP was performed as described before [1,3]. MBP was stained on optic nerve cross sections 1200–1400 μm caudal to the retina. Sources of reagents, of antibodies and clones of the antibodies were the same as described [1,3].

Assessment of demyelination

Myelin damage in optic nerve cross sections was assessed by measuring MBP negative areas, data were displayed as a percentage of the total area. For this, we used a Zeiss Axiophot2 microscope at a final magnification of 300×. The area was measured using digital images acquired via a CCD- camera and ImagePro 4.0 software.

Additionally, myelin damage was semiquantitatively rated as described before [1], with score 1 depicting homogeneous MBP distribution, score 5 massive myelin loss.

Tissue preservation for light microscopy of semithin sections

Optic nerves from transcardially perfused mice were processed for light microscopy of semithin sections as recently reported [1]. Tissue damage was assessed by quantification of axonopathic vacuoles >6 μm.

Quantification of immune cells in the CNS

Longitudinal sections of the optic nerve of 2, 6 and 12 months old wildtype, PD-1-/-, PLPtg and PLPtg/PD-1-/- mice and 10 months old bone marrow chimeras were analysed. Quantification of CD11b+ and Sialoadhesin (Sn)+ cells was performed as described before [1] in the rostral region. CD4+ and CD8+ T-cells were quantified in total longitudinal optic nerve sections, using a Zeiss Axiophot2 microscope and measurement tools as described above.

Detection of cytokines by ELISA and ELISPOT

1×10^6/ml splenocytes were cultured unstimulated or stimulated with ConA (2 μg/ml, Sigma, Schnelldorf, Germany) or CD3/CD28 coated microspheres (Dynal, Invitrogen, Karlsruhe, Germany). After 48 hours, supernatant was harvested and ELISA for IFN-γ, IL-2 or IL-10 (R&D Systems, Minneapolis, MN, USA) was performed according to the manufacturers instructions.

Assessment of interferon-gamma (IFN-γ) producing cells was performed by ELISPOT. $1x10^4$ CNS lymphocytes or 1×10^5 splenocytes per well were incubated for 24 hours, unstimulated or stimulated with PMA (20 ng/ml)/Ionomycin (500 ng/ml, both Sigma), or a mixture of class one PLP, MOG and MBP peptides (Genscript Corp, Piscataway, NJ, USA) as previously described [4]. ELISPOT assay was performed according to the manufacturers instructions (BD Pharmingen). Spots were quantified by CTL Europe (Aalen, Germany) using ImmunoSpot 4.0.17.

CDR3 Spectratyping

The CDR3 spectratyping was performed as described previously [4,21] using an ABI Prism 3130 capillary sequencer (Applied

Biosystems) to determine length and distribution, using a module for fragment-analysis. As an internal length standard, 500-ROX (Applied Biosystems) was used.

Statistical analysis

Quantified profiles were tested with two-tailed student's t-test, scores were analyzed by the nonparametric Mann-Whitney-U test and Kruskal-Wallis test.

Results

The role of PD-1, a co-inhibitory molecule critical for immune homeostasis and tolerance, was tested in a model of CNS-myelinopathy associated with secondary, low grade inflammation of high pathological relevance.

Numbers of CNS immune cells are significantly elevated in PLPtg/PD-1-/- double mutants

To obtain myelin mutants with inactivated PD-1 function, two strategies have been chosen. First, double mutants have been created in analogy to previous experiments [1,3] by crossbreeding the corresponding single mutants. This strategy has the advantage that all cells of the organism lack PD-1. Complementarily, bone marrow chimeric mutants have been generated using either irradiated or RAG-1-deficient PLPtg mice as recipients and PD-1-/- mice as donors [1,18]. The latter strategy has the advantage that unexpected side effects or influences of the systemic PD-1-inactivation (e.g. influences of PD-1 deficiency on thymic maturation or neonatal tolerance) can be circumvented.

PLPtg/PD-1-/- double mutants and their respective controls (wt, PD-1-/- and PLPtg mice), were examined at the age of 2, 6 and 12 months ($n = 3–7$). To quantify immune cells in the CNS, we focussed on longitudinal sections of the optic nerve, an already established read out technique for the scoring of inflammation in PLP mutant CNS [1,3,22].

At the age of two months, there was no significant difference between wt, PD-1-/-, PLPtg and PLPtg/PD-1-/- mice. At 6 months, however, there was a slight elevation of CD8+ T- cells in the optic nerve of PLPtg mice in comparison to wildtype mice, consistent with our previous observations. PD-1-/- also showed a mild elevation, whereas PLPtg/PD-1-/- double mutant mice exhibited a robust upregulation of CD8+ T-cells in optic nerve sections (approximately 35-fold increase compared to wildtype mice and a more than 8 fold increase in comparison to PLPtg mice, Figure 1A). In 12 months old mice, the general pattern of CD8+ lymphocyte numbers in optic nerves was similar in the different genotypes, but proportions had shifted a little: wt and PD-1-/- mice were now at similar levels (with no marked increase of profiles in PD-1-/- mice compared to the 6 months old group), and PLPtg mice displayed significantly more CD8+ profiles than the former two genotypes. Strikingly, these CD8+ T-lymphocytes were again clearly outnumbered by those from PLPtg/PD-1-/- mice (Figure 1B, C).

CD4+ T-lymphocytes are rarely present in the CNS of PLPtg and wt mice [1]. At the age of two months, no differences were detectable in all investigated groups.

At the age of 6 months a significant elevation of CD4+ lymphocytes was already visible in PLPtg/PD-1-/- compared to wt, PD-1-/- and PLPtg mice and there was no marked increase until the age of 12 months (Figure 1D). Similar changes in T-lymphocyte numbers were detected in the spinal cord (data not shown).

Furthermore, we investigated the number of CD11b+ macrophage-like cells in the optic nerve. We did not detect significant differences at the age of 2 or 6 months, although a trend of increased numbers was already detectable in PLPtg mice and PLPtg/PD-1-/- double mutants at 6 months (data not shown). In 12 months old mice, however, a difference was detectable. While wt and PD-1-/- mice showed a common low level of cells, both PLPtg and PLPtg/PD-1-/- mice had elevated numbers of CD11b+ cells (Figure 1E).

We additionally investigated the number of Sialoadhesin (Sn) expressing macrophage like cells in 12 months old PLPtg/PD-1-/- mice and detected a very low amount of positive profiles in both wildtype and PD-1-/- mice. In PLPtg, a more than ten-fold increase was detectable, but there was no significant difference between PLPtg and PLPtg/PD-1-/- mice (data not shown).

Numbers of immune cells are significantly elevated in PLPtg PD-1-/- transplanted bone marrow chimeras (BMCs)

Another strategy to investigate the role of PD-1 in PLPtg mice was to examine PLPtg mice which were transplanted with bone marrow from either PD-1-/- mice (PLPtg BMC PD-1-/-) or wildtype mice (PLPtg BMC wt). Wildtype mice which were transplanted with either wildtype or PD-1-/- bone marrow served as controls. These animals never displayed myelin pathology or CNS inflammation in any experiment.

The bone marrow chimeric mice faithfully reflected the findings described for the double mutants. For example, both irradiated and PLPtg/RAG-1-/- recipients showed a robust upregulation of CD8+ lymphocytes, when bone marrow was derived from PD-1-/- mice (Figure 1F). Similar observations were made for the low but significantly elevated amount of CD4+ cells (data not shown). Increased elevation of CD8+ and CD4+ cells in the absence of PD-1 was significant both in irradiated and in RAG-1-deficient PLPtg mice.

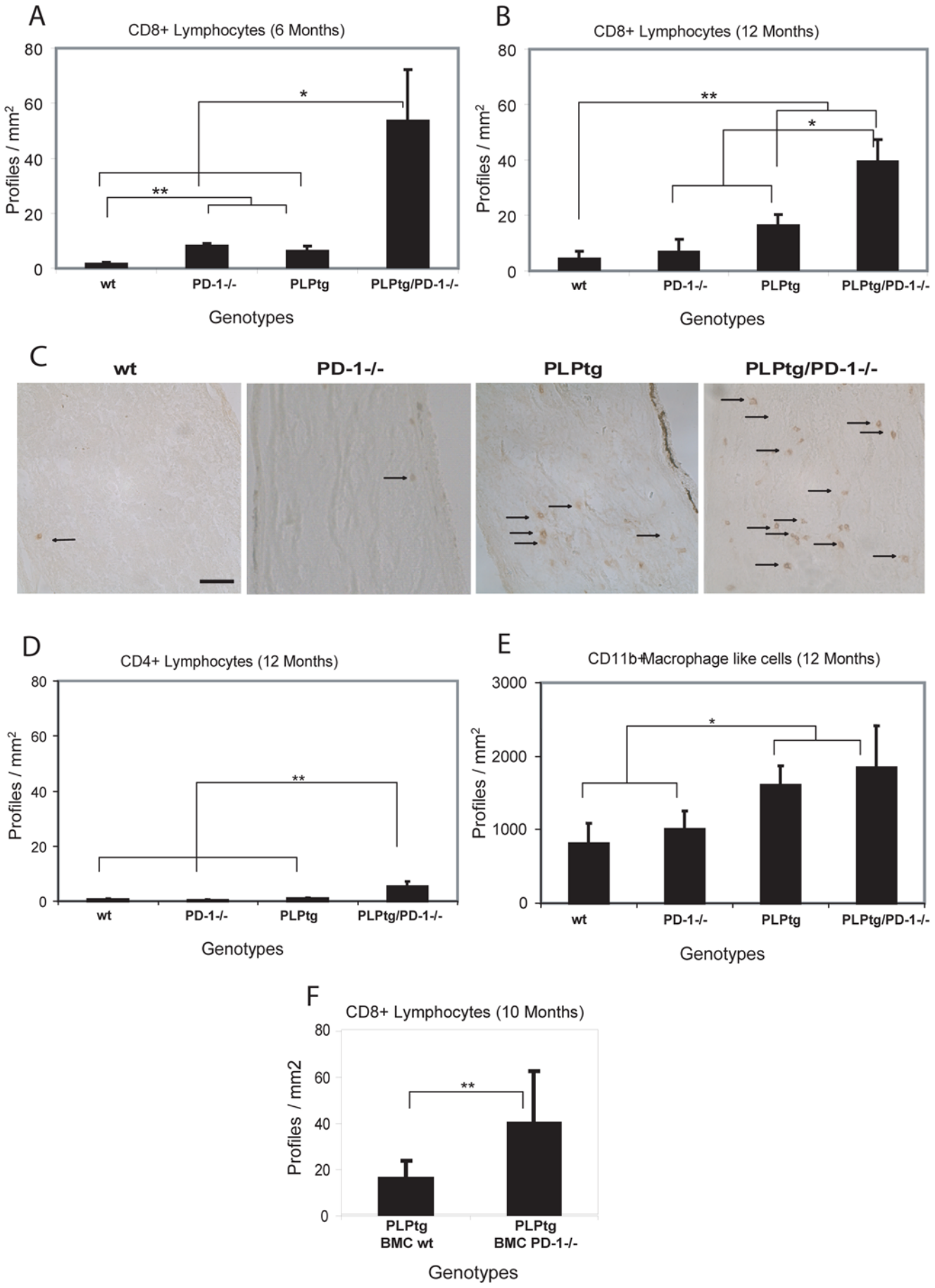
Analysis of CD11b+ macrophage like cells depicted a small, significant increase in PLPtg PD-1-/- transplanted chimeras compared to recipients that received bone marrow from wildtype mice (data not shown). In the irradiated PLPtg mice statistical significance was reached, while the PLPtg/RAG-1-/- mice showed a trend into the same direction but did not reach the level of significance ($p = 0.06$).

Pathological features are enhanced in PLPtg/PD-1-/- double mutated mice

MBP immunohistochemistry revealed that, while wildtype and PD-1-/- mice always showed a homogeneous distribution of myelin, PLPtg mice displayed a more patchy and inhomogeneous MBP staining. Compared to that, PLPtg/PD-1-/- mice showed an even less homogeneous MBP distribution, reflecting extensive myelin loss (Figure 2A).

Interestingly, in one PLPtg/PD-1-/- optic nerve we observed an extended and sharply confined area of MBP-loss, reminiscent of a demyelinated lesion common to active or inactive MS plaques (Figure 2B). This lesion was associated with an accumulation of CD8+ lymphocytes (Figure 2C) and hematoxylin-stained cells of probably inflammatory character (Figure 2D).

To quantify the demyelinating phenotype of the different mutants, MBP-negative areas of optic nerve cross sections were determined as measure for demyelination. Neither wildtype nor PD-1-/- mice showed any MBP-negative areas, while demyelination was present in PLPtg mice and further significantly increased in PLPtg/PD-1-/- mice (Figure 2E). Additionally, myelin integrity-related MBP distribution was analysed by semi-quantitative scoring. Again, wt and PD-1-/- mice showed healthy myelin


A
CD8+ Lymphocytes (6 Months)
Profiles / mm2
80
60
40
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**
wt
PD-1-/-
PLPtg
PLPtg/PD-1-/-
Genotypes
B
CD8+ Lymphocytes (12 Months)
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CD4+ Lymphocytes (12 Months)
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Genotypes

Figure 1. Quantification and immunohistochemical detection of CD8+ and CD4+ lymphocytes and CD11b+ macrophage-like cells in longitudinal sections of optic nerves of wt, PD-1-/-, PLPtg and PLPtg/PD-1-/- mice and of CD8+ cells in bone marrow chimeric mice - A, B. Quantification of CD8+ lymphocytes in 6 (A: n = 3) and 12 (B: n = 3–7) months old mice of different genotypes. C. Immunohistochemical detection of CD8+ lymphocytes in the optic nerve of 12 months old mice of different myelin and immunological genotypes. Arrows indicate positively labelled CD8+ lymphocytes. D. Quantification of CD4+ lymphocytes in 12 months old mice (n = 3–7). Note that in the myelin mutants, both CD8+ and the generally scarce CD4+ lymphocytes are substantially increased in the absence of PD-1. E. Quantification of CD11b+ macrophage-like cells in 12 months old mice (n = 3–6). Note that the number CD11b+ cells does not differ significantly in PLPtg/PD-1+/+ and PLPtg/PD-1-/- double mutants. Similar results are obtained in irradiated and non-irradiated (RAG-1-/-) PLPtg bone-marrow recipients. The rather small increase of CD11b+ cells is not surprising, since the molecule in focus (PD-1) is a component of predominantly T-lymphocytes rather than of macrophages/microglial cells. F. Quantification of CD8+ cells in the CNS of 10 months old PLPtg bone marrow chimeras (BMCs) which were transplanted with either wt or PD-1-/- bone marrow (n = 8–9). Note that also in bone marrow chimeras, CD8+ T-lymphocytes are substantially elevated in the CNS of PLPtg mutants in the absence of PD-1. Error bars represent standard deviations. * p- value$<$0.05, ** p - value$\leq$0.01. Scale Bar: 50 μm.
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(score 1) while PLPtg mice had an average score of 3±0.81 and PLPtg/PD-1-/- mice showed a higher score (3.83±0.75). PD-1-deficiency leads to the highest MBP loss in the myelin mutants and the most seriously affected mutants belong to the PD-1-deficient group. Generally, the persons investigating the histopathological features (A. K., R. M.) were not aware of the respective genotypes. Similar to these results in double mutant mice, 10 months old PLPtg BMCs reconstituted with PD-1-/- bone marrow showed a more disrupted state of MBP distribution than PLPtg mice transplanted with BMCs from wt mice (data not shown).

We also investigated periaxonal vacuoles in semithin cross sections of the optic nerve as another reliable pathological marker [1,3] (age of 12 months in double mutants, age of 10 months in bone marrow chimeras). PLPtg/PD-1-/- double mutants, compared to PLPtg mice, showed a clear trend of increased vacuole numbers (13.3±7.9 versus 7.95±3.6), while wt and PD-1-/- mice never displayed any vacuoles (data not shown).

PLPtg BMCs transplanted with PD-1-/- bone marrow showed a highly significant increase of axonal damage (Figure 2F, G, H). In the optic nerves of wildtype mice, we never detected any vacuoles, regardless what kind of bone marrow had been transplanted.

T-cell CDR3 spectratype analysis: robust clonal expansions in the CNS of PLPtg/PD-1-/- mice

Evidence for monoclonal T-cell expansions as revealed by single Vβ-Jβ peaks in the corresponding PCR-diagrams could be detected (one Vβ peak per animal) in 12-month-old PLPtg mice corroborating these previous findings [4]. Corresponding expansions could not be detected in spectratyping analyses from lymphocytes of wild type mice. Spleens of the same animals displayed the expected Gaussian distribution of Vβ profiles [4].

CDR3 spectratype in PD-1-/- mice (n = 7) showed more than one Vβ peak (Figure 3). Similarly, multiple Vβ peaks were visible in the CNS of PLPtg/PD-1-/- double mutants (n = 3) (Figure 3), as well as in 10 months old PLPtg/PD-1-/- BMCs (n = 5). The clonal expansions occurred widely distributed over different Vβ and Jβ regions, although some domains seemed to be prone for clonal expansions in different mutant mice. Notably, approximately 30% of the individual clonal expansions were detected in both spleen and brain, 70% of the expansions were exclusively present in CNS tissue.

We then sequenced some clones to 1) demonstrate that the specific PCR fragment represents one TCR (a readable sequence proves the existence of an expansion), and 2) to detect similarities between the CDR3s of different clonal expansions. For example, by analysing the sequences of 2 individuals (mouse A and B) we identified two expansions with the same VβJβ combination and one expansion with a different VβJβ combination in the two mice (Table S1). While those D segments with flanking N sequences (= NDN) amino acids, responsible for connecting with the MHC-bound antigen, show some similarities, the lengths of the CDR3 are not identical, showing that these clones are not specific for the exact same antigen.

Peripheral immune parameters do not differ between mutant mouse strains

In order to exclude that peripheral immune parameters in the mutant mouse strains could account for the different numbers of immune cells and the aggravated pathological features in double mutated mice, we analyzed (i) phagocytic capability of macrophages, (ii) inducibility and rate of stimulation-induced apoptosis of splenocytes, (iii) immune subset distribution (CD4+, CD8+, CD11b+, B220), and (iv) levels of stimulation induced IL-2 production between the different groups (wt, PD-1-/-, PLPtg, PLPtg/PD-1-/-).

The phagocytic capacity of peritoneal macrophages was similar in all genotypes. Furthermore, investigation of splenocytes showed no significant differences between the different genotypes used in this study in regard of inducibility of apoptosis, cell subsets and production of cytokines (see Material S1, Figure S1).

CNS T-cells are prone to IFN-γ secretion in the absence of PD-1

Polyclonal immune responses in the periphery do not differ between mouse mutants. We therefore tested whether CNS cells show altered production of inflammatory cytokines upon stimulation. IFN-γ secretion of CNS-derived T-lymphocytes was measured after addition of PMA/ionomycin or upon antigenic stimulation.

Interestingly, CNS lymphocytes of PD-1-/- and PLPtg/PD-1-/- mice showed strong IFN-γ secretion upon PMA/ionomycin challenge, while CNS T-cells from PLPtg mice or wt showed only minimal or no cytokine production (Figure 4A). Of note, such differences have not been observed in T-cells from spleen (Figure 4B). Antigenic stimulation with a number of MHC class I related myelin peptides [4] did not lead to IFN-γ production of CNS T-cells under any condition (ELISPOT, data not shown).

Discussion

Recent data from human studies in distinct leukodystrophies and some forms of MS together with investigations in myelin mutant mice indicate that a primary glial injury can be causative for neuroinflammation of substantial pathological and clinical relevance [1,3,4,23,24,25]. Further characterization of "secondary" inflammatory responses in a model of PLP overexpression identified CD8+ T-lymphocytes of effector cell phenotype as crucial mediators of demyelination and axon damage [1]. The finding that CD8+ T-cells in the CNS of myelin mutants are clonally expanded [4,25] further supports a

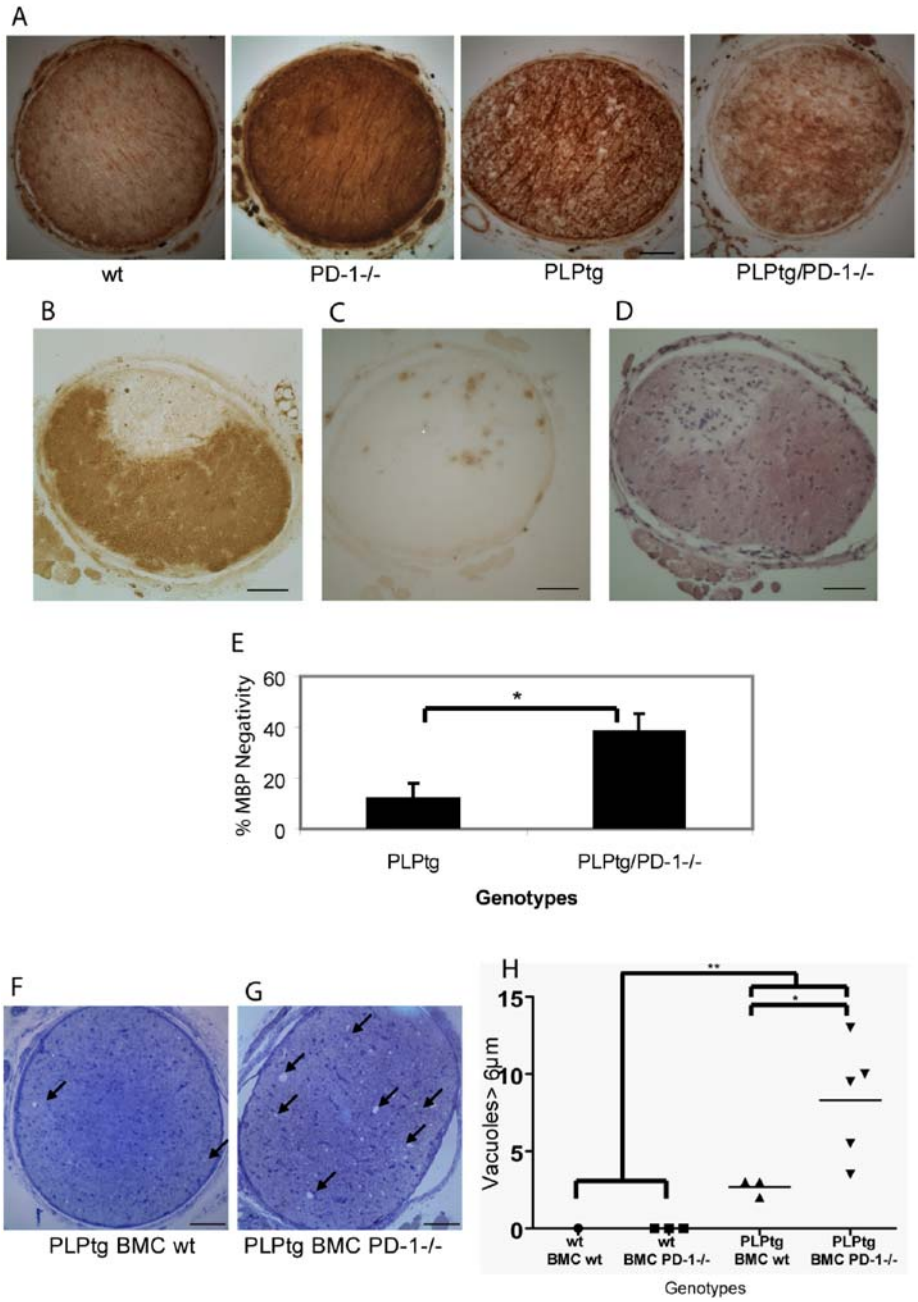

Figure 2. Quantification of pathological features in various myelin and PD-1 mutants and BMC mice A. MBP immunohistochemistry of optic nerve cross sections of 12 months old wt, PD-1-/-, PLPtg and PLPtg/PD-1-/- mice. Note homogeneous MBP distribution in wt and PD-1-/- mice compared to more disrupted myelin (inhomogeneous labeling) in PLPtg and, more pronounced, PLPtg/PD-1-/- mice. B.-D. MBP immunohistochemistry (B), immunohistochemical detection of CD8+ cells (C) and hematoxylin eosin staining (D) in a plaque like demyelinating lesion in a 12 months old PLPtg/PD-1-/- mouse. Note sharply confined lesion (B) with accumulated T-cells (C) and other, probably inflammatory, cell nuclei (D). E. Quantification of demyelination by measuring MBP-negative areas. PD-1-deficient myelin mutants (PLPtg/PD-1-/-) show more severe MBP loss than PLPtg mice expressing PD-1 (PLPtg; n = 4–6). F.,G. Semithin optic nerve sections sections of 10 months old PLPtg mice which were transplanted with wt (F) or PD-1-/- (G) bone marrow. Arrows indicate periaxonal vacuoles which are more numerous in PD-1-/- BMCs. H. Quantification of vacuoles >6 µm in bone marrow chimeras. PD-1-deficiency leads to the most robust histopathological alterations in the myelin mutants and the most seriously affected mutants belong to the PD-1-deficient group (n = 3–5). * p- value<0.05, ** p - value≤0.01. Scale bars: 50 µm.
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pathogenetic concept that primary myelin damage in the CNS can be associated with secondary reactivity of the adaptive immune system against a still unknown antigen(s). Another key question in this scenario is which factors control tissue homeostasis of immune cells. We therefore investigated the impact of immune-regulatory mechanisms on the adaptive immunity in PLP overexpressing mutants focussing on the role of the co-inhibitory molecule PD-1. Recent data in mice as well as in humans demonstrate that PD-1 is substantially involved in the control of T-cell homeostasis under physiological and pathological conditions [26] by preventing

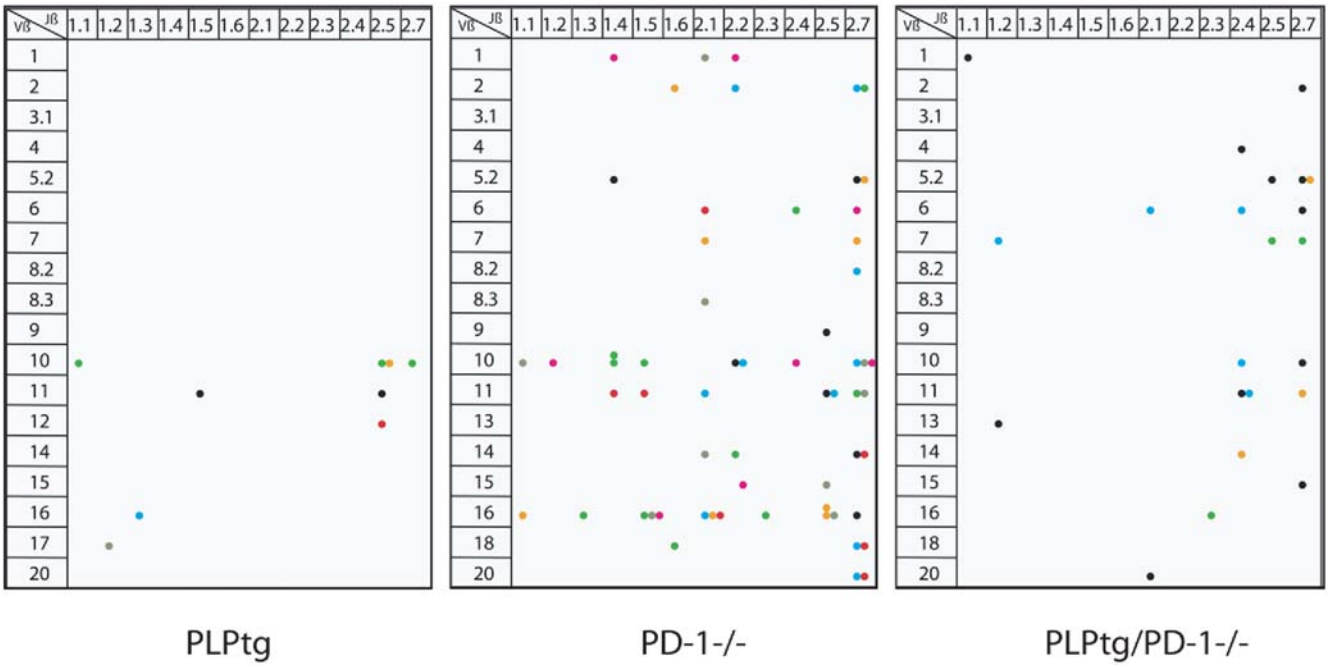

Figure 3. Spectratyping of CNS derived lymphocytes - Lymphocytes from the CNS of 12 months old PLPtg (n = 7, as adopted from [4], PD-1-/- (n = 7), and PLPtg/PD-1-/- (n = 4) mice were analysed for disturbances in the T-cell receptor repertoire by spectratyping. Clonal expansions (visible as single peaks in the fragment analysis) are shown as colored dots (different colors indicate individual animals). The expanded T-cells are characterized by their Vβ- and Jβ-chains. The clonal expansions occurred widely distributed over different Vβ and Jβ regions, although some domains seemed to be prone for clonal expansions in different mutant mice. Note that different numbers of experimental mice contribute to the different numbers of dots.
doi:10.1371/journal.pone.0004405.g003

uncontrolled proliferation of autoreactive T-cells [27]. In accordance with these functional data, certain polymorphisms in the PD-1 gene are associated with human autoimmune disease including MS [12,28].

Our present experiments show that PD-1 plays a major role in modulating numbers of CD8+ cells in the demyelinating model of PLP overexpressing mice.

The most relevant finding of our study was that in PLPtg mice the histopathological phenotype was much more severe when PD-1 was absent. Interestingly, not only CNS damage was more pronounced per individual, but most severely affected individuals always belonged to the group of PLPtg mice in combination to functional disruption of PD-1.

How does ablation of the PD-1 pathway affect CNS pathology? PD-1-deficiency alone only transiently affected the number of CD8+ cells found in the CNS and, more importantly, the corresponding mice showed normal histological features in the CNS. While it has been reported that PD-1-/- mice on a C57/Bl6

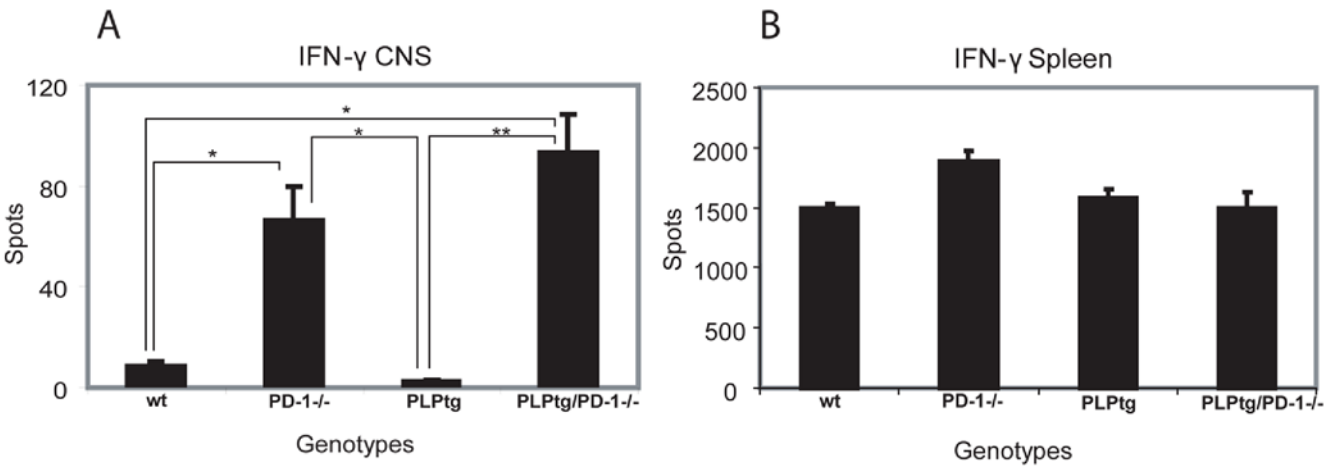

Figure 4. IFN-γ ELISPOT assay on spleen and brain derived lymphocytes - IFN-γ ELISPOT assay after stimulation with PMA/ionomycin on CNS derived lymphocytes (A) and splenocytes (B) from wt, PD-1-/-, PLPtg and PLPtg/PD-1-/- mice. Note elevated spot numbers in CNS T-lymphocytes taken from PLPtg/PD-1-/- mice in comparison to wt and PLPtg mice, reflecting a higher susceptibility to activation. Error bars represent standard deviations. * p - value$<$0.05, ** p - value$\leq$0.01
doi:10.1371/journal.pone.0004405.g004

background show lupus-like glomerulonephritis when aging [16] we did not observe any obvious organ pathology, urinal glucose or protein content (not shown) or spontaneous autoreactions associated with loss of PD-1. Furthermore, the peripheral immune "status" was not changed in the tested groups (wt, PD-1-/-, PLPtg, PLPtg/PD-1-/-). However, we found clonal expansions of T-cells in the periphery in PD-1-/- mice using CDR3 spectratyping analysis. While numerically not elevated in the CNS at 12 months, the PD-1-deficient CD8+ cells of PLPwt/PD-1-/- mice showed multiple clonal T-cell expansions, as opposed to PD-1-expressing CD8+ cells of normal wild type mice which do not show any repertoire perturbations. The corresponding CD8+ cells of PLPtg mice display their characteristic mono- or oligoclonal expansion in the CNS as previously described [4]. The combination of the PLP transgene with PD-1 deficiency led to significantly higher numbers of T-cells within the CNS. Moreover, CD8+ T-lymphocytes of PD-1 mutants are aberrant with regard of their strong numerical increase and prominent clonal expansions. This suggests that PD-1 prevents a large number of possible clonal expansions of a variety of T-cell clones in PLPtg mice. PD-1 signalling is known to attenuate signals of the T-cell receptor such as PKCtheta and ZAP-70/CD3ζ [29]. This could also be the explanation that only CNS T-cells deficient of PD-1 show markedly enhanced INF-γ secretion after stimulation, whereas CNS CD8+ cells from genuine PLPtg mice show no relevant production of this proinflammatory cytokine. These findings suggest that although prominently expanded and highly susceptible to become activated, PD-1-deficient T-lymphocytes appear to be pathologically "silent" in a healthy environment.

Due to its inhibitory properties the cognate ligand PD-L1 has been proposed to contribute to maintaining peripheral tolerance and limiting inflammatory damage [20,27,30,31,32]. Parenchymal PD-L1 contributes to the limitation of insulinitis and the resolution of inflammation [33]. We and others recently reported that PD-L1 is expressed and upregulated on CNS cells (e.g. microglia cells) under inflammatory conditions [20,34], restricts parenchymal neuroantigen-specific T-cell responses and confines inflammatory CNS damage in experimental autoimmune encephalomyelitis [35]. One therefore might assume that PD-L1 - PD-1 interactions counteract T-cell mediated pathology observed in PLPtg mice by limiting clonal expansion and cytokine release of detrimentally self-reactive low avidity clones.

A synoptic view summarizing our recent and previous observations may be as follows: overexpression of PLP may induce intracellular stress that causes several immune-relevant glial reactions, such as expression of cytokines and upregulation of MHC-I molecules on oligodendrocytes [1] and Sn on the surface of macrophage-like cells. It is of note that the latter reaction is an important prerequisite for CD8+ cell activation in the present model [3]. Furthermore, supraphysiological concentrations of myelin antigens associated with PLP-overexpression could promote reactivity of low-avidity T-cell clones that survived clonal deletion or ignorance in the thymus [4]. CNS-derived, but not spleen-derived CD8+ cells show mono- or oligoclonal expansions, further suggesting CNS-restricted specificity against yet unidentified CNS-antigens. PD-1 is critically involved in these processes: in the presence of PD-1 on CNS CD8+ cells, activation and proliferation is limited, whereas absence of PD-1 leads to substantial increase of CD8+ cells, a higher propensity to secrete proinflammatory cytokines, multiple clonal expansions and an aggravation of neural damage.

Taken together, our study demonstrates the important role of a co-inhibitory molecule, PD-1, in modulating glial-injury-related immune responses in the CNS. This impressively reflects the high and obviously wide-range relevance of immunmodulatory mechanisms under various pathological conditions and should be particularly considered when seeking for mechanisms leading to the high clinical variability of polygenic or even monogenic disorders of the nervous system.

Supporting Information

Figure S1 Exclusion of differences in the peripheral immune systems of wt, PD-1-/-, PLPtg and PLPtg/PD-1-/- double mutants. Peritoneal macrophages were incubated with fluorescent latex beads and the percentage of macrophages which ingested beads was found similar in all genotypes (A). Differences in the apoptosis rate were excluded by flow cytometry of annexin V and PI positive splenocytes under highly stimulatory conditions (B). Analysis of immune cell subsets (CD4+, CD8+, CD11b+ and B220+ cells) showed similar distribution in splenocytes of all investigated genotypes (C). Exclusion of deviations in peripheral proinflammatory activation using unstimulated and stimulated splenocytes from different genotypes, by examining IL-2 in the corresponding supernatants by ELISA (D). Error bars represent standard deviations.
Found at: doi:10.1371/journal.pone.0004405.s001 (0.39 MB TIF)

Material S1 Material and Methods S1
Found at: doi:10.1371/journal.pone.0004405.s002 (0.03 MB DOC)

Table S1 CDR3 sequences. Sequencing analysis of two clones with identical VβJβ combinations from PLPtg/PD-1-/- mice A and B and of one clone with a different VβJβ combination from mouse A. Note identical TCRVβ and TCRJβ regions surrounding the CDR3 region, which not only differs in aminoacids but also, more importantly, in length, thus indicating recognition of different antigens.
Found at: doi:10.1371/journal.pone.0004405.s003 (0.03 MB DOC)

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Author Contributions

Conceived and designed the experiments: AK MM HW RM. Performed the experiments: AK NS CWI CL. Analyzed the data: AK NS CWI CL HW RM. Contributed reagents/materials/analysis tools: CL KAN HW RM. Wrote the paper: AK HW RM.

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CD8+ T-cell clones dominate brain infiltrates in Rasmussen encephalitis and persist in the periphery

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Rasmussen encephalitis (RE) is a rare neurological disorder of childhood characterized by uni-hemispheric inflammation, progressive neurological deficits and intractable focal epilepsy. Destruction of neurons and astrocytes by cytotoxic CD8 T cells has been proposed as a pathogenic mechanism underlying this enigmatic disorder. We tested this hypothesis by analysing the clonal composition and T-cell receptor (TCR) repertoire of CD4+ and CD8+ T cells using complementarity determining region 3 (CDR3) spectratyping from peripheral blood and corresponding CNS specimens. Severe perturbations of the TCR repertoire were found in brain infiltrates from all specimens ($n = 5$). Clonal expansions, as evidenced by peripheral blood analysis ($n = 14$), belonged to the CD8+ T-cell subset, while CD4+ cells showed normal distributions. Some of those expansions were analysed in the respective CNS specimens by histochemistry. The stainings showed Vβ specific T cells containing the cytotoxic molecule granzyme B and lying in close appositions to NeuN+ neurons and GFAP+ astrocytes. Analysis of corresponding CNS/blood specimens revealed overlapping but also CNS-restricted expansions of certain TCR clonotypes suggesting expansions of T cells within the target organ itself. Longitudinal analysis of peripheral blood samples ($n = 5$) demonstrated dominance but also longitudinal persistence of specific CD8 T-cell clones over time. The Vβ/Jβ usage, length of the CDR3, and biochemical characteristics of the CDR3 amino acids suggested high similarities putatively related to common driving antigen(s) without shared clones. Taken together, our data strongly support the hypothesis of an antigen-driven MHC class-I restricted, CD8+ T cell-mediated attack against neurons and astrocytes in the CNS dominating the pathogenesis in RE.

Keywords: Rasmussen encephalitis; CDR3 spectratyping; CD8 cytotoxicity; clonal expansion

Abbreviations: CDR3 = complementarity determining region 3; HD = healthy donor; NDN = D segment with flanking N sequence; TCR = T-cell receptor; PBMC = peripheral blood mononuclear cells; RE = Rasmussen encephalitis

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Introduction

Rasmussen encephalitis (RE) is a very rare chronic progressive neurological disorder affecting mostly children and associated with hemispheric atrophy, focal epilepsy, intellectual decline and progressive neurological deficits (Rasmussen *et al*., 1958; Bien *et al*., 2005; Dubeau *et al*., 2007). The aetiology and pathogenesis of this severely disabling inflammatory disease are still enigmatic. An intriguing feature of RE is the restriction of the inflammatory process to one brain hemisphere, setting it apart from any other inflammatory disease of the CNS. Histopathological findings in RE comprise lymphocytic infiltrates, microglial nodules, neuronal and astrocytic loss, and gliosis of the affected hemisphere (Robitaille, 1991; Farrell *et al*., 1995). The hypothesis of RE as a primarily antibody-driven attack against neuronal structures [e.g. the glutamate receptor GluR3 (Rogers *et al*., 1994)] could not be confirmed in larger cohorts (Wiendl *et al*., 2001; Watson *et al*., 2004). Active brain inflammatory lesions contain large numbers of T lymphocytes, which are recruited early within the lesions suggesting that a T cell-dependent immune response contributes to the onset and evolution of the disease (Farrell *et al*., 1995). Moreover, the histopathological observation of granzyme B-containing CD8+ T cells in direct apposition to MHC class I positive neurons raised the hypothesis of a CD8+ T cells-mediated neuronal attack as a key pathogenetic mechanism underlying RE (Bien *et al*., 2002). Apart from neuronal cell death, CD8 cells may also be responsible for the degeneration of astrocytes found in RE lesions (Bauer *et al*., 2007).

The antigens of these brain-infiltrating lymphocytes are still unknown. It is not even clear yet, whether the CNS-directed T-cell response is focused towards particular antigens. Experimentally, this could be proven by demonstrating that individual clones are expanded in the tissue (Dornmair *et al*., 2003). This was possible in multiple sclerosis (Oksenberg *et al*., 1993; Skulina *et al*., 2004; Junker *et al*., 2007), but so far, only one study on the T-cell receptor (TCR) repertoire in RE has been published (Li *et al*., 1997). No information is available regarding the putatively pathogenic CD8+ T cells and longitudinal behaviour of T cells in RE patients.

Here we provide a detailed analysis of the TCR repertoire from RE patients' brain biopsy specimens as well as from time-matched and non-time-matched peripheral blood samples. CD4+ and CD8+ T-cell CDR3 spectratypes and sequences of dominant expansions in the CNS were determined in CNS samples of RE patients and compared with their peripheral T-cell repertoire, if available in serial peripheral blood samples. Additionally, morphological studies have been performed using immunohistochemistry and visualizing expanded T-cell populations in the brain specimens, their position and granzyme B positivity. We provide strong evidence for an antigen-driven MHC class-I restricted, CD8+ T cell-mediated attack against antigens presented on neurons and astrocytes in the CNS as the prevalent pathogenic process in RE.

Materials and methods

Patients and diagnosis

The local Ethics Committee (Ethikkommision der Universität Bonn) approved all studies and clinical investigations were conducted according to the Declaration of Helsinki. Informed consent was obtained from all participants or their parents or legal guardians. Fourteen patients (age 6–59, mean age 11) diagnosed with RE according to the typical clinical, MRI and neuropathological findings as proposed in (Bien *et al*., 2005) were enrolled in this study. From all RE patients, peripheral blood samples were obtained when patients were not receiving immunotherapy. Serial blood samples were available from five patients all receiving either tacrolimus or i.v. immunoglobulin treatment when these follow-up samples were taken. CNS tissue was obtained during neurosurgical operations (diagnostic open brain biopsies or functional hemispherectomies) from 12 RE patients. Material from all patients was paraffin-embedded. From five of these 12 patients, parts of the brain samples were cryoprotected in addition. As controls, peripheral blood was obtained from healthy volunteers and CNS tissue from autopsies in persons without known neurological disease, stroke victims and neurosurgical tissue from glioblastoma patients (Neuropathology University Wuerzburg, Prof. Roggendorf).

Immune cell purification and isolation

Peripheral blood mononuclear cells (PBMC) were isolated via density gradient centrifugation using lymphocyte separation medium (PAA Laboratories, Linz, Austria) within 24 hs after blood draw. CD4+ or CD8+ T cells were negatively isolated from PBMC using magnetic bead isolation (negative isolation procedure, MACS®, Miltenyi Biotec, Bergisch Gladbach, Germany), following the manufacturer's instructions. Purified T-cell subsets were over 95% pure as controlled by flow cytometry analysis.

Immunohistochemistry

Four-micrometre thick paraffin sections were studied by light-microscopy after staining according to established techniques using antibodies to the lymphocytic markers CD3, CD8, granzyme B, CD68, the neuronal marker NeuN and the astrocytic marker glial fibrillary acidic protein (Bauer *et al*., 2007). Densities of CD3+ and CD8+ cells were determined as described earlier (Bien *et al*., 2002). When clonal expansions of Vβ2 and Vβ3 were found in the brains of patients #03 and #10, 10-μm sections of cryoconserved brain material of these patients were double-stained immunohistochemically for light microscopy [antibodies to other Vβ families for immunohistochemistry use are either not available or do not give reliable staining results as reported by others (Bosboom *et al*., 2001) or tested in our own laboratory]. For the Vβ chains, the following antibodies were used: (1) Vβ2 (mouse-IgG antibody, clone MPB2D5, from Beckman Coulter, Marseille, France, dilution 1:50) and (2) Vβ3 (mouse-IgM, clone CH92, from Beckman Coulter, 1:100). Co-stainings were done using the following antibodies: (A) CD8 (mouse-IgG, clone C8/144B, from DakoCytomation, Glostrup, Denmark; 1:50); (B) CD3 (rabbit-IgG, clone SP7, from Lab Vision, Fremont, CA, USA, 1:500); (C) NeuN (mouse-IgG, clone A60, from Millipore, Billerica, MA, USA, 1:50); (D) glial fibrillary acid protein (GFAP, mouse-IgG,

Ab-6, clone ASTRO6, Lab Vision, 1:50); (E) GFAP (polycloncal rabbit-IgG, DakoCytomation, 1:7000); (F) granzyme B [polyclonal rabbit-IgG, ab4059, Abcam plc, Cambridge, UK, 1:2000, enhanced with biotinylated tyramine (King *et al*., 1997; Bien *et al*., 2002)]. The following species-divergent antibody combinations were applied: 1B, 1E, 1F, 2A, 2C, 2D, 2F. Antibody binding was detected by the following secondary antibodies: (1, A, C, D): alkaline phosphatase (AP)-conjugated anti-mouse-IgG antibody (made in goat, from Jackson ImmunoResearch, Newmarket, Suffolk, UK, 1:100); [2 (in combinations with C, E)] biotinylated anti-mouse-IgM antibody (made in goat, from LINARIS, Wertheim-Bettingen, Germany, 1:200); (2 in combination with F): AP-conjugated anti-mouse-IgM antibody (made in donkey, from Jackson ImmunoResearch, Newmarket, Suffolk, UK, 1:100); (B, E, F): biotinylated anti-rabbit-IgG antibody [made in donkey, GE Healthcare Europe (Amersham Biosciences), Munich, Germany, 1:200]. AP was visualized by Fast Blue and biotin by avidin-peroxidase and 3-amino-9-ethyl-carbazol (red staining) using standard protocols.

RNA isolation

RNA from peripheral blood T cells was isolated using standard methods (Quiagen RNeasy kit; Quiagen, Hilden, Germany) according to the manufacturer's instructions. Isolation of RNA from tissue specimens was performed from cryo-preserved CNS tissue of patients with the confirmed clinical and histopathological diagnosis of RE (see above).

To acquire statistically significant data, we included 1×10^5 CD8+ T cells in the analysis of each sample. We quantified the number of CD8+ T cells in 4 μm sections of each RE sample by immunohistochemistry. From this number the total amount of CNS tissue (as $n \times 50$ μm slices) was calculated to be used for RNA-isolation ($\sim 10^5$ CD8+ T cells) to make sure that we included roughly the same number of cells in each analysis. Slices were directly transferred into TriZol (Invitrogen, Mannheim, Germany), homogenized and then processed according to the manufacturer's protocol.

cDNA synthesis

cDNA synthesis from peripheral blood T-cell RNA was performed using standard methods as previously described (Wiendl *et al*., 2002). Reagents were obtained from Applied Biosystems (Foster City, CA, USA). For each sample 250 ng of RNA was transcribed, using random hexamers and M-MLV reverse transcriptase.

For RNA isolated from CNS samples, a previously published sensitive TCR β-chain-specific cDNA primer 'Cβ-RT' (Seitz *et al*., 2006) was used, as well as SuperScriptIII™ reverse transcriptase (Invitrogen, Karlsruhe, Germany), according to the manufacturer's instructions.

CDR3 spectratyping

For the CDR3-spectratyping, we used the Vβ forward primers as described in Monteiro *et al*. (1996), the Jβ reverse primers as described previously (Puisieux *et al*., 1994), and two different Cβ reverse primers: 'SpTy-β-out' (Junker *et al*., 2007) and 'Cβ-R' (Monteiro *et al*., 1996). The Vβ nomenclature according to Arden *et al*. (1995) is used throughout the manuscript.

For the peripheral blood derived samples, the following protocol was applied: First, the cDNA was used in 25 Vβ-Cβ reactions: 1.25 μl 10 pmol/μl Vβ primer, 1.25 μl 10 pmol/μl Cβ-R primer, 0.5 μl cDNA, 0.25 μl 2.5 mM dNTPs (Promega, Mannheim, Germany), 2.5 μl 10× buffer, 0.1 μl TaqPolymerase (both Applied Biosystems), 19.15 μl DEPC-H_2O. PCR conditions: 94°C, 6 min; 94°C, 1 min, 59°C, 1 min, 72°C, 1 min (40×); 72°C, 7 min. After those first-round PCRs, every PCR product was subjected to 13 individual Vβ-Jβ 'run-off' reactions (modified from Pannetier *et al*., 1993; Batliwalla *et al*., 1996) with 13 5′-fluorescence-tagged Jβ primers to differentiate between individual TCR Jβ-regions and also with a 5′-fluorescence-tagged Cβ-R primer. The length of these fluorescence-labelled PCR products was then analysed on an ABI3130 genetic analyser (Applied Biosystems), applying a module for fragment analysis. 500-ROX (Applied Biosystems) was the internal standard in each sample.

In the case of the CNS-samples, we used a more sensitive protocol (Junker *et al*., 2007) to compensate for the low T-cell numbers in the CNS specimens. Briefly, we introduced a semi-nested pre-amplification PCR step before the PCR described above. There, we used the same forward primers as above, but employed the Cβ-specific reverse primer 'SpTy-β-out' (10 pmol/μl) that hybridizes downstream of CβR, but upstream of the RT-primer Cβ-RT. Samples were incubated for 5 min at 94°C. Then PCR was run for 30 cycles of: 94°C, 1 min, 56°C, 1 min, 72°C, 1 min, followed by an incubation of 72°C, 10 min. From this PCR, 1 μl/reaction was used as template in the protocol mentioned before. NED-tagged primers were bought from Applied Biosystems (Foster City, CA, USA) and all other primers were provided by Metabion (Martinsried, Germany).

Data analysis

Data were processed by GeneMarker® software (SoftGenetics, State College, PA, USA). For all PCR products of Vβ- and Cβ-primers, or Vβ- and Jβ-primers, respectively, we plotted the peak intensities versus the fragment lengths. Unskewed repertoires yield Gaussian length distribution, whereas skewed repertoires show distortions (Pannetier *et al*., 1995). To evaluate the relative skew of the repertoires, we used the data obtained from blood of the healthy control persons as a standard: for each Vβ-Cβ-combination, we measured the average fragment lengths, i.e. we determined the positions of the maxima of the Gauss distribution. At these length positions, the peak intensity of the corresponding Vβ-Cβ product from the patients was defined as '1.0'. A Vβ-Cβ product from a patient was considered skewed, if a peak exceeded the intensity on this semi-quantitative scale for a factor of greater than two. If the factor was between 1.0 and 2.0, the repertoire was considered slightly disturbed. Very high peaks were reamplified with unlabelled Vβ and Jβ primer and the PCR products were sequenced directly. If a peak was not high enough for direct sequencing, the PCR product was sequenced after using the TA cloning kit pGEM-T (Promega).

Results

For patients' demographical and sample collection data, see Table 1.

Basic immunopathology of brain specimens

For comprehensive quantitative immunopathological work-up, paraffin-embedded specimens were available for analysis. They revealed a mean density of 20 CD3+ T cells/mm^2 on

Table 1 Clinical details of the patient cohort

Patient #	Gender	Age at onset of neurological symptoms (prodromal stage) (years)	Age at onset of acute stage (years)	Peripheral blood sampling (months after onset of acute stage)				Brain biopsy (months after onset of acute stage)	Number of CD8+ T cells/mm^2 in brain parenchyma
				#01	#02	#03	#04		
01*	M	10.8	10.8	15	23	30		15	53
02	F	5.7	5.7	12				12	n.a.
03*	F	3.6	5.0	20				11	3
04*	M	2.7	2.9	2				0	n.a.
05	F	4.5	4.5	20				7	4
06	M	6.1	6.1	26				1	n.a.
07*	F	17.8	18.1	7	16	16	18	6	n.a.
08	F	6.5	6.5	24	36				
09	F	1.1	2.1	14				6	n.a.
10*	M	26.0	50.6	−4	21			6	4
11	F	9.3	11.4	28				3	n.a.
12	M	12.7	15.5	36				10	n.a.
13	F	8.4	55.5	28					
14	M	3.8	4.5	21	33			8	n.a.

*Patients with available corresponding brain tissue and blood samples.

4 μm sections. A mean of 84% of them was CD8+, and 42% was granzyme B positive, respectively. The mean proportion of CD8+ cells lying in close apposition to neurons was 5%. CD68+ cells were observed at a density of 147/mm^2, 1% showing macrophage morphology (the rest to be considered microglial cells, partly forming nodules). These figures are highly similar to those obtained from a previous RE cohort (Bien *et al*., 2002). There was neuronal and astrocytic cell loss (not quantified).

Expanded T-cell clones infiltrate the CNS

We analysed the CDR3 length distribution for all Vβ families by CDR3 spectratyping from cryoprotected biopsy samples of five RE patients. In all patients, the TCR Vβ repertoire of the infiltrating T cells revealed clonal perturbations. Some examples are shown in Fig. 1A. Oligoclonally expanded T-cell clones were detected in the CNS of all patients (Fig. 1B). In 15–50% of the assessed Vβ families we detected oligoclonal expansions.

We also analysed various CNS control specimens. The first group represented CNS tissue specimens, in which neuropathological alterations were absent. In four out of five cases, we could not get a sufficient spectratyping signal due to the very small number of T cells in the CNS of these non-inflammatory controls (only few Vβ families were detectable). The fifth case, however, showed the complete Vβ repertoire with few clonal expansions (<20% of the assessed Vβ families, data not shown). Additionally, we analysed CNS specimens from three stroke patients. These samples are rich in mononuclear cells, but the accumulation is random and without any role for antigen-specific T cells. The TCR repertoire of the invading T cells in the detectable Vβ families was normally distributed (data not shown). As a third control group, we analysed tissue specimens from glioblastoma patients ($n=5$). These samples showed a very strong PCR signal due to the high amount of T cells in the tissue. Several of the Vβ families showed signs of clonal expansions (examples shown in Fig. 1C), which is in line with previous studies (Ebato *et al*., 1993). Interestingly, there were identical peaks in different patients (Fig. 1C, Vβ13.1).

Inter-individual analysis of T-cell expansions as analysed by Vβ/Jβ spectratyping suggested similarities in the antigen specificity of the expanded T-cell clones. To characterize the expanded clones, the TCR β-chains were sequenced. The deduced amino acid sequences of the CDR3 regions of CNS expanded T-cell clones are listed in Fig. 2, Supplementary Table 1. In the five patients, with CNS and peripheral blood samples available, no shared 'public' clones were found. The Vβ-usage and the CDR3 lengths and amino acid sequences were diverse, except some similarities (Vβ/Jβ/CDR3) between the Vβ18 clones of patients #01 and #07 (Fig. 2A). There, both chains are of the Vβ18 family, thus they carry identical CDR1 and CDR2 loops, their CDR3 loops are of the same length and consist exclusively of small or hydrophilic amino acids (with the exception of a single leucine in patient #07). Thus, even if, for example, there was a Vβ11-Jβ2.7 expansion in the CNS of patients #01, #04 and #10, with identical lengths of the CDR3 regions, the amino acids of the NDN regions did not show high homology (Fig. 2B). This is most strikingly evident from the positions of the arginine residues that introduce permanent positive charges into the CDR3 loop.

Skewed TCR repertoire of peripheral blood CD8 T cells parallels the repertoire in brain

To assess whether the clonal expansions of T cells were restricted exclusively to the CNS or were also present in the periphery, we

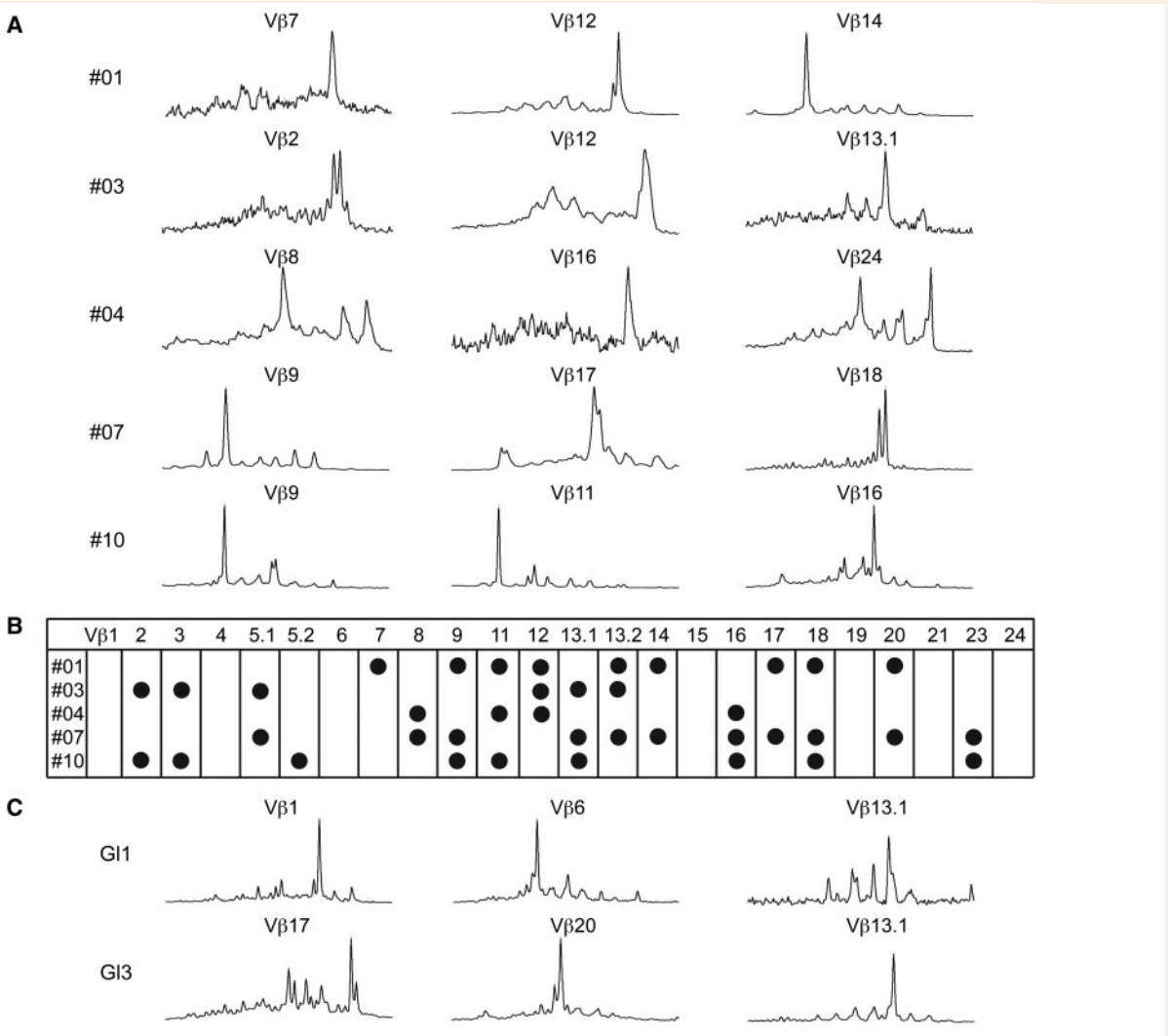

Fig. 1 CNS infiltrating T cells within active lesions of RE patients are clonally expanded. CNS brain specimens from five RE patients (#01, #03, #04, #07, #10) and five glioblastoma patients (GI1-5) were subjected to CDR3 spectratyping analysis. (**A**) Exemplary CDR3 spectratyping profiles of T cells in the CNS specimens of RE patients, showing clonal expansions. (**B**) Overview of clonal expansions in the Vβ segments of 5 RE patients. The *x*-axis shows the Vβ region of observed CNS expansions of T cells, while the *y*-axis shows the identification numbers of the five RE patients, where CNS tissue samples were available for analysis. A dot represents an expansion with a value of more than two in the respective Vβ region (for calculation see Materials and methods). (**C**) Exemplary CDR3 spectratyping profiles of T cells in the CNS specimens of two glioblastoma patients, showing clonal expansions.

purified CD4+ and CD8+ T cells from the peripheral blood of RE patients ($n = 14$) and from healthy volunteers as controls ($n = 7$) were analysed for their TCR repertoire. CD8+ T cells from RE patients showed strong skewings (Fig. 3A), with several clonal expansions of many Vβ families. In contrast, the repertoire of CD4+ T cells revealed only marginal deviations from the profile found in healthy individuals. As expected, spectratyping of CD4+ and CD8+ T cells in healthy volunteers revealed repertoire patterns that closely resembled Gaussian CDR3 length distributions of all Vβs (data not shown). Figure 3B summarizes the results of the repertoire analysis of CD8+ T cells from all RE patients. Strong clonal expansions in peripheral blood were detected in all patients (red dots). Inter-individual comparison of CDR3 length distributions among all RE patients in our cohort revealed that peripheral clonal expansions of some Vβ segments were more prevalent than others, i.e. 8 of 14 patients had clonal expansions in Vβ11 and Vβ15, whereas disturbances in the Vβ1 or Vβ7 segments were never detected (Fig. 3B).

Correspondence between TCR repertoire in CNS and peripheral blood

In all five RE patients with brain samples available, we also had access to blood samples. This allowed us to compare the TCR repertoire in CNS infiltrating versus peripheral blood derived CD4+ and CD8+ T-cell populations. We observed shared expansions (patient #01: 12%, #03: 10%, #04: 25%, #07: 23%, #10: 45%) with the same Vβ segment and identical CDR3 length in

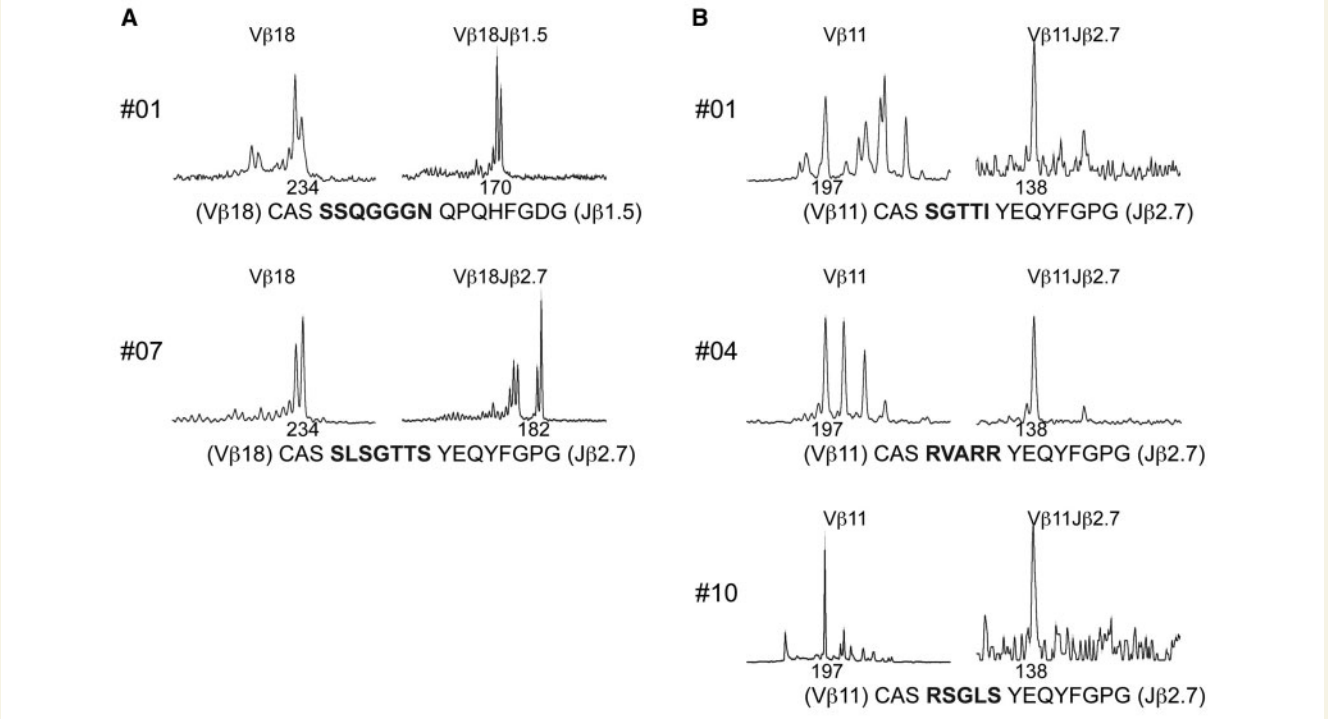

Fig. 2 Comparison of clonal CNS expansions in different patients. Clonally expanded T cells from the CNS of RE patients were analysed by CDR3 spectratyping and sequencing of the PCR products. The deduced amino acid sequences of the CDR3 are shown below the spectratyping profiles (NDN region in bold). The peak numbers are the exact length of the PCR fragment (in bp). (A) Vβ18 in the CNS of two patients (#01, #07) as well as Vβ18Jβ1.5 in the CNS of patient #01 and Vβ18Jβ2.7 in the CNS of patient #07. (**B**) Vβ11 and Vβ11Jβ2.7 in the CNS of three patients (#01, #04, #10).

CNS and parallel peripheral blood samples within the CD8+ T-cell subset, but never in the CD4+ population (Fig. 3B). To make sure that these clones in the CNS and periphery are identical, we sequenced exemplarily the respective clones in the patients #01 and #10. These clones turned out to have the same CDR3 nucleotide sequence in the CNS and in the peripheral CD8+ T-cell compartment (one example is given for patient #01, Vβ11Jβ2.7, Fig. 3B). Apart from the similarities of TCR expansions shared between CNS samples and peripheral blood, analysis of five patients with parallel CNS and blood samples revealed numerous clonal expansions restricted to either the CNS (23–59%) or the periphery (18–45%).

Morphological studies of expanded T-cell populations in CNS specimens

To confirm the presence of clonally expanded CD8+ T-cell clones in CNS and to support their putative pathogenic role, we exemplarily performed immunohistochemical double stainings for T-cell markers and Vβ2 and Vβ3 families which were clonally expanded in the CNS of patients #03 and #10. Figure 4 demonstrates the presence of clonally expanded autoinvasive T-cell clones co-expressing CD8 and Vβ3 and clones co-expressing CD3 and Vβ2 in both patients' brains. Figure 5 gives double stainings showing Vβ2 and Vβ3 positive cells containing granzyme B and lying in close apposition to neurons and astrocytes. This corroborates the hypothesis that these T cells act in a cytotoxic way against these cells (Bien *et al*., 2002; Bauer *et al*., 2007).

Persistence of the CD8 TCR repertoire in peripheral blood over time

To further support the hypothesis that CD8+ T cells play a key role for the self-perpetuating nature of the CNS immunopathology, we next investigated whether individual T-cell expansions persist over time or would be subject to change. Spectratyping analysis of CD8+ T cells was therefore performed on five RE patients at several time points after the first blood sample was taken (9–25 months). We found that particular CD8+ T-cell clones persisted in individual RE patients over periods of more than 1 year. The relative height of the peaks, however, varied over time, indicating fluctuations in absolute numbers in peripheral blood. This pattern was observed in all five patients. Exemplary results of three patients are shown in Fig. 6A. As an additional point, there was no significant correlation between disease onset and strength of the clonal expansions.

One patient (#07) underwent immune ablation and stem cell reconstitution with CD34+ haematopoetic stem cells from her identical twin. Accompanying immune reconstitution of this patient, the long-term persisting CD8+ T-cell clones reappeared within a period of a few months after ablation (data not shown).

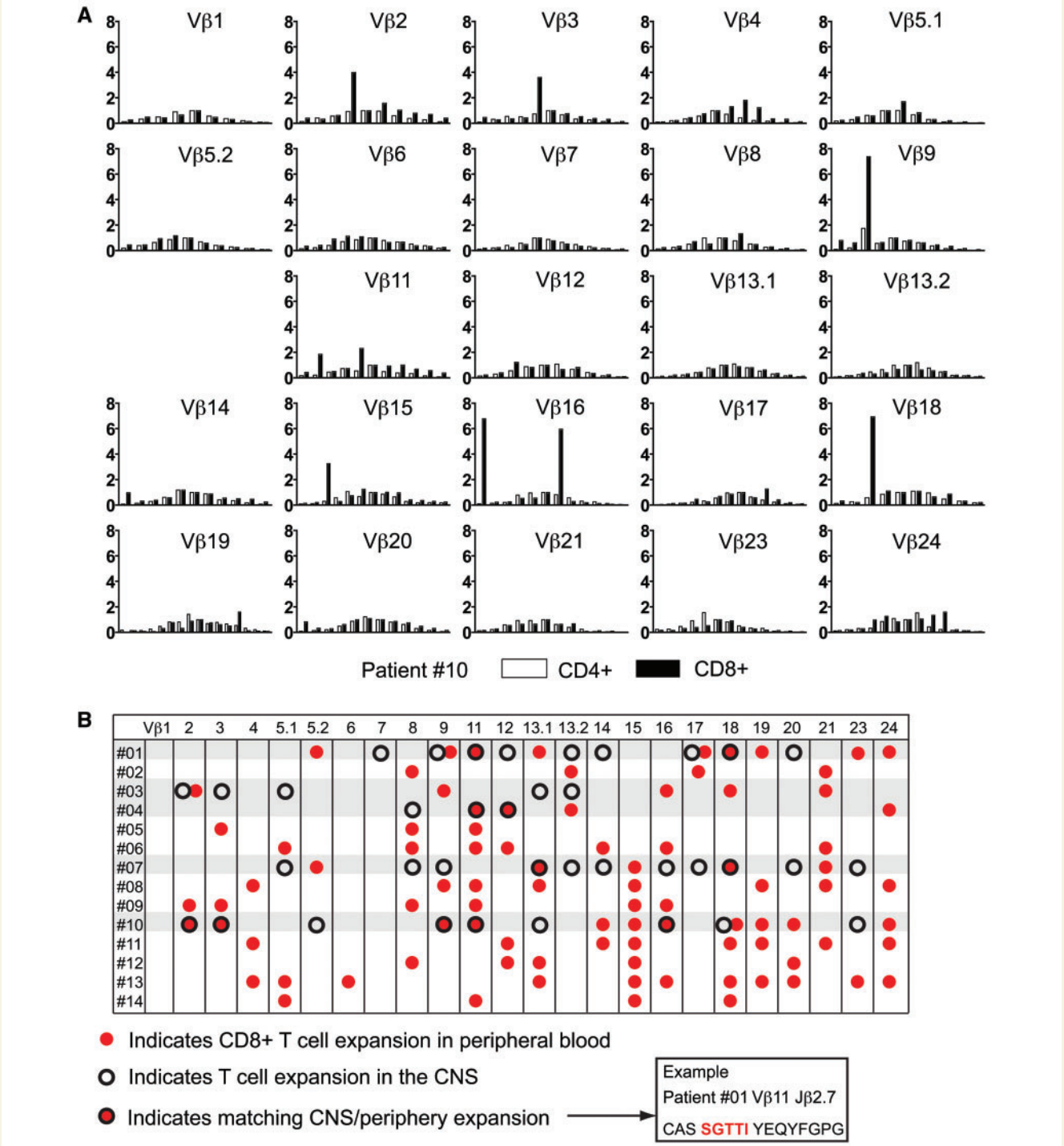

Fig. 3 The TCR repertoire of peripheral blood CD8+ but not CD4+ T cells is skewed in RE patients PBMC from 14 RE patients, magnetically separated into CD4+ and CD8+ T cells, were subjected to spectratyping analysis. (A) Representative example of the peripheral TCR repertoire of a RE patient (#10). White bars represent CD4+ T cells, black bars CD8+ T cells. The *x*-axis shows the CDR3 length of each possible TCR in the assessed Vβ regions, while the *y*-axis shows the normalized peak height (for the calculation see Materials and methods). (**B**) The *x*-axis shows the Vβ region of observed peripheral expansions of CD8+ T cells, the *y*-axis shows the number of 14 RE patients. An expansion is indicated by a red dot. Patients, where a CNS sample was available, are tinted grey. If an expansion (same Vβ and CDR3 length) also exists in the corresponding CNS sample (Fig. 1B), the dot is marked with a black line. An empty black line represents an expansion in the CNS, which is not present in the periphery. The amino acid sequence of the CDR3 of one exemplary matching clone is given (NDN region in red).

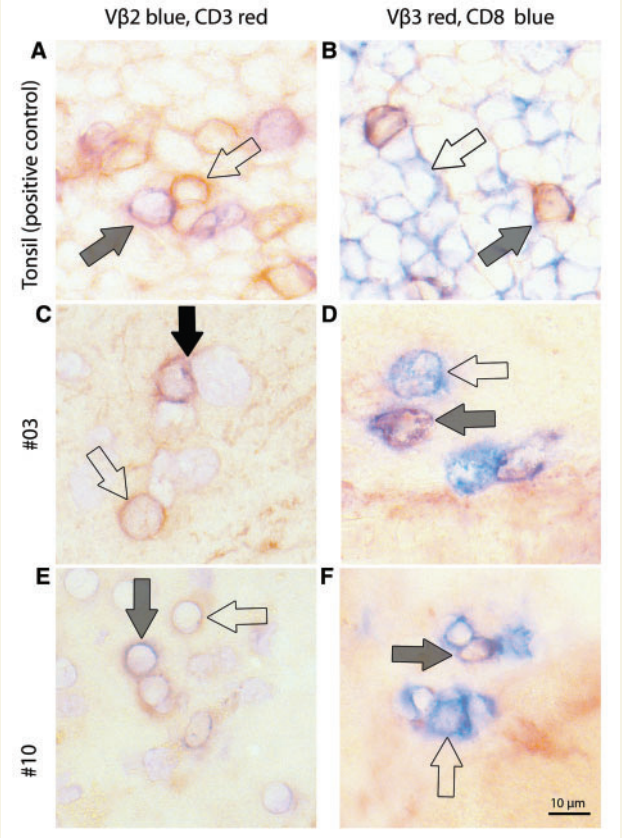

Fig. 4 Immunohistochemical stainings for expanded Vβ populations in the CNS of RE patients. Double staining for CD3/Vβ2 and CD8/Vβ3 T cells from cryoconserved biopsy specimen from tonsil (positive control), patient #03 (hippocampus) and patient #10 (temporal neocortex). C–F are very mildly counterstained with haematoxylin. Open arrows indicate CD3 or CD8 positive T cells negative for the respective TCR, filled arrows point to double positive cells, i.e. those that express Vβ2 or Vβ3. Note the close apposition between a double positive lymphocyte and a cell with a large, irregular shaped nucleus probably belonging to a neuron. The 10 μm bar is valid for all images.

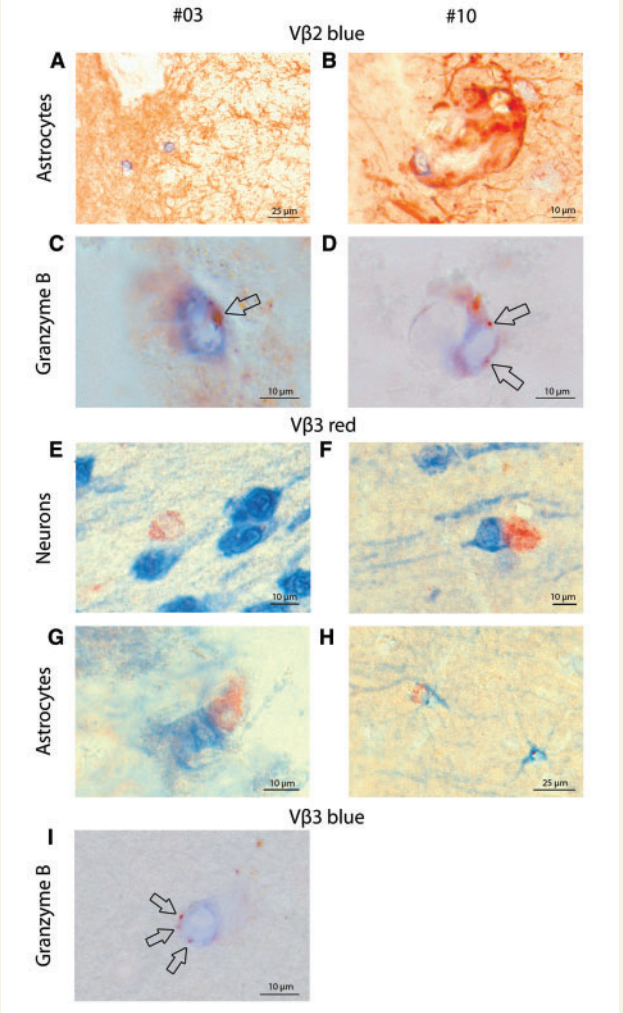

Fig. 5 Double stainings for Vβ2 (A–D) and Vβ3 T cells (E–I) with astrocyte marker glial fibrillary acid protein (GFAP, A, B, G, H), neuronal marker NeuN (E, F) and cytotoxic protein granzyme B (C, D, I) on cryoconserved biopsy specimens of patient #03 (hippocampus, **A, C, E, G, I**) and patient #10 (temporal neocortex, **B, D, F, H**). (**A**) Two Vβ2 positive T cells at the borderzone between an area with dense fibrillary astrogliosis (left part of the image) and an area with reduced astrocyte density (at these borderzones, cytotoxic T cells and apoptotic astrocytes are frequently found [see Figs 1 A–E and 4 N in (Bauer et al., 2007)]. (**B**) Vβ2 positive T cell closely attached to a (perivascular) astrocyte. (**C** and **D**): Vβ2 positive T cells with granula containing granzyme B (arrows). (**E** and **F**) Close apposition of Vβ3 positive T cells with neurons. (**G** and **H**) Vβ2 positive T cell closely attached to astrocytes. (**I**) Vβ3 positive T cell with granula containing granzyme B (arrows). (No granzyme B positive Vβ3 positive cell was found in the section from patient #10 studied this way.)

Figure 6B demonstrates the persistence of an expanded T-cell clone in the peripheral CD8+ T-cell compartment of patient #01 (time period of 15 months), confirmed by sequencing. This identical clone was also strongly expanded in two independent areas of the CNS from the same patient: one part of the biopsy comprised mainly cortical tissue, the other one subcortical white matter (both within the superior frontal gyrus), and both showed typical histopathological characteristics of active RE. Interestingly, the TCR repertoires from these two brain regions in patient #01 were identical, suggesting a homogenous clonal distribution in the affected hemisphere.

Discussion

Destruction of neurons and astrocytes by cytotoxic CD8+ T cells has been proposed as pathogenic mechanism underlying RE. We tested this hypothesis by analysing the clonal composition and TCR repertoire of CD4+ and CD8+ T cells using CDR3 spectratyping from peripheral blood and corresponding CNS specimens. We show here that the existing clonal expansions in RE patients, at least those in peripheral blood, are predominantly due to CD8+ cells and that they may persist for at least 1–2 years.

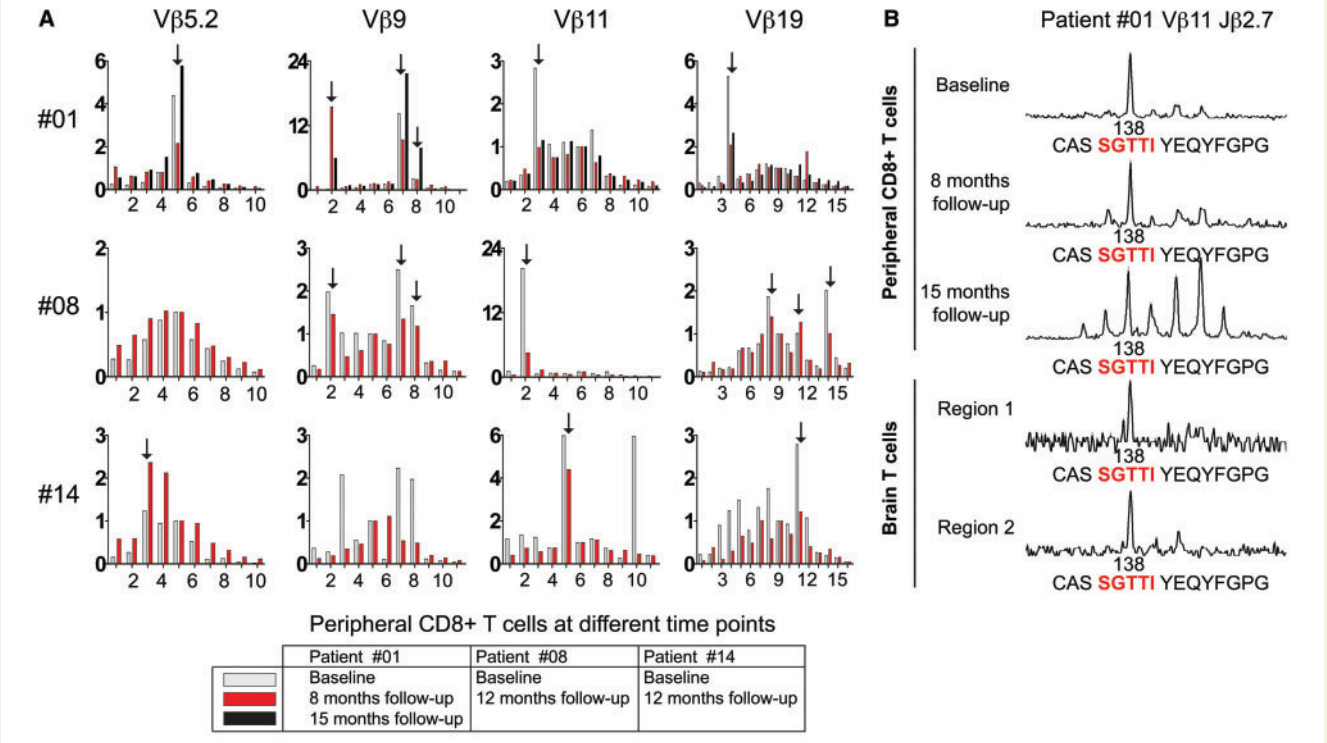

Fig. 6 Long-term persisting peripheral CD8+ expansions are shared with CNS T-cell expansions in different brain regions. (A) The TCR repertoire of the peripheral CD8+ immune subset of five RE patients was followed over time. The figure shows the spectratyping analysis of three patients (#01, #08, #14) followed up to 15 months. The *x*-axis shows the CDR3 length of the respective TCR, while the *y*-axis shows the relative strength of the expansion (for the calculation see Materials and methods). Arrows mark long-term persisting expansions. Baseline spectratyping (the first spectratyping of this patient) is represented by grey bars, first follow-up by red bars, and the last follow-up (if applicable) by black bars. (**B**) One representative example of a matched peripheral/CNS expansion (long-term persisting): the Vβ11Jβ2.7 TCR in patient #01 in peripheral CD8+ T cells from three different time points and in T cells from two different brain regions. The given peak numbers are the exact length of the PCR fragment (in bp). The CDR3 sequence is given in amino acids, the NDN region is indicated in red.

All CNS specimens from RE patients showed severe perturbations of the TCR repertoire. All patients showed clonal T-cell expansions, which strongly supports the hypothesis of an antigen-driven, T cell-mediated autoimmune process in contrast to a random attraction of cells as part of a secondary immune response. This has been shown in many other autoimmune diseases and it confirms previous findings in RE (Li *et al*., 1997). Upon exemplary morphological assessment of expanded Vβ families in the brains of two patients, T cells expressing these Vβ families were readily found. These lymphocytes contained the cytotoxic molecule granzyme B and were found in close appositions to neurons and astrocytes (Figs 4 and 5) thereby extending previous pathogenetic findings on RE T cells in general to lymphocytes with individually overexpressed Vβ clones (Bien *et al*., 2002; Bauer *et al*., 2007).

Brain specimens from stroke victims showed normal distribution of the TCR repertoire. This is expected due to the unspecific T-cell influx occurring on the site of a stroke lesion. In the one case of non-pathological CNS autopsy tissues allowing us to get an adequate PCR signal we found slight TCR perturbations, which is not surprising, because the person was 64 years of age, where age-related clonal expansions are quite common (Messaoudi *et al*., 2004). As an even more meaningful control experiment, we investigated tissue specimens from glioblastoma patients. It has been published previously that lymphocytes in glioma tissue show skewed distributions of the TCR repertoire (Ebato *et al*., 1993) and even contain the same amino acids in the NDN region in some cases (Ebato *et al*., 1994). In our analyses we also found several clonal expansions in the glioblastoma patients' CNS, confirming the above studies. Strikingly, we found matching Vβ13.1 peaks in two patients (Fig. 1C). Of note, it is known that Vβ13.1 is preferentially expanded in glioma patients (Ebato *et al*., 1993). Both, healthy control subjects and glioma patients confirmed the results expected from previous studies and therefore provided evidence that our method may reveal clonal expansions without introducing particular biases.

When we detected peaks similar in Vβ/Jβ composition and CDR3 length in several patients, we sequenced their CDR3 regions. Although there are some similarities (Fig. 2A), the majority of the clonal expansions are too different to be able to assume common triggers. However, thus far identical TCR expansions have only been found in very few diseases, e.g. paroxysmal nocturnal haemoglobinuria (PNH) (Gargiulo *et al*., 2007), where the finding of identical TCRs (so called public clones) can be considered as an unambiguous indication for the recognition of similar epitopes, albeit it is probable that these TCRs do not

recognize the same peptide under the given different MHC restrictions, especially because the patients in this study did not share HLA genes. Shared (public) clones with the same CDR3, however, can only be assumed if one of the HLA genes of different individuals matches. In the mouse system with a given inbred identical MHC locus, such public clones against single peptides have been observed (Menezes *et al*., 2007). Similar findings in humans are so far very scarce. On the other hand, the similarity in the Vβ/Jβ composition and CDR3 length (Fig. 2A) should be considered as too high to be explained by pure coincidence in the composition of these expansions, as the theoretical size of the T-cell repertoire is $\sim 10^{15}$ (Davis and Bjorkman, 1988).

Surprisingly, we found very strong clonal expansions exclusively in the peripheral CD8+ T-cell compartment of RE patients providing evidence for an ongoing CD8+ T cell-mediated immune reaction in the periphery of all RE patients. Interestingly, the expansions showed skewed Vβ segments. While this bias towards certain Vβ segments could be interpreted as an indication of common antigenic triggering leading to similar expansions in different patients, the Vβ segment alone cannot prove antigenic similarities. Also, it has been shown that CD8+ T cells are more prone to antigen-driven or spontaneous age-related expansions than CD4+ T cells. This is especially observed in older aged human individuals (Messaoudi *et al*., 2004). The mean age of RE patients at the time of the analysis was 11 years; age-related expansions therefore seem highly unlikely as an explanation of our data. As expected, the peripheral TCR repertoire of the CD8+ T cells of healthy individuals was normally distributed with only insignificant aberrations that reached a very small fraction of the patients' disturbances, if they occurred at all.

We found that 10–45% of the clonal expansions in the brains of RE patients were shared with the peripheral CD8+ T-cell repertoires, while there were no shared expansions within the CD4+ T-cell compartment. Of note, many shared TCR sequences were validated by DNA sequencing (exemplary in Fig. 6B). In addition to the low number of parenchymal CD4 T cells in RE biopsy specimens, these data provide very strong evidence that the CNS infiltrating, oligoclonally expanded T-cell clones are CD8+ T cells.

We also found several expansions that were restricted to the brain or to the periphery in all five patients. This is not surprising, since the peripheral TCR repertoire in inflammatory CNS disorder only partly reflects the repertoire in the CNS. Examples from other chronic progressive inflammatory CNS disorders, i.e. multiple sclerosis has revealed similar findings (Skulina *et al*., 2004).

We found that several CD8+ T-cell expansions persisted for up to 15 months. The fact that these CD8+ T-cell clones stay expanded for more than 1 year strongly supports their assumed pathogenic role in RE. We speculate that these cells (re)encounter their specific antigen during this time because T cells that are not confronted with their antigen for a certain time after an expansion will be subject to regulatory mechanisms to ensure homeostasis, mainly over the Fas/FasL pathway (reviewed by Lenardo *et al*., 1999).

In the case of the stem cell transplantation, the re-occurrence of the clones might possibly be explained by assuming that T cells residing in the CNS are not sufficiently eradicated by the immunablative regimen. The detection of expanded T-cell clones in peripheral blood thus could indicate either a strong persisting memory response or, much more likely, hint towards an ongoing exposure to the yet unknown antigen(s). Of note, the peripheral TCR repertoire of CD4+ and CD8+ T cells in the patient's identical twin was completely normally distributed (data not shown), proving that RE is not (solely) of genetic origin, but dependent on an exogenous trigger, which might possibly be a virus. Also, the application of various immunotherapeutic regimes (e.g. intravenous immunoglobulins or tacrolimus) did not seem to affect the persistence of expanded individual T-cell clones in the patients whom we examined over a longer period of time. An alternative explanation, which we cannot rule out completely, might be that the persistence of these clones results from this therapy. However, it seems highly unlikely that drugs used for immunoregulatory purposes promote the survival of specific clones.

Our observations clearly favour the hypothesis of an ongoing pathogenic process triggering survival or continuous activation of pathogenic CD8+ T-cell clones detectable in the peripheral blood of RE patients. Exposure to either CNS-derived autoantigen(s) or a persisting viral infection would be compatible with such a hypothesis.

It is tempting to speculate that in RE patients the common trigger of the focused, dominant CD8 response is a similar antigen, which persists over a long period of time and sustains the stimulus necessary for the perseverance of these putatively pathogenic clones. The long-term persistence (or re-occurrence) of putatively pathogenic T-cell clones despite therapy (or even immunoablation) may indicate an ongoing exposure of the immune system to the antigenic trigger. This trigger (autoantigen or virus) could very well reside within the CNS. The finding of identical TCR clones between the CNS and peripheral blood compartment in patients with matching CNS-blood samples is in line with this assumption. Furthermore, TCR clones were found between individual patients demonstrating high biochemical similarities in their CDR3, albeit not fully matching on the CDR3 amino acid level. The antigenic specificity of these CNS-infiltrating CD8+ T cells in RE patients, however, remains elusive at present.

Taken together, our findings support the assumption that local clonal expansions of CD8+ T cells represent a pathogenic feature of active RE lesions. Partly, expanded, putatively pathogenic CD8+ T-cell clones can also be found in the peripheral blood of RE patients, where a severely perturbated TCR repertoire of CD8 but not CD4 cells TCR repertoire is constantly found in all patients.

We provide strong evidence for an antigen-driven MHC class-I restricted, CD8+ T cell-mediated attack against antigens presented on neurons and astrocytes in the CNS dominating the pathogenesis in RE. While further work is warranted to characterize the nature of the recognized antigenic structure(s) by putatively pathogenic T-cell clones, our study has significant implications relating to our understanding of RE pathogenesis.

Supplementary material

Supplementary material is available at *Brain* online.

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Neurobiology

Accelerated Course of Experimental Autoimmune Encephalomyelitis in PD-1-Deficient Central Nervous System Myelin Mutants

AQ: A

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It is assumed that the onset and course of autoimmune inflammatory central nervous system (CNS) disorders (eg, multiple sclerosis) are influenced by factors that afflict immune regulation as well as CNS vulnerability. We challenged this concept experimentally by investigating how genetic alterations that affect myelin (primary oligodendrocyte damage in PLPtg mice) and/or T-cell regulation (deficiency of PD-1) influence both the onset and course of an experimental autoimmune CNS inflammatory disease [MOG_{35-55}-induced experimental autoimmune encephalomyelitis (EAE)]. We observed that double pathology was associated with a significantly earlier onset of disease, a slight increase in the neurological score, an increase in the number of infiltrating cells, and enhanced axonal degeneration compared with wild-type mice and the respective, single mutant controls. Double-mutant PLPtg/PD-$1^{-/-}$ mice showed an increased production of interferon-γ by CNS immune cells at the peak of disease. Neither PD-1 deficiency nor oligodendropathy led to detectable spread of antigenic MHC class I- or class II-restricted epitopes during EAE. However, absence of PD-1 clearly increased the propensity of T lymphocytes to expand, and the number of clonal expansions reliably reflected the severity of the EAE disease course. Our data show that the interplay between immune dysregulation and myelinopathy results in a stable exacerbation of actively induced autoimmune CNS inflammation, suggesting that the combination of several pathological issues contributes significantly to disease susceptibility or relapses in human disease. *(Am J Pathol 2009, 174:000–000; DOI: 10.2353/ajpath.2009.081012)*

Multiple sclerosis (MS) is a relatively frequent neurological disorder of young adults characterized by demyelination, inflammation, and axonal damage of the central nervous system (CNS).[1] Experimental autoimmune encephalomyelitis (EAE) in rodents is a widely used animal model for MS mimicking typical neurological symptoms such as paralysis and ataxia. Experimental disease as well as MS lesions are characterized by inflammation, demyelination, and accompanying axonal damage.[2] Active immunization with CNS-specific protein antigens such as myelin antigens induces $CD4^+$ Th1- and interleukin (IL)-17-dominated immune response.[3–5] Fn1

A broad variety of factors modulate the disease courses of MS and EAE. Histopathology as well as clinical course of MS shows considerable heterogeneity. Different histopathological patterns have been proposed,[6] some of which, namely pattern III and IV, include primary oligodendrocyte damage or death.[7] In the last years, several cases were described, in which mutations in the most abundant myelin protein of the CNS, proteolipidprotein (PLP), were associated with primary progressive or relapsing remitting MS.[8,9] It is therefore highly interesting to investigate how alterations in myelin proteins interplay with the immune system. Genetic variations of myelin

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proteins might be modulators or even causative for the disease pathogenesis.

Recently, we investigated a mouse model, the transgenic PLP-overexpressing (PLPtg) mouse,[10] which spontaneously develops CNS inflammation with accumulation of both $CD11b^+$ microglial cells and $CD8^+$ T lymphocytes. The pathogenetic relevance of these cells was proven when crossbreeding PLPtg mice with RAG-1-deficient mice, which lack mature B or T lymphocytes. Absence of T lymphocytes lead to an amelioration of the phenotype,[11] pathogenetically relevant lymphocytes were clonally expanded $CD8^+$ T cells.[12]

Being aware of this context, it was interesting to investigate the influence of immune modulators in this model of primary oligodendropathy. As important candidates responsible for tissue immune homeostasis and the maintenance of tolerance we assumed members of the B7/CD28 family: T cells express co-stimulatory and co-inhibitory molecules. A co-stimulatory signal delivered by ligation of CD80 or CD86 on antigen-presenting cells (APCs) and CD28 on T cells is essential for the activation of T cells and maturation toward effector function. Other molecules are responsible for the termination of immune responses and induction or maintenance of tolerance.[13] Important members of this family of co-inhibitory molecules are programmed death (PD)-1 (CD279) and its ligands PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273). PD-1 is expressed on activated T and B lymphocytes and myeloid cells. Its ligand B7-H1 is expressed on a broad variety of cells, including lymphocytes, APCs, and tissue cells such as pancreatic islet cells,[14] endothelial cells,[15] liver cells,[16] or microglial cells.[17] In contrast, PD-L2 was mainly detected on dendritic cells.[18] The PD-1/B7-H1 pathway has been studied extensively, implying a predominantly negative regulatory role. PD-1-deficient mice develop spontaneous strain-dependent autoimmune diseases such as lupus-like disease in C57/BL/6 mice[19] or autoantibody-mediated cardiomyopathy in BALB/c mice.[20] Similarly, blockade and deletion of PD-1 has been shown to result in exacerbation of diseases such as EAE,[21,22] inherited peripheral nerve,[23] and CNS demyelination,[24] diabetes,[25,26] or allograft rejection.[27]

Our group could demonstrate that a PD-1 polymorphism was associated with a progressive disease course,[28] indicating that PD-1 acts as a disease-modifying factor in human autoimmunity. Genetic ablation of B7-H1 (PD-L1) is associated with an accelerated and worsened EAE disease course,[29] a consequence of the limited ability of CNS parenchymal cells to restrict CNS-specific immune responses via the B7-H1/PD-1 pathway.[29,30] Thus, a functional PD-1/B7-H1 pathway is an important regulator of immune homeostasis and tissue tolerance.

In the present study, we investigated how the combination of genetic alterations affecting myelin (primary oligodendrocyte damage in PLPtg mice) and/or affecting T-cell regulation (PD-1 deficiency) influence the onset and course of an experimental autoimmune CNS inflammatory disease [myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ induced EAE]. This experimental setup could mimic a situation in which genetics predispose a combination of a CNS vulnerability factor (myelinopathy) together with an immune regulatory impairment (PD-1-dysfunction) and encounter an acute CNS-directed inflammatory response. We demonstrate that combined pathology leads to a very stable and accelerated experimental autoimmune CNS inflammation.

Materials and Methods

Animals and Determination of Genotypes

PLP transgenic mice[10] were bred and genotyped as described previously.[11] In short, the transgene was polymerase chain reaction (PCR)-amplified using the primer set 5′-CAGGTGTTGAGTCTGATCTACACAAG-3′ and 5′-GCATAATACGACTCACTATAGGGGATC-3′ with an annealing temperature of 55°C and an elongation time of 45 seconds at 72°C. PD-$1^{-/-}$ mice[31] were genotyped as previously described with the primer pair 5′-CCGCCTTCTGTAATGGTTTG-3′ and 5′-TGTTGAGCAGAAGACAGCTAGG-3′ for the wild-type allele, an annealing temperature of 54°C, and an elongation time of 45 seconds. The knockout allele was detected with the primer pair 5′-GCCCGGTTCTTTTTGTCAAGACCGA-3′ and 5′-ATCCTCGCCGTCGGGCATGCG CGCC-3′ at an annealing temperature of 60°C and an elongation time of 45 seconds. PCR products were visualized on an agarose gel with the wild-type band at 690 bp and the knockout band at 400 bp. PLPtg mice were crossbred with PD-$1^{-/-}$ mice. All mice were bred on a C57/BL/6 background for more than 10 generations and all animal experiments were approved by the local authorities (Regierung von Unterfranken, experiment number 54-2531.01-36/06).

Induction and Scoring of EAE

Active EAE was induced in age- and sex-matched littermate mice by subcutaneously injecting 100 μg of MOG_{35-55} peptide (EVGWYRSPFSRVVHLYRNGK; synthesized and high performance liquid chromatography-purified by R. Volkmer, Charite, Berlin, Germany) as described previously.[29] MOG_{35-55} peptide was emulsified with an equal volume of complete Freud's adjuvant, containing *Mycobacterium tuberculosis* H37RA (Difco, Detroit MI) at a final concentration of 1 mg/ml. Additionally, the mice received two intraperitoneal injections of pertussis toxin (400 ng per mouse; List Biological Laboratories, Campbell, CA) at the time of immunization and 48 hours later. Mice were weighed and observed daily for clinical signs and scored based on the following scale (EAE score): 0, no disease; 1, limp tail; 2, hind limp weakness; 3, hind limp paralysis; 4, hind and fore limp paralysis; 5, moribund or death. Scores are shown as mean daily clinical scores for all mice per group (n = 4 to 6 per group).

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Purification of Splenocytes

Splenocytes were harvested as described before[11,12] by passing them through a 70-μm cell strainer (BD Biosciences Pharmingen, San Jose, CA). Erythrocyte lysis was performed with a hypo-osmolar lysis buffer (150 mmol/L NH_4Cl_2, 10 mmol/L $KHCO_3$, 0.1 mmol/L ethylenediaminetetraacetic acid in distilled water at pH 7.3) for 5 minutes at room temperature. Afterward, cells were washed and processed for consecutive experiments.

Purification of Mononuclear Cells from Cervical Lymph Nodes

Cervical lymph nodes were prepared and the tissue was disrupted by grinding between object trays. Cells were then passed through a 40-μm cell strainer (BD Biosciences Pharmingen). Afterward, cells were washed and processed for the respective experiments.

Preparation of CNS Mononuclear Cells

Mice were euthanized with CO_2 and transcardially perfused with cold 0.1 mol/L phosphate-buffered saline (PBS). The brains were dissected and the spinal cords were flushed out with PBS by hydrostatic pressure. Afterward, CNS tissue was homogenized, washed, and separated over a cold Percoll (Amersham Biosciences, Freiburg, Germany) gradient. Cells were harvested from the 30 to 50% Percoll interface, washed, and used for following experiments.

Tissue Preparation and Immunohistochemistry

For identification of $CD11b^+$ macrophage-like cells, mice were transcardially perfused with 4% paraformaldehyde in 0.1 mol/L cacodylate followed by tissue dissection of optic nerves and spinal cords from the brainstem to the lumbar regions, postfixation in the same fixative for 2 hours, and cryoprotection in 30% sucrose overnight. Alternatively, mice were perfused with 0.1 mol/L PBS followed either by direct snap-freezing of the dissected tissue (for $CD4^+$ and $CD8^+$ T-lymphocyte immunohistochemistry) or postfixation in 4% paraformaldehyde in PBS for 2 hours (for neurofilament immunohistochemistry) and then subsequent embedding in Tissue-Tek OCT compound (Miles Laboratories, Elkhart, IN). After snap-freezing, 10-μm-thick longitudinal sections of the optic nerves and transverse sections of the spinal cords were cut. For immune cell analysis, lumbar spinal cords were used, whereas for neurofilament immunohistochemistry, sections were taken from the beginning of the cervical enlargement to ensure that the same regions were analyzed in all mice.

Immunohistochemical stainings were performed as described before.[11,32] Nonspecific binding was blocked for 30 minutes in 5% normal bovine serum and then sections were incubated with the primary antibodies diluted in 1% normal bovine serum (overnight, 4°C). Activated macrophage-like cells were detected with rat anti-mouse CD11b (Serotec, Oxford, UK); rat anti-mouse CD4 (Serotec) and rat anti-mouse CD8 (Chemicon, Temecula, CA) antibodies were used for the identification of T lymphocytes. To identify all axons (myelinated and demyelinated), sections were incubated with antibodies against phosphorylated neurofilament (SMI-31; Sternberger Monoclonals, Lutherville, MD) and nonphosphorylated neurofilament (SMI-32) in combination or alone. Detection of primary antibodies was achieved by using a biotinylated secondary antibody to rat Igs (macrophage-like cells, T-cell antibodies) or mouse Igs (SMI-31 and SMI-32) for 1 hour, followed by avidin/biotin reagent (DAKO, Carpinteria, CA) before incubation and staining with diaminobenzidine-HCl (DAB) and H_2O_2. The specificity was controlled by omission of the primary antibodies.

Quantification of Immune Cells and Axons in the CNS

Lumbar spinal cord cross sections and longitudinal sections of the optic nerve of wild-type, PD-$1^{-/-}$, PLPtg, and PLPtg/PD-$1^{-/-}$ mice were examined. Quantification of $CD4^+$, $CD8^+$, and $CD11b^+$ cells was performed in longitudinal sections of the complete optic nerve or lumbar sections of the spinal cord using a Zeiss (Thornwood, NY) Axiophot2 microscope at a final magnification of ×300. The number of positively labeled cells was calculated per mm^2. The area was measured using digital images acquired via a charge-coupled device camera and ImagePro 4.0 software. Axonal density was determined within preselected fields (500 μm^2 in area) at specific sites within the dorsal column (cuneate fasciculus), dorsal corticospinal tract, as well as in ventral and lateral fasciculus. Stained axons were counted using MetaVue Software (Molecular Devices, Downingtown, PA). AQ: C

Enzyme-Linked Immunosorbent Assay (ELISA)

Splenocytes (1×10^6) were cultured overnight with and without MOG_{35-55} peptide (10 μg/ml). After 24 hours, the supernatant was collected and analyzed by ELISA (R&D Systems, Minneapolis, MN) according to the manufacturer's recommendations. Readout was performed with an Original Multiskan EX ELISA reader (Labsystems, Helsinki, Finland).

ELISPOT Assays

Splenocytes, lymph nodes, and CNS-derived lymphocytes were investigated during the priming phase (day 7 after immunization), the peak of disease (approximately day 15), and in the late phase of disease (day 20). CNS lymphocytes (5×10^4) or 1×10^5 lymph node lymphocytes or splenocytes per well were incubated for 24 hours in provided 96-well plates, unstimulated or stimulated with either MOG_{35-55} peptide (10 μg/ml), mixed MHC class I restricted peptides (PLP peptides: VCGSNLLSI, AATYNFAVL, ATYNFAVL, NYQDYEYL; MOG peptides: LIICYNWL, VGLVFLFL, SPGKNATGA, FYWVNPGVL; one

MBP peptide: ADPGNRPHL; from Genscript Corp., Piscataway, NJ, used at 200 nmol/L) (Leder et al[12]) or five mixed MHC class II restricted peptides: $MOG_{113-127}$ (LKVEDPFYWVSPGVL), $MOG_{120-134}$ (YWVSPGVLTLIALVP), $MOG_{183-197}$ (FVIVPVLGPLVALII), MBP_{54-72} (SHHAARTTHYGSLPQKSQR), $PLP_{178-191}$ (NTWTTCQSIAFPSK) (from EMC Microcollections, Tuebingen, Germany). Interferon (IFN)-γ and IL-17 ELISPOT assays were performed according to the manufacturer's instructions (BD Pharmingen, San Diego, CA; R&D Systems). Spots were quantified by CTL Europe (Aalen, Germany) using ImmunoSpot 4.0.17.

Spectratyping

The CDR3 spectratyping with the corresponding primers was performed as described previously.[12,33] Briefly, PCR with a Cβ-specific reverse primer and 24 Vβ-specific forward primer was performed on cDNA transcribed from 500 ng of leukocyte mRNA derived from spleens or CNS. The following PCR steps were applied: 94°C, 1 minute; 94°C, 1 minute 10 seconds; 60°C, 1 minute; 72°C, 4 minutes (40 steps); 72°C, 10 minutes. Thirteen fluorescence-labeled reverse primers (12 Jβ, 1 Cβ) were used to label each Vβ-Cβ PCR product during PCRs. The PCR steps were: 94°C, 2 minutes, 60°C, 1 minute, 72°C, 15 minutes (five steps). The labeled PCR products (Vβ-Jβ, Vβ-Cβ) were analyzed on an ABI Prism 3130 capillary sequencer (Applied Biosystems, Foster City, CA) regarding their length and distribution, using a module for fragment analysis. As an internal length standard, 500-ROX (Applied Biosystems) was applied for every sample.

Statistical Analysis

The unpaired two-tailed Student's *t*-test was used for comparison of quantified profiles. Scores were analyzed by using the nonparametric Mann-Whitney *U*-test and the Kruskal-Wallis test.

Results

Absence of PD-1 Alters Onset of EAE

To investigate the influence of combined primary oligodendrocyte damage and a defective T-cell inhibition on the EAE disease course, we immunized PLPtg/PD-$1^{-/-}$ mice with MOG_{35-55} peptide. As controls we used wild-type, PD-$1^{-/-}$, and PLPtg mice. After titration experiments with different doses of antigen (data not shown), experiments were performed with a relatively low dose of MOG_{35-55} peptide (100 μg) to detect putatively higher susceptibilities for the disease, especially because PD-1 is known to act mainly under submaximal stimulatory conditions.

Mice with a combined pathology (PLPtg/PD-$1^{-/-}$ mice) had an earlier onset of disease, as reflected by the disease course, the relative number of normal appearing mice after immunization, and the mean day of onset (Table 1), which was significant in all experiments compared with wild-type mice. In addition, assessment of the EAE score always revealed a significantly more pronounced disease onset in PLPtg/PD-$1^{-/-}$ double mutants compared with the wild-type control group (Table 1; Figure 1, A and B).

Table 1. Significantly Earlier Onset of Disease in PLPtg/PD-$1^{-/-}$ Mice

Group	Mean day of onset*	Mean maximal score†	Median‡
wt	13.3 ± 2.2	2.9 ± 0.3	3.0
PD-$1^{-/-}$	12.4 ± 1.7	3.6 ± 1.1	4.0
PLPtg	11.8 ± 2.6	2.0 ± 1.2	2.5
PLPtg/PD-$1^{-/-}$	10.6 ± 0.8§**	2.6 ± 0.0	2.5
wt	17.0 ± 3.3	2.8 ± 0.5	2.0
PD-$1^{-/-}$	12.5 ± 2.6§**	3.3 ± 0.9	3.0
PLPtg	14.0 ± 3.1	2.6 ± 0.4	2.5
PLPtg/PD-$1^{-/-}$	10.5 ± 2.3§††	2.8 ± 0.3	3.0
wt	13.3 ± 2.3	2.1 ± 1.2	2.5
PD-$1^{-/-}$	11.5 ± 1.1	1.9 ± 1.1	2.5
PLPtg	13.3 ± 2.3	1.7 ± 1.1	2.5
PLPtg/PD-$1^{-/-}$	10.9 ± 1.8§¶**	2.8 ± 0.4¶‖**	3.0
wt	12.5 ± 0.6	1.1 ± 1.0	1.0
PD-$1^{-/-}$	10.6 ± 1.8	2.9 ± 0.4§**	3.0
PLPtg	11.2 ± 1.3	3.0 ± 0.5§**	3.0
PLPtg/PD-$1^{-/-}$	10.6 ± 0.9§††	3.4 ± 0.2§‖‡‡	3.5
wt	12.8 ± 1.6	0.8 ± 0.6	1.0
PD-$1^{-/-}$	11.3 ± 2.2	2.2 ± 0.8§‡‡	2.5
PLPtg	11.2 ± 2.4	1.7 ± 0.6§‡‡	1.5
PLPtg/PD-$1^{-/-}$	10.6 ± 2.1§††	2.3 ± 0.8§‡‡	2.5
wt	13.3 ± 1.5	1.8 ± 1.4	1.8
PD-$1^{-/-}$	12.8 ± 2.1	2.0 ± 1.1	2.0
PLPtg	13.0 ± 1.0	1.0 ± 0.6	1.0
PLPtg/PD-$1^{-/-}$	11.6 ± 0.6§¶‖**	2.7 ± 0.6‖††	2.5
wt	12.4 ± 1.5	1.7 ± 1.4	2.0
PD-$1^{-/-}$	12.4 ± 1.4	2.6 ± 1.2	3.3
PLPtg	11.3 ± 1.7	1.9 ± 0.9	2.3
PLPtg/PD-$1^{-/-}$	9.5 ± 1.2§¶‖†††‡‡	3.4 ± 0.8§‖††	3.5

*Mean day of disease onset was calculated only for the mice that developed EAE during the time of investigation. Note significantly earlier onset of disease in PLPtg/PD-$1^{-/-}$ mice compared with wild-type (wt) mice. Mean maximal score (†) and median (‡) were calculated for all mice in the group.

§Significant differences compared with wild-type mice (Student's *t*-test).

¶Significant differences compared with PD-$1^{-/-}$ mice.

‖Significant differences compared with PLPtg mice. Data represent mean ± SD, n = 3 to 8 per group.

P values ≤0.05 were considered significant,**P value <0.05, ††P value ≤0.01, ‡‡P value ≤0.001.

PD-$1^{-/-}$ mice usually showed a similar disease course like PLPtg/PD-$1^{-/-}$ mice, but displayed much broader interindividual variations with a slightly delayed disease onset compared with double mutants. Initially, the slope of the disease curve developed similarly in wild-type and PLPtg mice. After ~15 to 20 days, all groups exhibited comparable disease scores (Table 1, Figure 1A) with slightly higher levels in the absence of PD-1. Nevertheless, when comparing the mean maximal score, in five of seven experiments, PLPtg/PD-$1^{-/-}$ mice showed a significantly higher mean maximal score. Disease incidence and mortality rate, which was generally low, did not differ significantly between the groups (Table 1). Taken together, PLPtg/PD-$1^{-/-}$ mice developed disease significantly earlier and reached a higher mean maximal score compared with wild-type mice.

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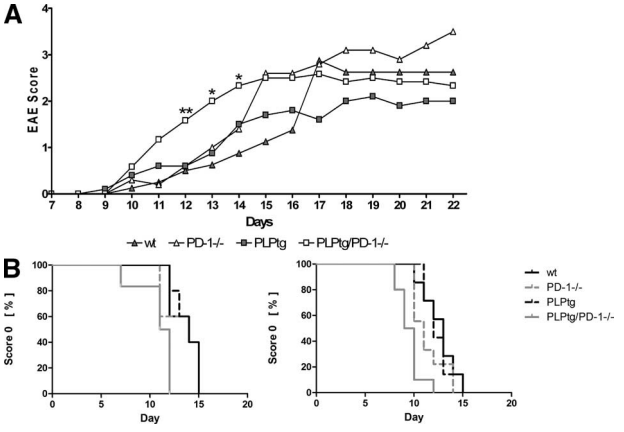

Figure 1. PLPtg/PD-1$^{-/-}$ mice show earlier and more pronounced onset of EAE. **A:** Wild-type (wt), PD-1$^{-/-}$, PLPtg, and PLPtg/PD-1$^{-/-}$ mice were immunized with MOG_{35-55}. PLPtg/PD-1$^{-/-}$ show a significantly earlier onset and stronger disease course compared with wild-type mice. At later time points, EAE disease scores are similar in all groups, as reflected by lack of statistical significance. The figure shows one representative of seven experiments. $^{*}P < 0.05$, $^{**}P = 0.01$. $n = 5$ mice per group. **B:** Relative number of mice displaying no clinical alterations (score 0) after immunization. These two representative experiments clearly reflect early onset disease in PLPtg/PD-1$^{-/-}$ mice ($n = 5$ mice per group).

Absence of PD-1 and Overexpression of PLP Is Associated with Increased Numbers of Immune Cells in the CNS

To quantify lymphocytes and macrophage-like cells in neural tissue, immunohistochemistry for CD4, CD8, and CD11b was performed on cross sections of the lumbar spinal cord and longitudinal sections of the optic nerve during EAE. CD4^{+} cells were significantly more frequent in spinal cords of PLPtg/PD-1$^{-/-}$ mice compared with wild-type mice. PLPtg and PD-1$^{-/-}$ mice both showed higher amounts than wild-type mice, but differences were not statistically significant (Figure 2A). A similar trend lacking statistical significance was detectable in optic nerves (Figure 2B). In contrast, CD8^{+} lymphocytes were significantly elevated both in spinal cord (Figure 2C) and optic nerve (Figure 2D) in PLPtg/PD-1$^{-/-}$ mice compared with wild-type mice. Regarding the spinal cord, PLPtg and PD-1$^{-/-}$ single mutants showed a stronger accumulation of cells than wild types. CD11b^{+} macrophages were also significantly more frequent in spinal cords of PLPtg/PD-1$^{-/-}$ mice compared with wild-type mice (Figure 2, E and F), which was neither detectable in PLPtg and PD-1$^{-/-}$ mice nor in optic nerves of all groups (Figure 2G). Healthy mice which were investigated at the same age (2 months) did not show significant differences between the genotypes for CD4^{+} (Figure 2H) and CD8^{+} (Figure 2I) lymphocytes and CD11b^{+} macrophage-like cells (Figure 2J). Taken together, combined pathology is associated with higher numbers of CD4^{+} and CD8^{+} T cells and higher numbers of macrophages in the spinal cord of EAE mice.

Double-Mutant PLPtg/PD-1$^{-/-}$ Mice Exhibit Highest Axonal Loss during EAE

To determine whether differences in the CNS-infiltrating T cells and macrophages in double mutants were associated with more pronounced axonal damage we performed quantitative immunohistochemistry against phosphorylated and nonphosphorylated neurofilament in the cervical spinal cord 50 days after induction of EAE. A significant reduction of total neurofilament was observed in the absence of PD-1 (independent from the absence or presence of the PLP transgene) in the ventral, lateral, and dorsal funiculus and in the corticospinal tract. Interestingly, the amount of defective, nonphosphorylated neurofilament was highest in PLPtg/PD-1$^{-/-}$ mice, which was significant compared with wild-type mice in all investigated areas and for the dorsal funiculus and in the corticospinal tract compared with PD-1$^{-/-}$ and PLPtg mice, thereby reliably reflecting the clinical score in these mice (Figure 3, A and B).

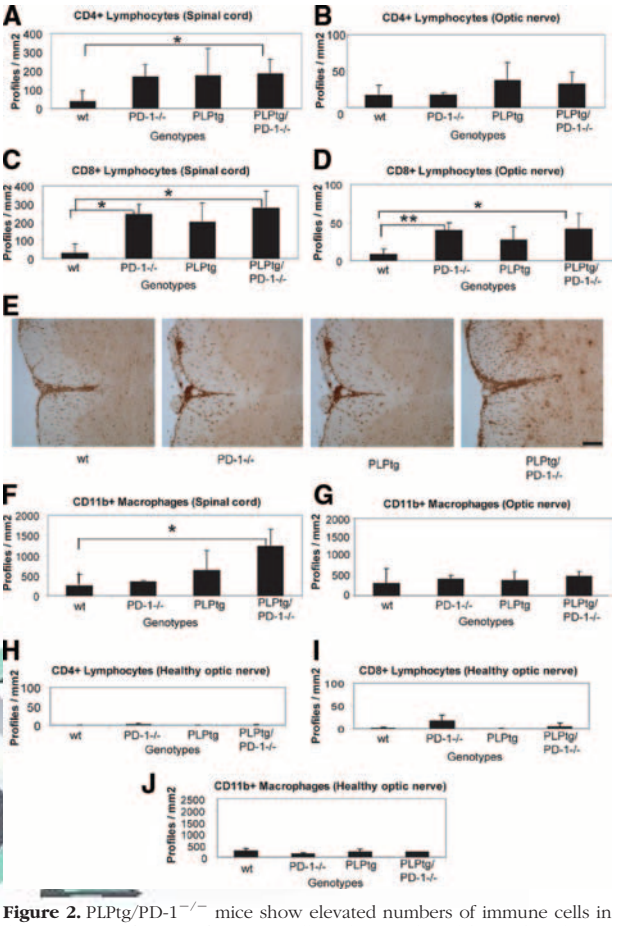

Figure 2. PLPtg/PD-1$^{-/-}$ mice show elevated numbers of immune cells in the CNS. Quantification of immune cells in the spinal cord (**A**, **C**, **F**) and optic nerve (**B**, **D**, **G**) of wild-type (wt), PD-1$^{-/-}$, PLPtg, and PLPtg/PD-1$^{-/-}$ mice at the peak of disease ($n = 3$ to 5). Quantification includes CD4^{+} (**A**, **B**) and CD8^{+} (**C**, **D**) lymphocytes and CD11b^{+} macrophages (**E–G**). **F:** Representative CD11b immunohistochemistry on cross-sections of the lumber spinal cord. **H–J:** Quantification of CD4^{+} (**H**) and CD8^{+} (**I**) lymphocytes and CD11b^{+} macrophage-like cells (**J**) in healthy mice of the same age. Error bars represent standard deviations. $^{*}P < 0.05$, $^{**}P \leq 0.01$. Scale bar = 200 μm.

Absence of PD-1 Is Associated with Higher Production of IFN-γ by Splenocytes during the Early Phase of Disease

We next investigated whether differences in the production of proinflammatory cytokines were associated or re-

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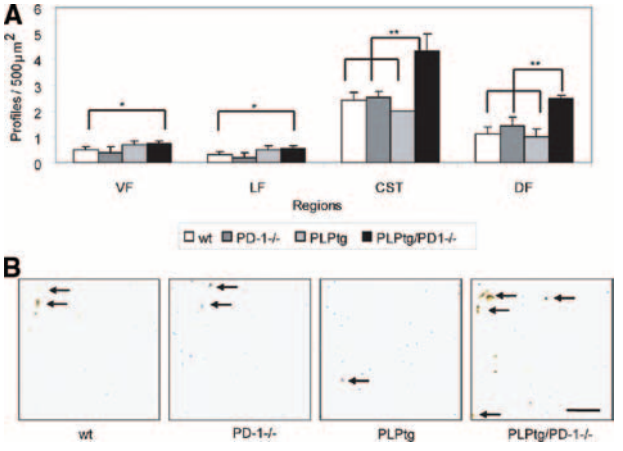

Figure 3. PLPtg/PD-1$^{-/-}$ mice show elevated numbers of dephosphorylated neurofilament in the spinal cord. **A:** Quantification of nonphosphorylated neurofilament in the cervical spinal cord of wild-type (wt), PD-1, PLPtg, and PLPtg/PD-1$^{-/-}$ mice (n = 3 to 4). Investigated areas include ventral funiculus (VF), lateral funiculus (LF), corticospinal tract (CST), and dorsal funiculus (DF). **B:** Representative examples of SMI32^{+} profiles. Error bars represent standard deviations. $^{*}P < 0.05$, $^{**}P \leq 0.01$. Scale bar = 10 μm.

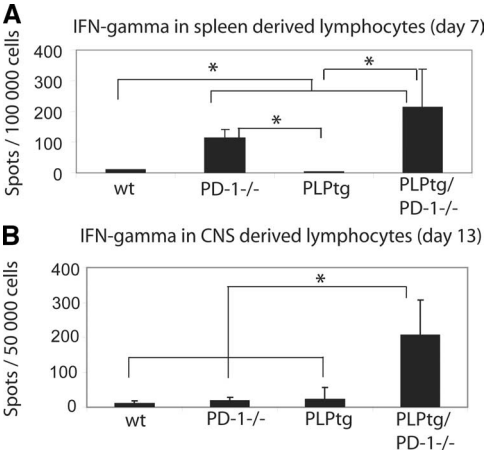

Figure 4. CNS-derived cells from PLPtg/PD-1$^{-/-}$ mice produce higher amounts of IFN-γ^{+} cells at the peak of disease. IFN-γ ELISPOT assays were performed on splenocytes (**A**) and CNS-derived lymphocytes (**B**) under restimulation with MOG_{35-55}. **A:** Higher amounts of IFN-γ^{+} cells were detected in PD-1$^{-/-}$ and PLPtg/PD-1$^{-/-}$ mice in the early phase of EAE (day 7). **B:** IFN-γ^{+} cells are significantly more frequent in PLPtg/PD-1$^{-/-}$ at the peak of disease (day 13). In these experiments, lymphocytes were pooled from three mice per group. $^{*}P < 0.05$.

sponsible for the observed differences. IFN-γ was measured as an indicator cytokine strongly regulated during EAE and closely correlated to CNS damage.[34] To first elucidate if PD-1 influences the secretion of IFN-γ during EAE, we investigated the IFN-γ levels by ELISA in the supernatant of splenocytes from wild-type and PD-1$^{-/-}$ mice at different time points of disease. At day 7 after immunization, before the actual onset of disease, significantly more IFN-γ was secreted by PD-1$^{-/-}$ splenocytes after re-stimulation with MOG_{35-55} peptide (see Supplemental Figure S1A at *http://ajp.amjpathol.org*).

At the peak of disease (day 13), there was still a trend toward a higher production of IFN-γ by PD-1$^{-/-}$ splenocytes and the amounts of secreted IFN-γ had substantially increased, but this was no longer significant (see Supplemental Figure S1B, see *http://ajp.amjpathol.org*). In the late phase of the disease (day 17), PD-1$^{-/-}$ splenocytes did not produce detectable levels of IFN-γ and also the amount in wild-type splenocytes dropped to levels comparable with day 7 (see Supplemental Figure S1C, see *http://ajp.amjpathol.org*).

Double-Mutant PLPtg/PD-1$^{-/-}$ Mice Show Increased Production of IFN-γ in the CNS at the Peak of Disease

To investigate the production of IFN-γ in detail, we harvested lymphocytes from the CNS, cervical lymph nodes, and the spleen at various time points after immunization in the different genotypes. Moreover, we used a highly sensitive Elispot assay. In the early phase of disease (day 7), when stimulated with MOG_{35-55}, IFN-γ production was significantly elevated in splenocytes of PD-1$^{-/-}$ and PLPtg/PD-1$^{-/-}$ mice compared with wild-type and PLPtg mice (Figure 4A). At later phases of disease (day 15 after immunization), splenocytes of all groups exhibited a strong IFN-γ response when stimulated with MOG_{35-55}, which did not differ significantly between the groups (data not shown).

A delayed pattern of IFN-γ production could be observed in lymphocytes derived from cervical lymph nodes restimulated with MOG_{35-55}. At day 7, only low amounts of cells produced IFN-γ and the amount of cells did not differ between the genotypes. After the onset of disease (day 10 after immunization) the IFN-γ production was primarily increased. At that time point, PLPtg/PD-1$^{-/-}$ mice show significantly more IFN-γ^{+} cells than wild-type mice. Later, at day 15 after immunization, PLPtg/PD-1$^{-/-}$ mice still produce significantly more IFN-γ than wild-type and PLPtg mice but the total number of positive cells was reduced to very low levels (data not shown).

Interestingly, at the peak of disease (day 13), the amount of IFN-γ^{+} cells on MOG_{35-55} stimulation in CNS-derived lymphocytes was significantly higher in cells from PLPtg/PD-1$^{-/-}$ mice than in all other groups (Figure 4B), which might reflect the highest maximal score in these mutants. In the later phase of the disease, the IFN-γ production in the CNS was reduced in double mutants while it increased in the other genotypes, leading to a significantly higher amount in wild-type and PD-1$^{-/-}$ cells compared with PLPtg/PD-1$^{-/-}$ (data not shown).

These data indicate that absence of PD-1 is associated with higher peripheral MOG-specific IFN-γ secretion. This systemic difference is only observable in the preclinical phase of EAE. However, combined pathology is associated with a significantly higher secretion of IFN-γ by CNS-MOG-specific T cells at peak of disease.

Absence of PD-1$^{-/-}$ or Oligodendropathy Does Not Lead to Detectable Spread of Antigenic MHC Class I or Class II Restricted Epitopes during EAE

We reasoned whether EAE in PLPtg mice or in PD-1$^{-/-}$ mice would be associated with early epitope spreading

against various CNS antigens, either restricted by MHC class I or MHC class II molecules. This has been elegantly shown in several models of EAE, especially in SJL mice immunized with PLP peptides.[35] We therefore tested animals at the peak of disease for reactivity against a number of MHC class II-related myelin peptides ($MOG_{113-127}$, $MOG_{120-134}$, $MOG_{183-197}$, MBP_{54-72}, $PLP_{178-191}$).[36] However, no relevant MHC class II restricted responses against other myelin peptides than MOG_{35-55} were detectable (data not shown). Because the pathogenic inflammatory response in PLPtg mice is mediated by $CD8^+$ T cells[11,12] we also tested for responses after antigenic stimulation with a number of MHC class I myelin peptides mentioned in the Materials and Methods.[12] However, no responses were measured (data not shown).

Absence of PD-1 Increases Clonal Expansions of T Cells during EAE

To assess the T-cell receptor repertoire during MOG EAE in BL/6 wild-type, PD-$1^{-/-}$, PLPtg, and PLPtg/PD-$1^{-/-}$ mice, we used CDR3 spectratyping analysis on CNS- and spleen-derived lymphocytes.[12] This PCR-based method allows detection of mono- and oligoclonal expansions of T cells in different tissues.

CNS-derived lymphocytes were assessed for clonal expansions and distributions ($n = 3$ mice per group). In wild-type mice peak numbers of 8 ± 1.7 were detected. This was similar to the number found in EAE of PLPtg mice (9.3 ± 4.7). Absence of PD-1 clearly increased the propensity of T lymphocytes to expand: In PD-$1^{-/-}$ mice, 17 ± 7.8 peaks were detected compared with 15.3 ± 2.5 in PLPtg/PD-$1^{-/-}$ double mutants. When applying statistical analysis to peak numbers, a significant difference was only detectable between wild-type and PLPtg/PD-$1^{-/-}$ mice ($P = 0.01$), which was attributable to high variations in PD-$1^{-/-}$ mice and again a sign of very homogeneous disease in the double mutants. Additionally, the number of clonal expansions reliably reflected the severity of the EAE disease course.

In other mouse strains (eg, B10.PL), it has been shown that so called public clones, which are T cells with the same Vβ, Jβ, CDR3 length and the same or similar CDR3 amino acid sequence, which are present in each animal, are responsible for driving EAE.[37] Our analysis firstly describing CDR3 spectratyping in MOG_{35-55}-induced EAE in C57/B6 mice revealed a higher variability of clonal expansions in different mice.

Here, the expansions clustered in several Vβ regions: Vβ 1: 23%; Vβ 5.2: 8%; Vβ 10: 16%; Vβ 11: 22%; Vβ 16: 9%; of all observed expansions, meaning that 5 Vβ regions (of 24) included 78% of all expansions (Figure 5). F5 However, no defined Vβ/Jβ expansion was present in all analyzed animals. In examples of Vβ/Jβ combinations, which could be found in several animals (eg, Vβ1/Jβ2.5, found in 7 of 12 animals), the CDR3 length varied slightly and proved the difference on the clonal level. Interestingly, we found the Vβ/Jβ combination (Vβ11/Jβ1.1) of the MOG-specific 2D2 T cells[38] in the CNS of 5 of 12 animals. However, the CDR3 length of these clones was not identical, suggesting differences in the antigen specificity. When compared with the spleen TCR repertoire, it became evident that the vast majority of peaks were CNS-restricted. The corresponding spleen spectratypes also always showed peaks, but the peak number was ~20% of that in the CNS (data not shown).

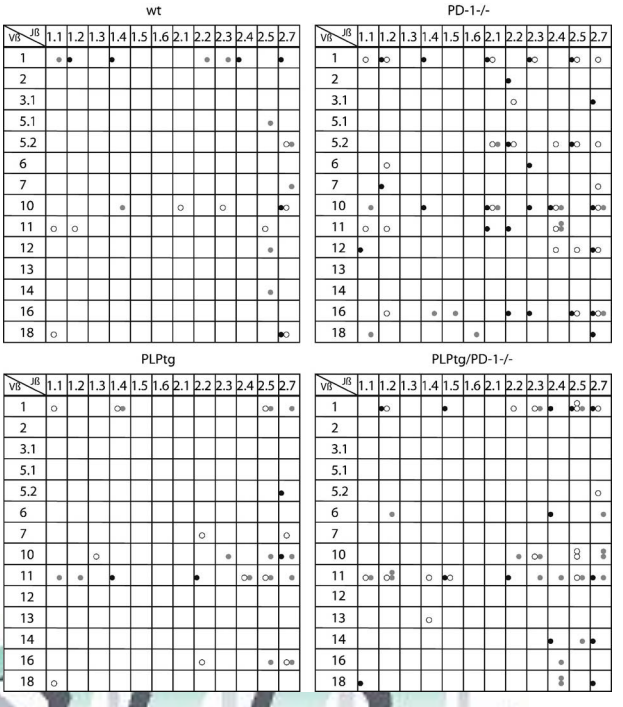

Figure 5. Spectratyping analysis of T-cell receptor in CNS-derived lymphocytes. The T-cell receptor repertoire of lymphocytes from the CNS of wild-type, PD-$1^{-/-}$, PLPtg, and PLPtg/PD-$1^{-/-}$ ($n = 3$) mice in the late phase of EAE (day 18) was analyzed by spectratyping. Clonal expansions (visible as single peaks in the fragment analysis) are shown as dots (different gray scales indicate individual animals). The expanded T cells are characterized by their Vβ- and Jβ-chains (Vβ, rows; Jβ, columns). The clonal expansions occurred widely distributed over different Vβ and Jβ regions, although some domains seemed to be prone for clonal expansions in different mutant mice.

Discussion

We here investigated how genetic predisposition for myelin damage (PLPtg overexpression) combined with aberrant T-cell regulation (PD-1 deficiency) influence the onset and course of an experimental autoimmune CNS disease (MOG_{35-55}-induced EAE). We observed that double pathology was associated with a significantly earlier onset of EAE, a slight increase in the neurological score, an increase of infiltrating cells, and enhanced neuronal degeneration compared with wild-type mice. Thus PLP overexpression and PD-1 deficiency have a confounding effect on CNS inflammation. The double mutants also displayed an earlier onset in comparison with PD-$1^{-/-}$ and PLPtg single mutants (Figure 1B), but because of high individual variability of the single mutants, the corresponding differences failed to reach statistical significance.

The less homogenous disease onset and course in PD-$1^{-/-}$ single mutants and the lack of statistically significant differences compared with wild-type mice might

be caused by the low dose of antigen we used to induce the disease. Under these conditions, only the presence of two predisposition factors led to homogenous disease course. Most likely, at least two different disease mechanisms were acting in the immunized double mutants. First, the underlying primary oligodendrocyte damage might have induced an enhanced vulnerability and, possibly, tissue instability. Second, the absence of the co-inhibitory molecule PD-1 induced a stronger inflammatory stimulus, and the combination of both culprits resulted in the accelerated, stable, homogenous exacerbation of disease. Importantly, the combination of the two factors without EAE induction already led to an aggravation of myelin and axon damage[24] but at a much later time point. It is tempting to speculate that, in the young double mutants, a not yet visible predisposition for severe inflammatory disease progression already exists and becomes triggered by EAE induction. It is of note that the earlier and stronger onset of disease is followed by a parallel disease course of all groups and absence of significant differences. Nevertheless, the mean maximal score was significantly higher in PLPtg/PD-1$^{-/-}$ double mutants compared with wild-type mice in the vast majority of experiments.

The significance of combined pathology is reflected not only by the disease course, which is most prominent in the double mutants, but also by the accumulation of CD4$^+$ and CD8$^+$ lymphocytes and CD11b$^+$ macrophages in the CNS. Here, the highest amount is always detected in double mutants, whereas the presence of one single pathological feature (PLPtg or PD-1$^{-/-}$) is sufficient to induce increased cell numbers compared with the wild-type level.

In this context, it is interesting that the demyelination in PLPtg mice is clearly mediated by CD8$^+$ lymphocytes,[11] whereas EAE is mainly depending on CD4$^+$ lymphocytes. It is therefore plausible to assume that the increased cell numbers in PLPtg compared with wild-type mice are mainly triggered by the underlying oligodendrocyte damage. Recent studies have shown a predominance of CD8$^+$ T lymphocytes in MS lesions.[39] The fact that EAE is predominantly mediated by CD4$^+$ T lymphocytes supports the view that a direct comparison with MS is a limited possibility.[40] It would, therefore, be challenging to perform similar experiments by using a CD8$^+$ T-lymphocyte-mediated MS model.

It was another important finding that the immune response in the investigated mice was always directed primarily against MOG$_{35-55}$, which had been used for immunization, and we could not detect any reactivity toward other myelin peptides. This does not entirely exclude the presence of epitope spreading, but at least none of the predicted MHC class I or II peptides we investigated was recognized by CNS-infiltrating T cells within the experimental window.

Another important feature, which faithfully reflected the clinical phenotype of the mice, was the amount of non-phosphorylated neurofilament (reflecting axon injury[41]), which was highest in PLPtg/PD-1$^{-/-}$ mice and resembles enhanced neuronal damage in the presence of mice with double pathology. The stronger susceptibility of PD-1-deficient immune cells to proliferate and produce cytokines was reflected not only by the higher amount of IFN-γ production by PD-1-deficient compared with wild-type splenocytes but also by the increased number of IFN-γ producing CNS cells in ELISPOT assays in PLPtg/PD-1$^{-/-}$ mice. Disruption of the PD-1/PD-L1 pathway may therefore be a critical event for the production of IFN-γ within the CNS, as it is for the disease score in EAE.[21,22,29]

The strong influence of PD-1 on T-cell homeostasis is also reflected by the number of peaks in our clonality analysis (spectratyping analysis). Oligoclonal expansions, which can account for strong immune activation against a specific antigen, were present in all investigated mice and genotypes. We have shown in previous experiments that healthy 12-month-old wild-type mice do not display any clonal expansions neither in spleen nor in CNS-derived lymphocytes whereas PLPtg mice always showed one Vβ expansion, which differed in size and location.[12]

When investigating healthy 12-month-old PD-1$^{-/-}$ and PLPtg/PD-1$^{-/-}$ mice, we always detected higher numbers of peaks,[6–9] indicating oligoclonal expansions, but no significant differences between these two groups.[24] To our knowledge, this is the first study describing clonal expansions in BL/6 mice during EAE. Interestingly, there was no difference in the number of oligoclonal expansions between PLPtg and wild-type mice, which both displayed approximately eight to nine oligoclonal expansions, whereas in the absence of PD-1, independent from an additional myelin mutation, the number was approximately twice as high (17 and 15, respectively). In line with our results describing the homogeneous disease course in PLPtg/PD-1$^{-/-}$ mice, these mice displayed only a very limited variability of different Vβ/Jβ types in the spectratyping analysis. Therefore one might speculate that the immunological and pathological response gets focused or stabilized in the presence of a factor predisposing for CNS vulnerability (PLPtg) in addition to factors impairing T-cell homeostasis (lack of PD-1). We suggest that transgenic overexpression of myelin molecules in PLP transgenic mice thus amplified and focused the CNS-directed immune response observed after immunization with MOG. This assumption is supported by the highly altered clonal expansion patterns observed in mice bearing a double pathology in comparison with PD-1-deficient mice alone. Of note, neither deficiency of PD-1, nor the combination with PLPtg influenced epitope restriction or promoted epitope spreading under the given experimental conditions.

This indicates that under the dominant immune stimulus of EAE induction, subtle differences (myelin) are no longer visible while a general immune dysregulation can enhance the severity of disease and immune reaction. Nevertheless, it is interesting that—as opposed to B10.PL mice[37]—the reaction toward a common antigen in BL/6 mice results in diverse and not identical clonal expansions.

Taken together, investigating the course and the immunopathogenetic features of MOG$_{35-55}$-induced EAE in a model combining both oligodendrocyte-related myeli-

nopathy and PD-1-dependent alteration of immune responses can provide interesting insights in the modulation of autoimmune diseases. Our data suggest that the interplay between immune dysregulation and myelinopathy results in a stable exacerbation of the disease. The PD-1$^{-/-}$ mutation alone led to a stronger exacerbation of EAE than in wild-type mice, but was characterized by a striking clinical and pathological variability within the group. This variability was not seen in mice bearing double pathology. Our observation might be of clinical relevance in that the combination of several subclinical pathological alterations (eg, CNS vulnerability, immune dysregulation, induced autoimmunity) may significantly contribute to disease susceptibility or relapses.

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The co-inhibitory molecule PD-1 modulates disease severity in a model for an inherited, demyelinating neuropathy

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ABSTRACT

We have previously shown that mice heterozygously deficient for P0 are characterized by a late onset myelin disorder implicating CD8+ T-lymphocytes and macrophages. We now investigated the impact of the co-inhibitory molecule "programmed death" (PD)-1 (CD279), a CD28-related receptor expressed on activated T- and B-lymphocytes on the pathogenic phenotype of CD8+ T-lymphocytes in the P0 myelin mutants. PD-1 deficiency in P0+/− mice leads to a stronger increase of CD8+ T-lymphocytes and a substantially aggravated histological phenotype in the PNS compared to P0+/− mice expressing PD-1. Correspondingly, functional down-stream features, such as electrophysiological parameters, walking coordination and mechano-sensation are more affected than in PD-1-expressing myelin mutants. Our study demonstrates that a monogenic nerve disorder can be substantially modified by immune-controlling mechanisms. Thus, understanding the implication of disease-modifiers in inherited demyelination could be of pivotal interest for limiting the detrimental impact of primarily genetically-mediated myelin disorders by fostering immuno-regulatory pathways.

Introduction

Inherited peripheral neuropathies form a genetically heterogeneous group of peripheral nerve disorders leading to muscle wasting, sensory dysfunction, progressive disability and, thus, to a substantial reduction of quality of life. Presently, there are no approved therapeutics for causative treatment of the human disorders. Mutations in several myelin genes, peripheral myelin protein (PMP) 22, myelin protein zero (P0/MPZ), the gap junction component connexin 32 (Cx32/GBJ-1) and periaxin, were identified as being responsible for the most frequent forms showing a predominantly demyelinating phenotype (Berger et al., 2006; Niemann et al., 2006). In spite of the substantial progress in the knowledge of the underlying gene mutations, the exact demyelinating mechanisms are only partially understood in most cases. They may comprise impaired stability of protein interactions among myelin components, the impaired association of Schwann cell molecules with extracellular matrix components, as well as different intracellular pathways of Schwann cell stress and injury (Berger et al., 2006; Niemann et al., 2006; Pennuto et al., 2008). Another possibility is the implication of the immune system in the demyelinating process, as has been previously shown in knock out mice heterozygously deficient for P0 (P0+/− mice) or homo/hemizygously deficient for Cx32 (Ip et al., 2006b). The significant impact of immune cells could unequivocally be demonstrated by cross breeding the myelin mutants with RAG-1-deficient mice which lack T- and B-lymphocytes resulting in lymphocyte-deficient myelin mutants with a substantially ameliorated phenotype (Kobsar et al., 2003; Schmid et al., 2000). Moreover, bone marrow reconstitution of the immune-deficient double mutants with wildtype bone marrow resulted in equal phenotypes as in genuine P0+/− mice (Maurer et al., 2001).

In the present study, we investigated how the regulation of immune homeostasis affects phenotype and pathogenic T-lymphocytes in P0+/− mice. A candidate molecule which might be involved in this regulation and keep the pathogenetic impact of the T-lymphocytes limited in the myelin mutants, is the co-inhibitory molecule "programmed death" (PD)-1 (CD279), a CD28-related receptor expressed on activated T- and B-lymphocytes (Okazaki and Honjo, 2007; Okazaki et al., 2002). PD-1 can react with two distinct ligands, PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273).While PD-L1 is expressed on a broad variety of cells, including lymphocytes, antigen-presenting cells and tissue cells like pancreatic islet cells (Keir et al., 2006), endothelial cells (Grabie et al., 2007) or microglial cells (Magnus et al., 2005), PD-L2 was mainly detected on dendritic cells (Latchman et al., 2001).

Reflecting the established inhibitory function of PD-1, the clinical and pathological outcome of some autoimmune disorder models, such as autoimmune diabetes or experimental autoimmune encephalomyelitis,

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are substantially aggravated in the absence of or by pharmacological modulation of PD-1 or the PD-L1/PD-1 pathway (Carter et al., 2007; Fife et al., 2006; Keir et al., 2006; Okazaki and Honjo, 2007; Salama et al., 2003; Ortler et al., 2008).

Most interestingly, we could recently show that the absence of PD-1 leads to an exacerbation of genetically-mediated demyelination in a CNS mutant overexpressing the major oligodendrocytic myelin component proteolipid protein (PLP; Kroner et al., submitted).

Our present work demonstrates the substantial impact of PD-1 in an established model for some forms of inherited peripheral neuropathies of the Charcot–Marie–Tooth (CMT) type 1, therefore highlighting the relevance of immune-homeostasis signals for the tissue damage and the clinical phenotype in this disorder.

Materials and methods

Animals and determination of genotypes

Mice heterozygously deficient for P0 (P0+/− mice, on a C57/Bl6 background; Giese et al., 1992) were bred and genotyped by PCR reaction as previously described (Schmid et al., 2000). Shortly, genotyping was performed using the oligonucleotides 5′-TCAGTTCCTTGTCCCCCGCTCTC-3′, 5′-GGCTGCAGGGTCGCTCGGTGTTC-3′ and 5′-ACTTGTCTCTTCTGGGTAATCAA-3′ leading to a 334 bp product for the P0 null allele and 500 bp for the wildtype allele, respectively (Schmid et al., 2000). Animals were kept under barrier conditions in the animal facility of the Department of Neurology. All animal experiments were approved by the Regierung von Unterfranken. All investigated mice were on a C57 BL/6 background.

Absence of the recombinase activating gene-1 (RAG-1) in P0+/−/RAG-1−/− double mutants was confirmed either by flow cytometric analysis of tail vein blood with CD4− and CD8− specific antibodies as previously described (Schmid et al., 2000; Kobsar et al., 2003; Ip et al., 2006a) or by PCR as provided by Jackson Lab (www.jax.org) using the primers 5′ TGGATGTGGAATGTGTGCGAG 3′, 5′ GAGGTTCCGCTACGACTCTG 3′ and 5′ CCGGACAAGTTTTTCATCGT 3′ with an annealing temperature of 58 °C and an elongation time of 45 s at 72 °C. On an agarose gel, the wildtype band could be detected at 474 bp and the knockout band was located at 530 bp. Absence of PD-1 in PD-1−/− mice (Nishimura et al., 1998) was also confirmed by PCR genotyping. The wildtype allele was amplified using the primer pair 5′ CCGCCTTCTGTAATGGTTTG 3′ and 5′ TGTTGAGCAGAAGACAGCTAGG 3′ with an annealing temperature of 54 °C and an elongation time of 45 s, the knockout allele was detected using the primer pair 5′ GCCCGGTTCTTTTTGTCAAGACCGA 3′ and 5′ ATCCTCGCCGTCGGGCATGCGCGCC 3′ with an annealing temperature of 60 °C and an elongation time of 45 s. PCR products were visualized on an agarose gel with the wildtype allele at 690 bp and the knockout allele at 400 bp.

Bone marrow chimerization

PD-1−/− or wildtype bone marrow was transplanted into P0+/−/RAG-1−/− recipients with P0+/+/RAG-1−/− as controls. Transplantation was performed as described before (Maurer et al., 2001; Ip et al., 2006a). Briefly, bone marrow from donor mice was isolated by flushing out femoral bone marrow with sterile PBS, washed and filtered through a mesh with 70 μm pore size to remove debris. Afterwards, cells were resuspended and approximately 2×10^7 cells were injected intravenously into recipient mice. Successful transplantation was confirmed by flow cytometry for CD4 and CD8 as described for definition of the RAG-1−/− status.

Tissue preparation and immunohistochemistry

After transcardial perfusion with 0.1 M phosphate buffered saline (PBS), femoral nerves were obtained and snap frozen immediately after dissection. 10 μm thick transverse sections of respective tissues were cut. Immunohistochemical stainings were performed as described before (Carenini et al., 2001; Ip et al., 2006a; Ip et al., 2007). Macrophages were detected using rat–anti mouse F4/80 antibody (Serotec, Oxford, UK). Rat–anti mouse CD4 (Serotec, Oxford, UK) and rat–anti mouse CD8 (Chemicon, Temecula, CA, USA) antibodies were used for the identification of T-lymphocytes. After rinsing with PBS, detection of primary antibodies was achieved by using a biotinylated secondary antibody to rat immunoglobulins (Igs) for 1 h. Colorimetric stainings were finalised with StreptABComplex kit (Dako, Hamburg, Germany) which contains horseradish peroxidase. Substrate visualization was performed using diaminobenzidine (DAB; 1 mg/ml) substituted with 0.02% H_2O_2.

Quantification of immune cells

Quantification of F4/80+ macrophages and CD4+ or CD8+ T-lymphocytes was performed as described before (Carenini et al., 2001; Fischer et al., 2008) on cross sections of the femoral nerve, comprising both quadriceps and saphenous branch. In general, quantification was performed by an investigator (A.K.) being blinded regarding the genotype of the investigated animals.

Assessment of demyelination and axonal damage

For the investigation of semithin or ultrathin sections, femoral and sciatic nerves and spinal roots were used. These tissues were processed for light microscopy of semithin sections as recently reported (Carenini et al., 2001; Ip et al., 2006a). Mice were transcardially perfused with 4% paraformaldehyde and 2% glutaraldehyde in 0.1 M cacodylate buffer. The tissue was postfixed in the same fixative overnight, followed by osmification with 2% osmiumtetroxide in 0.1 M cacodylate buffer for 2 h, dehydration in acetone and embedding in Spurr's medium. 0.5 μm thick semithin sections were cut for light microscopic analysis, and stained with alkaline methylene blue. Nerve damage was assessed by ranking nerves with regard of myelin abnormalities.

Electron microscopy

Ultrathin sections (100 nm) were contrasted with lead citrate and analysed using a ProScan Slow Scan CCD (Proscan, Lagerlechfeld, Germany) camera mounted to a Leo 906 E electron microscope (Zeiss, Oberkochen, Germany) and corresponding software iTEM (Olympus Soft Imaging Solutions, Muenster, Germany). All sections were analysed by an investigator unaware of the genotype.

Purification of splenocytes

Splenocytes were extracted as described before (Ip et al., 2006a) by passing the tissue through a 70 μm cell strainer (BD Biosciences Pharmingen, San Jose, CA, USA). After erythrocyte lysis cells were processed as indicated for the respective experiments.

Purification of PNS immune cells

Mice were killed with CO_2 and transcardially perfused with cold 0.1 M PBS. Sciatic, plantar and femoral nerves and spinal roots were dissected. PNS tissue was homogenized and cells were isolated from the interface of a 30 to 50% Percoll gradient. Mononuclear cells were washed, counted and used for subsequent experiments.

Flow cytometry

For flow cytometry, splenocytes and PNS derived lymphocytes were stained with the following directly labelled antibodies for 30 min

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at 4 °C in FACS buffer: CD4-PerCP, CD8-PE, CD62L-APC, CD44-FITC and CD69-FITC, PD-L1-PE or biotinylated PD-L2. In case of PD-L2, the antibody was visualized using Streptavidin-PE (all from BD Pharmingen, San Diego, CA, USA). Afterwards, cells were washed once, diluted in the desired volume and measured by flow cytometry (FACSCalibur, BD Biosciences Pharmingen, San Jose, CA USA). Specificity was assessed by application of isotype control antibodies. Analysis was performed using CellQuestPro software (BD Bioscience, San Diego, CA, USA) and FlowJo7 software (Tree Star Inc., Ashland, OR, USA).

IFN-γ ELISPOT assays

1×10^5 PNS lymphocytes or splenocytes were incubated for 24 h in provided 96-well-plates, unstimulated or stimulated with either phorbol myristate acetate (PMA, 20 ng/ml)/ionomycin (500 ng/ml, both Sigma, Munich, Germany). ELISPOT assays were performed according to the manufacturer's instructions (BD Pharmingen, San Diego, CA, USA). Spots were quantified by CTL Europe (Aalen, Germany) using ImmunoSpot 4.0.17.

Electrophysiology

Sciatic nerve conduction properties from P0+/−/RAG-1−/− mice which were either transplanted with wildtype or PD-1−/− bone marrow were determined at the age of 10 months by established electrophysiological methods as previously described (Samsam et al., 2003; Zielasek et al., 1996). Briefly, mice were anaesthetized and two electrodes were applied into the small foot muscles for motor response. For distal stimulation, two electrodes were placed behind the ankle and one more electrode pair was placed at the sciatic notch for proximal stimulation. Distal and proximal stimulation were performed with increasing strength until supramaximal stimulation with maximal motor response.

Behavioral tests

Sensitivity to mechanical stimuli was tested with von Frey hairs using the up-and-down method (Chaplan et al., 1994). Briefly, mice were placed on wire mesh in Plexiglas cages without bottom. When all four paws were placed on the wire, the plantar surface of one hind paw was touched with a von Frey monofilament. Hair values were beginning at 0.69 g, where pressure was administered for 5 s with a slightly bent filament. If the mouse reacted with withdrawal of the hind paw, the next finer von Frey hair was applied. If the animal did not react, the next stronger monofilament was used. The range of von Frey monofilament strength used was 0.07–1.20 g. Each hind paw was tested six times consecutively. The 50% withdrawal threshold (i.e., force of the von Frey hair to which an animal reacts in 50% of the administrations) was recorded as described before (Uceyler et al., 2006).

Subtle motor disturbances were investigated by gait studies as described previously (Kunkel-Bagden et al., 1993; Uceyler et al., 2006). For this, the hind paws were painted with ink and the mice were allowed to walk through a cardboard tunnel placed over two pieces of paper. Three consecutive footprint pairs were analysed regarding distinct gait parameters like stride width (distance between feet), stride length on the right and left sides, and limb rotation. The average values were calculated for each parameter. To rule out an influence of animal size, mice were weighed before the procedure.

Statistical analysis

The unpaired two-tailed students' *t*-test was used for comparison of quantified profiles. It may be used for parametric distributions. Scores and percentages were analysed by using the nonparametric Mann–Whitney-*U* test and the Kruskal–Wallis test.

Results

To analyse the pathogenetic impact of PD-1-deficiency in P0+/− mutants, we performed bone marrow chimerization experiments using wildtype (wt) or PD-1-deficient mutants (Nishimura et al., 1998) as donors and P0+/+ or P0+/− mice devoid of mature T- and B-lymphocytes (RAG-1−/− mice) as recipients. This approach enabled us to avoid irradiation of the recipients. Of note, PD-1 deficiency *per se* on a wildtype C57/BL6 background showed no alterations in the peripheral nerves. The resulting bone marrow chimeras (BMCs), P0+/−/RAG-1−/− BMC wt and P0+/−/RAG-1−/− BMC PD-1−/−, where investigated at the age of 10 months (8 months after transplantation). To control the impact of PD-1-deficiency in mice with normal myelin, we additionally investigated P0+/+/RAG-1−/− BMC PD-1−/− and P0+/+/RAG-1−/− BMC wt mice. In all transplanted mice investigated, the success in transplantation was controlled by flow cytometry of the peripheral blood and splenocytes of BMCs. Only individuals with normal amounts of CD4+ and CD8+ T-lymphocytes (approximately 10% and 12%, respectively, Maurer et al., 2001) were included in the study.

To detect which ligands of PD-1 were present in the investigated myelin mutants, we analysed splenocytes and immune cells pooled from peripheral nerves of one year old wildtype and P0+/− mice ($n=2$ per group) by flow cytometry. While PD-L1 was strongly expressed both in spleens and peripheral nerves, PD-L2 could not be detected, thus underlining its presence mainly described in dendritic cells (Latchman et al., 2001; Liang et al., 2003; see Supplementary Fig. 1).

CD8+ T-lymphocytes, but not macrophages, are significantly elevated in PD-1-deficient P0+/− bone marrow chimeras (BMCs)

We performed immunohistochemistry for F4/80+ macrophages, CD4+ and CD8+ T-lymphocytes as well as B-lymphocytes on frozen cross sections of the femoral nerve of myelin mutant and wt mice reconstituted with immune cells carrying PD-1 or not. Cell numbers in ventral roots were not quantified in this study but other experiments had shown similar amounts of immune cells in quadriceps nerves and ventral roots (unpublished data).

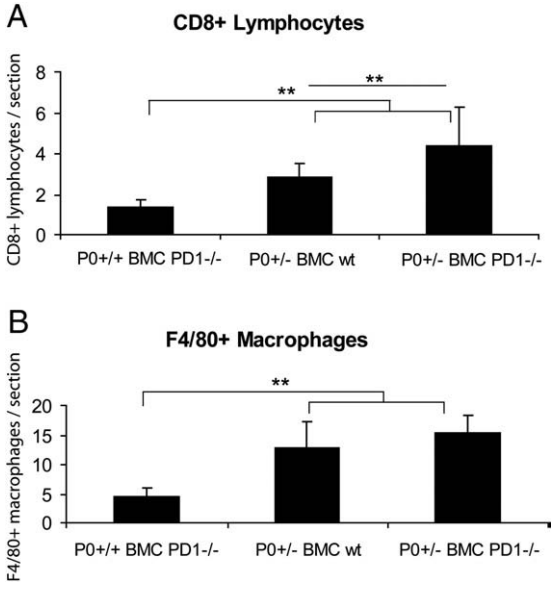

Fig. 1. PD-1-deficiency in P0+/−/RAG-1−/− BMCs leads to increased numbers of nerve T-lymphocytes, as revealed by quantification of CD8+ T-lymphocytes (A) and F4/80+ macrophages (B) in quadriceps femoral nerve of P0+/+/RAG-1−/− BMC PD-1−/− ($n=6$), P0+/−/RAG-1−/− BMC wt ($n=5$) or P0+/−/RAG-1−/− BMC PD-1−/− ($n=6$) mice. Note the significant increase of CD8+ cells in P0+/− compared to P0+/+ mice and the further increase in mice transplanted with PD-1−/− bone marrow. By contrast, PD-1-deficiency does not lead to a further increase in F4/80+ macrophages in the myelin mutants. Error bars represent standard deviation. *p-value ≤ 0.05, **p-value ≤ 0.01.

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CD8+ T-lymphocytes were elevated in the mainly motor part of the femoral nerve, the quadriceps nerve, in P0+/−/RAG-1−/− BMC wt mice compared to P0+/+/RAG-1−/− BMC (wt or PD-1−/−) mice ($p=0.002$), as expected from earlier investigations (Schmid et al., 2000). However, the absence of PD-1 in P0+/−/RAG-1−/− BMC PD-1−/− mice was associated with a significant increase of CD8+ cells compared to P0+/−/RAG-1−/− BMCs wt ($p=0.01$) (Fig. 1A). As reported previously, CD4+ T-lymphocytes were only scarcely present in the PNS sections and there were no significant differences between the wildtypes and both groups of transplanted myelin mutants (data not shown). Furthermore, as shown previously, B220+ B-lymphocytes were never detectable in peripheral nerves in either of the mice investigated (Schmid et al., 2000). F4/80+ macrophages were significantly elevated in the quadriceps nerve in P0+/−/RAG-1−/− BMC wt mice compared to P0+/+/RAG-1−/− BMC (wt or PD-1−/−) mice ($p=0.002$), as expected from earlier studies (Schmid et al., 2000). Neither in P0+/+/RAG-1−/− BMC nor in P0+/−/RAG-1−/− BMC the absence of PD-1 caused an increased number of macrophages (Fig. 1B). In the saphenous nerve, which is the mainly sensory part of the femoral nerve, macrophage numbers were not significantly altered in either of the groups investigated.

Remarkably, in one single P0+/−/RAG-1−/− BMC PD-1−/− mouse we detected abnormally high numbers of CD4+ (32 cells/section), CD8+ (41 cells/section) and F4/80+ (121 cells/section) profiles. Correspondingly, this animal also displayed robust myelin degeneration and axonopathic changes in the peripheral nerves. This is a unique example for how severe the inflammation can develop in the absence of PD-1 and, reciprocally, that only in the absence of PD-1 such extreme (inflammatory) phenotypes appear. Since this was a unique case, we excluded this mutant from statistical analysis (Supplementary Fig. 2).

Pathological features are enhanced in PD-1-deficient P0+/− BMCs

We investigated semithin sections of ventral roots, sciatic and femoral nerves of P0+/−/RAG-1−/− BMC PD-1−/− compared to P0+/−/

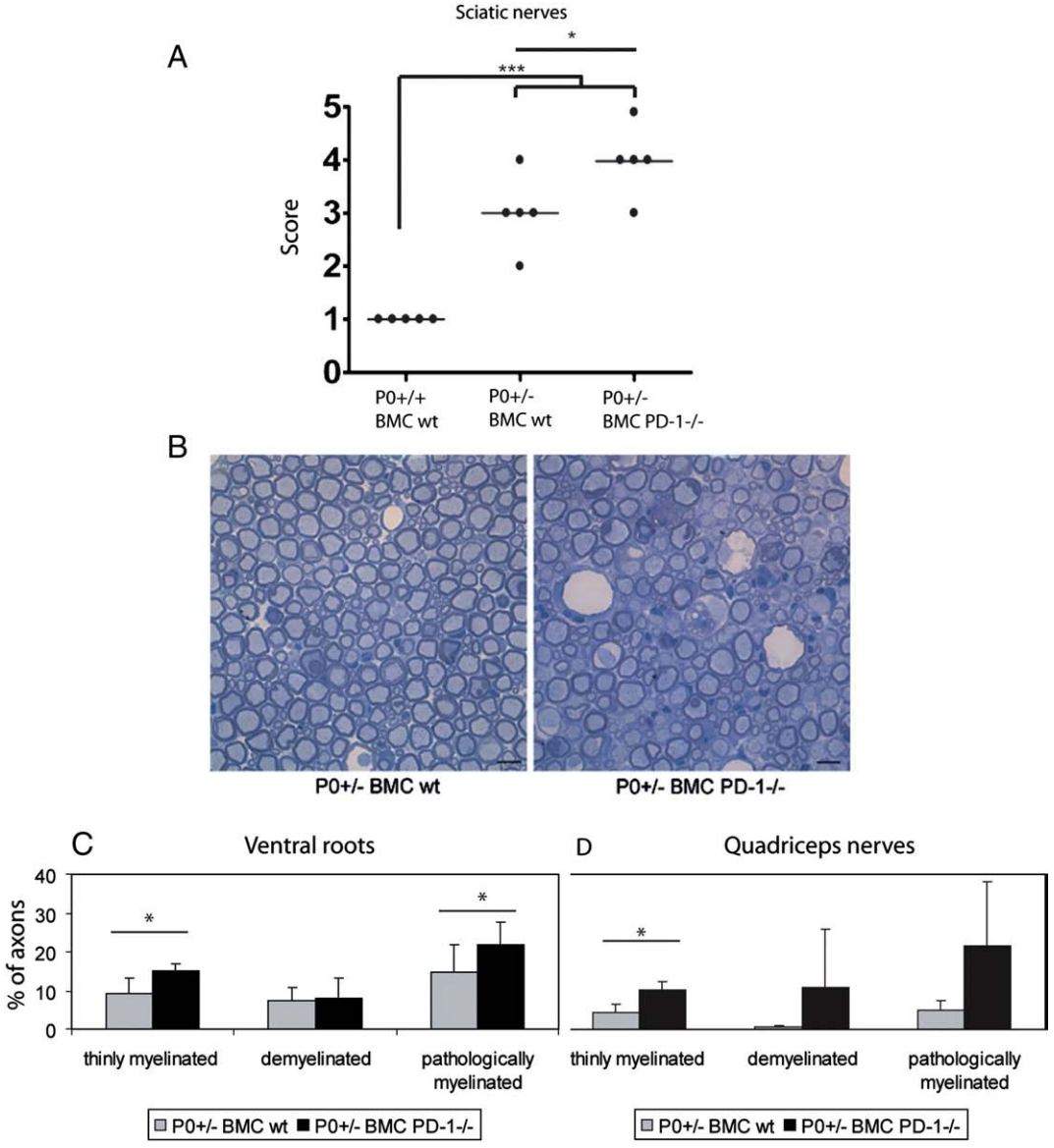

Fig. 2. PD-1-deficiency in P0+/−/RAG-1−/− BMCs leads to increased myelin degeneration in sciatic nerves (A) ventral roots (B, C) and quadriceps nerves (D). This is demonstrated by ranking (A) with score 1 representing intact myelin and 5 severe myelin damage, representative semithin sections (B) and quantification of myelin abnormalities, visualized by electron microscopy (C, D). Note the higher number of pathologically myelinated axons in P0+/−/RAG-1−/− BMC PD-1−/− ($n=5$) compared to P0+/−/RAG-1−/− BMC wt ($n=5$). Pathologically myelinated axons comprise thinly myelinated axons and demyelinated axons. Numbers represent percentages of total myelin-competent axon numbers (~1000/ventral roots and ~500/quadriceps nerves). Scale bar: 10 μm. Error bars represent standard deviations, $*p<0.05$, $***p$-value ≤ 0.001.

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Table 1
Electrophysiological features of P0+/−/RAG-1−/− BMC wt and P0+/−/RAG-1−/−BMC PD-1-/- mutants

	P0+/−/RAG-1−/− BMC wt	P0+/−/RAG-1−/−BMC PD-1−/−	*p*-value
Amplitude (distal)	6.32±1.89 mV	4.04±2.27 mV	0.03
Latency (distal)	0.94±0.09 ms	0.91±0.11 ms	n.s.
Amplitude (proximal)	4.71±1.5 mV	3.38±2.27 mV	n.s.
Latency (proximal)	1.56±0.09 ms	1.6±0.1 ms	n.s.
F-wave latency	5.76±0.67 ms	6.54±0.54 ms	0.02
NCV	31.77±5.83 m/s	27.16±1.84 m/s	0.048

P0+/−/RAG-1−/− BMC wt (n=5) and P0+/−/RAG-1−/− BMC PD-1−/− (n=4) mice were bilaterally electrophysiologically investigated. Distal and proximal amplitude (in mV), latencies (in ms), F-wave latencies (in ms) and nerve conduction velocities (NCV, in m/s) were analysed. Note decrease in amplitudes of compound muscle action potentials and nerve conduction velocities and increased F-wave latencies in the absence of PD-1. p-values<0.05 are considered significant, n.s.=not significant.

RAG-1−/− BMC wt. Demyelination was generally more pronounced in P0+/−/RAG-1−/− BMC PD-1−/− versus P0+/−/RAG-1−/− BMC wt (Fig. 2B). For quantitative analysis, myelin degeneration was ranked by two blinded investigators (A. K. and R. M.) with a scoring system from 1, representing a completely normal myelin status, to 5, characterizing nerves with massive demyelination. By both investigators, ranking revealed a healthy myelin status in all P0+/+/RAG-1−/− BMC PD-1−/− mice and a significantly more pronounced damage in sciatic nerves of P0+/−/RAG-1−/− BMC PD-1−/− with a mean value of 4±0.6 than in BMCs receiving bone marrow from wt mice (3±0.7; p=0.03, Fig. 2A). Ranking of ventral roots showed similar results.

To confirm these observations, we determined pathological alterations by ultrastructural analyses. Ventral roots from both P0+/+/RAG-1−/− BMC wt and P0+/+/RAG-1−/− BMC

PD-1−/− mice displayed a normal phenotype, with only 0.1% abnormally myelinated axons. In ventral roots from P0+/−/RAG-1−/− BMC PD-1−/−, there were significantly more thinly and abnormally myelinated axons (completely demyelinated and thinly myelinated axons) than in P0+/−/RAG-1−/− BMC wt (p=0.04, Figs. 2B, C).

Analysis of the quadriceps nerve as the mainly motor part of the femoral nerve also showed a significant elevation of thinly myelinated axons in P0+/−/RAG-1−/− BMC PD-1−/− compared to P0+/−/RAG-1−/− BMC wt mice. The number of completely demyelinated nerves was also far higher in PD-1−/− BMCs with an average of 10% of all myelinated axons versus 0.6% in the wt transplanted mice, but due to high interindividual differences, no statistical significance was reached. Similarly, the overall pathology affected 5% of all myelinated axons in wt BMCs compared to 22% in PD-1−/− BMCs (Fig. 2D). Interestingly, interindividual variations are generally higher in quadriceps nerves than in ventral roots, as reflected by enlarged standard deviations (Fig. 2D).

Axonopathic vacuoles and degenerating axons were also investigated in both ventral roots and quadriceps nerves. Only 0.33±0.57% and 0.58±0.89% of the axons showed these pathological features in P0+/−/RAG-1−/− BMC wt and P0+/−/RAG-1−/− BMC PD-1−/− mice, respectively.

Previous studies on P0+/− mice had shown an affection of mainly motor axons like in ventral roots and in the quadriceps part of the femoral nerve (Martini et al., 1995; Shy et al., 1997). Predominantly sensory parts of the peripheral nervous system, such as dorsal roots and saphenous nerves were much less affected and particularly profiles indicative of myelin degeneration were lacking. Since absence of PD-1 was associated with increased pathological alterations, we investigated whether under such disease-aggravating conditions the sensory parts of the PNS are still preserved from myelin degeneration. For this purpose, we selected the dorsal spinal roots, which, at this segmental level, contain approximately 2000 myelinated axons of predominantly smaller size, and a large number of unmyelinated axons in association with non-myelinating Schwann cells.

There was a trend towards higher amounts of pathologically altered profiles in the dorsal roots of P0+/−/RAG-1−/− BMC PD-1−/− compared to wildtype BMCs, but statistical significance was not reached, possibly due to high individual variations (Supplementary Table). In spite of this mild increase in pathological profiles in the absence of PD-1, they were far less frequent than in ventral roots or femoral nerves of these mutants.

Electrophysiological investigations reveal features indicative of increased axonopathy and myelin damage in P0+/−/RAG-1−/− BMC PD-1−/− mice

To investigate the functional consequences of the increased pathological alterations in P0+/−/RAG-1−/− BMC PD-1−/− mice, we performed electrophysiological investigations in 10 months old P0+/−/RAG-1−/− BMC wt (n=5) and P0+/−/RAG-1−/− BMC PD-1−/− (n=4) mice. In brief, sciatic nerves were stimulated at the level of the sciatic notch (proximal stimulation) or above the ankle (distal stimulation) and compound muscle action potentials were recorded from the small foot muscles as previously described. The present recordings showed reduced distal amplitudes as well as significantly delayed F-wave latency in P0+/−/RAG-1−/− BMC PD-1−/− versus P0+/−/RAG-1−/− BMC wt, reflecting increased axonopathy and myelin damage, respectively (Table 1).

P0+/−/RAG-1−/− BMC PD-1−/− mice show altered gait performance and reduced mechanical sensitivity in behavioral testing

To investigate motor behavior, gait tests were performed. For this, the hind paws of mice of similar body weight (29.4±3.5 g in wt BMCs versus 28.75±4.11 g in PD-1−/− BMCs) were stained with ink and mice were allowed to walk on white paper sheets. Distance between feet, stride length and rotation of feet was measured. This test revealed no differences in stride length or rotation but a significantly broader distance between feet in the P0+/−/RAG-1−/− BMC PD-1−/− group, suggesting difficulties in weight bearing (Fig. 3).

Further, we performed an established test for mechanical sensitivity of the hind paw (von Frey test). In a first test, P0+/−/RAG-1−/− BMC wt mice reacted at 0.16±0.13 g compared to P0+/−/RAG-1−/− BMC PD-1−/− mice whose withdrawal level was 0.39±0.22 g (p-value<0.02). A second test also showed higher mechanical withdrawal thresholds in the absence of PD-1: 0.18±0.18 g versus 0.61±0.36 g (p-value=0.005).

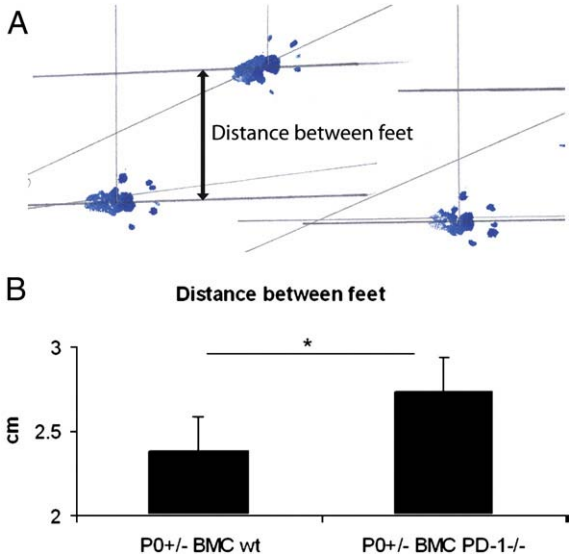

Fig. 3. PD-1-deficiency in P0+/−/RAG-1−/− BMCs leads to increased distance between feet, reflecting impaired straight performance, as demonstrated by a representative step print (A) and the corresponding quantitative analysis (B) in P0+/−/RAG-1−/− BMC wt (n=5) versus P0+/−/RAG-1−/− BMC PD-1−/− (n=4). Error bars represent standard deviations. *p-value<0.05.

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Isolated PNS derived T-cells show features indicative of activated effector cells

Isolation and flow-cytometry analysis of CD8+ cells from peripheral nerves of P0+/−/RAG-1−/− BMC wt and P0+/−/RAG-1−/− BMC PD-1−/− mice revealed that lymphocytes expressed the effector cell markers CD44 and CD69, but scarcely the marker of immature lymphocytes CD62L. There was a tendency towards an even higher activation status in P0+/−/RAG-1−/− BMC PD-1−/− mice. Splenocytes, in contrast, showed a more balanced composition of naïve (CD62L+) and effector T-lymphocytes (CD44+/CD69+) which did not differ significantly in the absence or presence of PD-1 (Fig. 4).

Isolated PNS derived T-cells are prone to IFN-γ secretion in P0+/− mice

In a next step IFN-γ Elispot assays were performed on 10^5 PNS-derived mononuclear cells from P0+/+ and P0+/− mice and from P0+/−/RAG-1−/− BMC wt or PD-1−/−. A higher number of IFN-γ expressing cells was present in peripheral nerves of the myelin mutants (P0+/+: 19±8.5; P0+/− 58±4.2), but not in spleens of P0+/− mice compared to P0+/+ mice under unspecific stimulatory conditions with PMA/ionomycin. In peripheral nerves of P0+/−/RAG-1−/− BMC PD-1−/−mice, there was a non-significant trend to an increased susceptibility of lymphocyte activation (72±9.9) in comparison to P0+/−/RAG-1−/− BMC wt (47±26.16).

Discussion

Several studies of our group have shown that in animal models for inherited demyelinating neuropathies, T-lymphocytes are involved in the disease mechanisms (Ip et al., 2006b). In the present study, we asked the question which factors control tissue homeostasis of the pathogenetically relevant immune cells. For this, we investigated the impact of the co-inhibitory molecule PD-1, a CD28-related molecule mainly expressed on activated T- and B-lymphocytes and controlling proliferation of autoreactive T-cells (Okazaki and Honjo, 2006; Sharpe et al., 2007).

The present study shows that the absence of PD-1 is associated with further elevated numbers of CD8+ T-lymphocytes in the PNS of

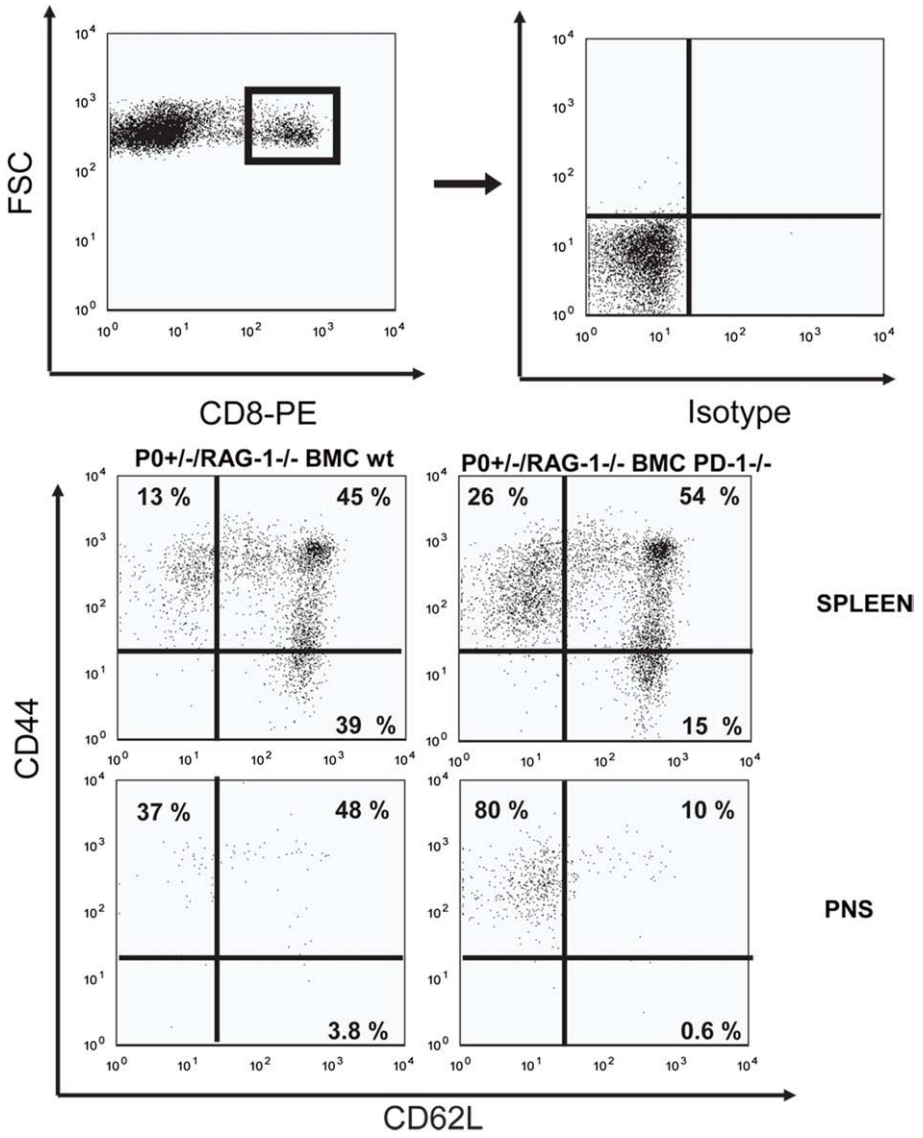

Fig. 4. CD8+ T-lymphocytes from peripheral nerves of P0+/−/RAG-1−/− BMC wt and P0+/−/RAG-1−/− BMC PD-1−/− mice display features of activated cytotoxic effector cells, as revealed by flow cytometry experiments. Cells are gated to CD8+ (upper diagrams) and investigated for CD44 (activation marker) and CD62L (marker for immature lymphocytes; lower diagrams). Note the similar distribution of naïve (CD62L) and effector cells in splenocytes of both groups, while the nerve derived lymphocytes from P0+/−/RAG-1−/− BMC PD-1−/− show a more activated (= CD44+) phenotype. For this experiment, representative examples of individual spleens are shown. Peripheral nerves of 2 mice per group at the age of 10 months were pooled due to the low amount of cells.

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P0+/− mice. Furthermore, the histological phenotype is substantially aggravated in PD-1−/− reconstituted bone marrow chimeras. Consequently, functional down-stream features, such as electrophysiological parameters, walking coordination and mechano-sensory functions are more affected than in P0+/− mice expressing the co-inhibitory molecule. In one case, we even saw an extreme increase of CD8+ T-lymphocytes, macrophages and of the usually rare CD4+ T-cells, combined with a severely aggravated phenotype. In a recent study dealing with CNS myelin mutant mice overexpressing the oligodendrocytic myelin component PLP, we found a similarly increased demyelinating phenotype when PD-1 was absent, and also there, one single PD-1-deficient myelin mutant was characterized by an extreme demyelinating lesion (Kroner et al., submitted). These observations do not only demonstrate that absence of PD-1 strictly leads to a more severe demyelinated phenotype but that there is a higher propensity for extreme disease aggravation.

In the PLP overexpression model, we characterized the pathogenic CD8+ T-lymphocytes as CD44+/CD69+/CD62L-activated effector cells (Ip et al., 2006a). In the P0+/− mutants expressing PD-1, we now show that the nerve-derived CD8+ T-cells display a similar phenotype and demonstrate – in comparison to P0+/+ mice – an enhanced propensity to secrete proinflammatory cytokines (IFN-γ) after unspecific stimulation with the PKC-activator PMA and the Ca^{++} ionophor ionomycin as revealed by ELISPOT-assays. This propensity of PNS-derived T-cells showed also a trend towards a further increase in the absence of PD-1. Additionally, PD-1-deficiency leads to a substantial increase of pathogenetically relevant immune cells. This has also been observed in PLP overexpressing mutants (Kroner et al., submitted), supporting the view that PD-1 is a regulator of tissue homeostasis of a wide range of disease-relevant conditions (Okazaki and Honjo, 2006, 2007; Sharpe et al., 2007). While, however, in the CNS mutants, the CD8+ effector cells were mono- or oligoclonally expanded, suggesting antigen-specificity (Leder et al., 2007), and displayed a massive polyclonal expansion in the absence of PD-1 (Kroner et al., submitted), no clonal expansions were seen in the peripheral nerves of P0 mutants, neither in the presence or absence of PD-1 (data not shown). This could, however, be due to technical limitations, related to the relatively small number of T-lymphocytes in the peripheral nerve tissue.

Another common feature of PD-1-deficiency in CNS and PNS mutants was that PD-1-deficiency alone neither affected the number of CD8+ cells nor the typical intact histological appearance of healthy myelinated nerve fibers. This is particularly remarkable, since PD-1-deficient mice show substantial systemic clonal expansions of T-lymphocytes and an elevated propensity to secrete proinflammatory cytokines (Kroner et al., submitted). Thus, PD-1-deficiency alone does not initiate disease when combined with a normal or healthy environment, at least under the present conditions (e. g. C57/Bl6 strain).

The contribution of the immune system in inherited peripheral neuropathies is of substantial clinical impact. Some reports demonstrated that the clinical phenotype of CMT patients sometimes varies considerably although being caused by identical mutations. This is particularly striking in patients belonging to one and the same family. It is plausible to assume that the high variability in the phenotype could be related to the immune system. The most striking examples are related to CMT patients that have been reported to develop a chronic inflammatory demyelinating polyneuropathy (CIDP), an idiopathic peripheral nerve disorder mediated by autoimmune mechanisms. These patients are characterized by features that are unusual for CMT, such as rapid disease progression and fulminant infiltrates comprising lymphocytes and macrophages (Martini and Toyka, 2004). It is not yet known why a subpopulation of CMT patients develops the CIDP-like features. Based on our present study, it is tempting to speculate that such patients may have impaired immune regulatory (co-inhibitory) pathways. Along these lines it is of note that some polymorphisms of PD-1 are linked to particularly severe forms of multiple sclerosis (Kroner et al., 2007). Based on these considerations, it might be of interest to investigate in CMT-patients with unusually rapid progression whether disease modifiers, such as PD-1 and other immune-modulating molecules, are altered.

In summary, our study demonstrated that a monogenic nerve disorder can be substantially modified due to impaired immune-controlling mechanisms. Understanding the implication of disease-modifiers in inherited demyelination might be of pivotal interest to develop strategies to limit the detrimental mechanisms inherent to the primarily myelin-related disorders by fostering immuno-regulatory pathways.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.nbd.2008.09.021.

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