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P0 specific T-cell repertoire in wild-type and P0 deficient mice

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To my biology teachers,
Valentina Ionita and Maria Hanu

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Introduction

1. T-cell receptor repertoire and self tolerance

T-cell repertoire and self tolerance are one of the most active areas of research as a consequence of the paradox that two opposite effects, namely positive and negative selection, originate in the same event: recognition of self MHC:self peptide ligand by the same T-cell receptor. How T-cells are selected for self-tolerance and not for self-reactivity during their maturation remains one of the most challenging problems in immunobiology.

1.1 T-cells develop in thymus

T cells develop from precursors arriving in the thymus from bone marrow. Here, in the unique microenvironment provided by the thymus, T-cells are educated towards a self-MHC restricted and self-tolerant T-cell repertoire. Thus, thymic selection becomes the first and most important checkpoint where the autoreactive T-cells are censored [42]. Thymus induced tolerance is referred to as **central tolerance** to differentiate it from **peripheral tolerance** which occurs in secondary lymphoid tissues after the export of mature T-cells from the thymus.

The thymus consists of an epithelial network known as the thymic stroma. With regard to its spatial distribution two types of thymic epithelial cells (TECs) can be distinguished: cortical thymic epithelial cells (cTECs) and medullary thymic epithelial cells (mTECs). The epithelial network is embedded with bone marrow derived cells (BMdC) that comprise, at a certain time point, distinct populations. The cortex is populated with immature thymocytes, while the medulla contains mature thymocytes, dendritic cells (DCs) and macrophages [35]. Thus, the thymus is formed by two groups of cells with different origin, structure and function – TEC and BMdC - both of which contribute to selection of a mature T-cell repertoire.

1.2 Positive selection

Upon arrival in the thymus T-cell precursors express neither of the two co-receptor molecules CD4 or CD8, nor CD3:T-cell receptor complexes that define mature T-cells. Owing to the absence of CD4 and CD8, such cells are called 'double-negative' thymocytes (DN; $CD4^-8^-$). These cells will give rise to large numbers of $CD4^+8^+$ cells [20, 66]. These 'double-positive' (DP) thymocytes form the vast majority of cells found in the cortical region of the thymus. At this site, DP thymocytes are in close contact with the dense network of epithelial cells. At this stage thymocytes are subjected to **positive selection**, which allows DP thymocytes to mature into CD4 or CD8 'single-positive' (SP) cells.

The major histocompatibility complex (MHC) molecules on epithelial cells play a decisive role in determining the fate of DP thymocytes. Typical cortical DP cells express low levels of T-cell receptor (TCR) molecules and are programmed to die within three to four days unless rescued by a TCR signal [8, 19, 72, 75]. Such signalling reflects TCR contact with specific self peptide:MHC complexes on cortical epithelial cells; the respective peptides are generated through breakdown and processing of various intracellular proteins, thus causing MHC molecules on epithelial cells to display a wide variety of self peptides [14, 23, 62, 63, 67].

Of the large numbers of DP cells in the cortex, only a small proportion (1-5%) have significant binding specificity for the various self peptide:MHC complexes expressed on adjacent epithelial cells. DP cells binding these complexes receive a low-level TCR signal that allows the cells to survive, up-regulate TCR expression and differentiate into mature 'single-positive' (SP) CD8 and CD4 cells after being screened in a **negative selection** processs.

The recognition of self MHC molecules ensures that the positively selected thymocytes can recognize foreign peptides presented only by self MHC (MHC restriction) in the periphery. This process of positive selection is aided and fine-tuned by the CD8 and CD4 molecules on DP cells. Conform to the instructive model of lineage commitment, DP cells recognizing peptide-MHC I molecules will result in retention of CD8 expression but downregulation of CD4 expression, thus generating $CD4^-8^+$ SP cells. Conversely, cells that recognize peptide-MHC II complexes will retain CD4 but lose CD8, thereby forming $CD4^+8^-$ cells [78]. The vast majority of DP cells (over 95%) have negligible affinity for thymic peptide-MHC complexes. These cells fail to undergo TCR signalling and, in the absence of this protective signal, the cells die by neglect.

1.3 Negative selection

The paradox mentioned before has been partially solved by the quantitative-avidity model [72] which suggests that overall avidity between thymocytes and self peptide:MHC complex on various thymic cells determines the fate of thymocytes. The overall avidity includes both the number of interactive events and the strength of the interactions (affinity) between the TCR and self peptide:self-MHC ligand.

Thymocytes which have low avidity for self peptide:MHC complex fail to signal through TCR and will undergo programmed cell death due to neglect. When TCRs interact weakly with selecting self peptide:MHC complexes, positive selection is promoted. Stronger avidity between the TCR and self peptide:MHC ligand induces apoptosis in thymocytes which are therefore said to be negatively selected [22].

Most of the self peptides controlling positive selection are interacting relatively weakly with T-cells. However, as T-cells continue to maturate, they up-regulate the TCRs at the cell surface [72]. These peptides or a spectrum of other MHC-associated self peptides have now the potential to bind strongly to the TCR. If T cells specific for these peptides were allowed to exit from the thymus, there would be a high risk that the cells would attack self-components and thereby induce autoimmune disease. Based on this line of reasoning it has long been argued that T-cells with high affinity for self antigens are deleted in the thymus.

Several different types of cells mediate the process of negative selection, mainly BM derived DCs and macrophages. These are the professional antigen presenting cells (APC) that also activate T-cells in peripheral lymphoid tissues. Self antigens presented by these cells must lead to T-cell deletion in the thymus in order to induce a state of self tolerance [35]. As BM derived DCs and macrophages are found almost exclusively in the medulla of the thymus, this place was regarded as the anatomical site of negative selection.

Cells that survive negative selection will leave the thymus and form the mature T-cell receptor repertoire in the periphery (Fig.1). Negative selection ensures that T-cells that escaped apoptosis (TCR repertoire) will not react to self-antigens and will be able to develop an immune response against non-self antigens.

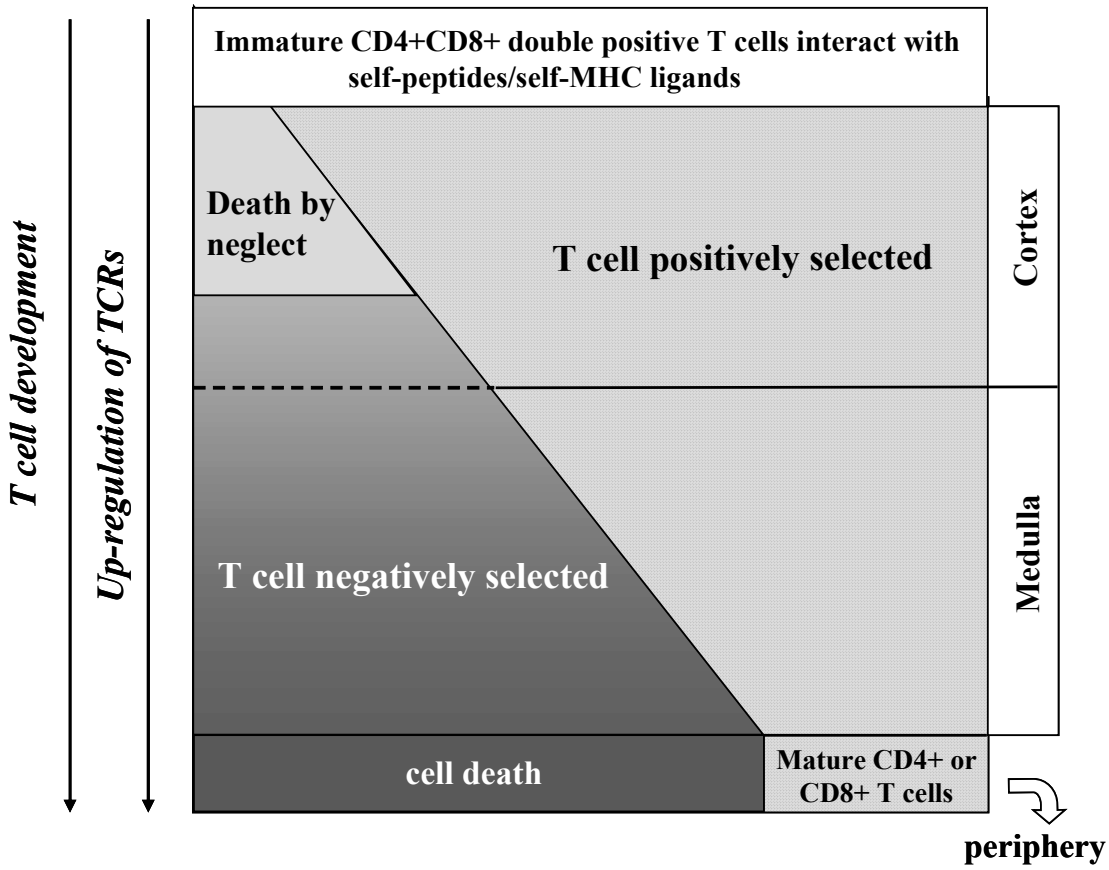

Figure 1. T-cells develop in thymus. Their development depends on interaction of the T-cell receptors with self peptide-self MHC on diverse thymic APCs present in cortex and medulla. Interaction failure leads to death due to neglect within 2-3 days. Weak interaction between T-cells and cortical epithelial cells provides a survival signal, which means that T-cells are positively selected. The anatomical localization of positive selection is the cortex of the thymus. As thymocytes mature, the TCR density increases on the cell surface [10, 28, 60] and so does the strength of the signal. If the signal increases beyond a certain threshold required for negative selection it would render T-cells apoptotic. Thus, negative selection can occur also in the cortical area. Since cell fate is largely dependent upon thymocyte-stromal cell avidity, this suggests that thymocytes that have initially undergone positive selection may be susceptible to negative selection via the same selecting ligand. The medulla is populated mainly by DCs and macrophages of BM origin. As these are professional APCs, they express a high number of self peptides-self MHC ligands at the cell surface. On the other hand, T-cells approaching the medulla express higher numbers of TCRs and thus interaction avidity between APCs and TCRs is higher. Many cells with high avidity for self ligands will receive a signal which triggers negative selection in order to clear the T-cell repertoire of autoreactive T-cells.

2. Dominance, subdominance and crypticity of T-cell epitopes

Native antigens cannot be recognized by T-cells. Instead they need to be taken up by APCs and processed to short peptides, which are displayed by MHC molecules at the cell surface. Only peptides with good trafficking within cell compartments will be displayed at the cell surface. From these peptides one or a very limited number will be recognized by specific T-cells and able to mount an immune response out of the entire amino acid sequence of a given protein antigen. These immunostimulatory peptides frequently contain an **immunodominant determinant**. In addition, there may be **subdominant determinants**, which mediate a less potent immune response, or **cryptic determinants**, which have virtually no impact on the immune response. Peptides derived from processed protein antigens that bind well to MHC molecules are called **agretopes**. Peptides which are specifically recognized by T-cells are called **T-cell epitopes** [15].

In order that a peptide sequence to be considered a T-cell determinant it must fulfill two conditions: to have an MHC binding site (agretypic features) and a T-cell receptor binding site (epitypic features).

It has been well established that synthetic peptides can be used to replace protein epitopes derived from endogenous or exogenous proteins and can load MHC class I and class II complexes which are then recognized by both cytotoxic and helper T-cells [71, 83]. Using synthetic peptides in immunization protocols together with adjuvants, T-cell epitopes can be identified in recall assays which measure proliferation or cytokine production such as in ELISpot assays.

In order to detect an immunogenic determinant, a native protein is injected together with an adjuvant and the response to its peptide determinants is assessed by subsequent in vitro T-cell proliferation or ELISpot, after a 10-day priming interval. Under such conditions, only a minority of epitopes, the dominant ones, induce proliferation or cytokine production. Subdominant determinants induce only a weak response. Their weak immunogenicity is due to either poor agretypic or poor epitypic features. Subdominant determinants can induce an immune response when injected in peptide form, which can be recalled in vitro by the peptide or the native protein from which the peptide derived. A cryptic epitope produces an immune response that can be recalled only with the peptide itself and not with the native proteins from which derives.

The notion of crypticity does not refer to the magnitude of the immune response. A cryptic determinant could be either more or less immunogenic than the dominant determinant. Because of insufficient processing or even excessive processing the cryptic epitope is not generated and thus not presented in the MHC binding groove. Thus, T-cells, which interact with their peptides only in context of self MHC, do not 'see' cryptic epitopes after natural processing of the native protein.

3. Myelin protein zero (P0)

P0 is an adhesive cell surface glycoprotein belonging to the immunoglobulin superfamily. It accounts for about 50-60% of the total proteins of the peripheral nerve myelin and has been proposed to mediate intracellular and extracellular myelin compaction. It is essential for normal spiralling, compaction and maintenance of peripheral myelin sheaths [50].

In adults, its expression is confined to myelinating Schwann cells whereas during embryonic development it is weakly expressed in precursors of Schwann cells independently of whether they will give rise to myelinating or non-myelinating Schwann cells [50]. P0 contains a high percentage of hydrophobic residues, which explains its low solubility in aqueous solutions.

The mouse protein has a molecular weight of 24.8 kDa corresponding to a 219 amino acids sequence. P0 contains three domains [90] (Fig.2):

- a 124 amino acid extracellular domain which is hydrophobic and acts as a homotypic adhesion molecule;
- a 26 amino acid transmembrane domain**,** which is highly hydrophobic and
- a 69 amino acid cytoplasmic domain, which is hydrophilic.

signal sequence

MAPGAPSSSP SPILAA LLFS SLVLSPALA **I VVYTDREIYG AVGSQVTLHC**

extracellular domain

SFWSSEWVSD DISFTWRYQP EGGRDAISIF HYA KGQPYID EVGAFKERIQ

WVGDPRWKDG SIVIHNLDYS DNGTFTCDVK NPPDIVGKTS QVTLYVFEKV

transmembrane domain

PTRYGVVLGA V IGGILGVV L LLLLLFYLIR YCWLRRQAAL RRLSAMEKG

cytoplasmic domain

RFHKSSKDSS KRGRQTPVLY AMLDHSRSTK AASEKKSKGL GESRKDKK

Figure 2. P0 amino acid sequence. P0 protein contains three domains: extracellular, transmembrane and cytoplasmic. The signal sequence is not part of the mature protein.

4. P0 knockout mice

Approximately 30 mutations have been described in the human P0 gene resulting in either Charcot-Marie-Tooth 1B (CMT 1B) disease and Dejerine–Sottas neuropathy or congenital hypomyelination. In order to evaluate the functional role of the molecule in vivo, a P0 knockout (P0 ko) mouse was generated as a model to study these diseases [24].

P0 ko mice are deficient in normal motor coordination and exhibit tremor. Body weight is lower than normal, forelimbs and especially hind limbs are weak and display reduced muscular mass. Yet they have normal life expectancy.

Peripheral nerves from homozygous P0 deficient mice ($P0^{-/-}$) are characterized by severe hypomyelination leading to compromised conduction properties. The number of axon-Schwann cell units entirely devoid of myelin increases with age. Heterozygous P0 deficient mice ($P0^{+/-}$) show an apparently normal phenotype at young age. However, at three months postnatally, histological analyses show clear signs of myelin degeneration, which mostly resembles demyelinating neuropathies.

The role of the immune system has been addressed in genetically induced myelin disorders, which surprisingly exhibit some features of human demyelinating neuropathies.

Schmidt and co-workers demonstrated an age-related increase in endoneurial CD8 positive T-cells in femoral nerves of heterozygous P0 mutant mice, which are an appropriate model for some forms of CMT 1B [69]. The functional relevance of this finding was demonstrated by crossbreeding of $P0^{+/-}$ deficient animals with mice deficient for the recombination-activating gene (RAG-1) that lack mature T- and B-cells [55]. These double mutants showed an ameliorated pathology in comparison to mutants with an intact immune system.

Macrophages are the primary effector cells in autoimmune neuropathies. In this model, the number of macrophages also shows an age-related increased in $P0^{+/-}$ animals that corresponds to the severity of myelinopathy (Fig. 3). The difference in the number of macrophages in the motor nerves of mutants is highly significant in comparison to wild type controls.

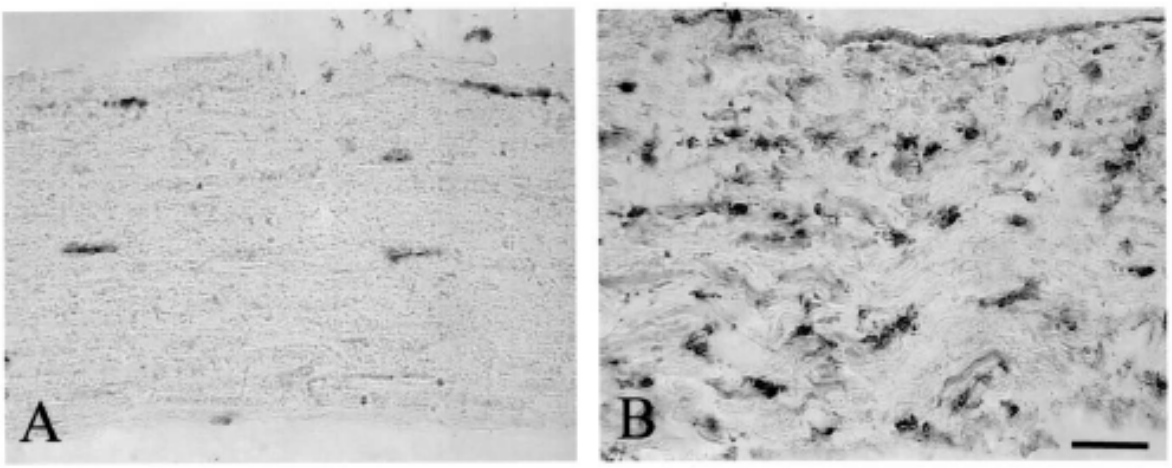

Figure 3. Immunohistological localization of macrophages in the PNS of mice heterozygously deficient for P0 at the age of 6 months using antibodies to the mouse macrophage surface marker F4/80. In comparison to wild type mice **(A)** the number of macrophages is clearly increased in $P0^{+/-}$ mice **(B).** Longitudinal cryosection of the quadriceps nerve. Bar 40 µm.

5. Experimental autoimmune neuritis – animal model for Guillain–Barré syndrome

5.1 Guillain–Barré syndrome (GBS)

Many insights into immune mechanisms of demyelination were primarily based on studying experimental autoimmune neuritis (EAN), the animal model for human Guillain–Barré syndrome [27, 84].

GBS is an acute inflammatory demyelinating polyradiculoneuropathy, which typically follows an acute course. Most cases are associated with a preceding bacterial and/or viral infection, although the list of reported triggering agents is diverse [25]. It is therefore less likely that a single antigen is involved in the autoimmune etiology that causes GBS.

Both cellular and humoral immunity may be involved in the pathogenesis of GBS. Abnormal cellular responses to P2 and P0 proteins have been reported in some patients with GBS [40]. Presence of antibodies to peripheral nervous system (PNS) myelin has repeatedly been demonstrated in GBS patients' sera [94]. Their pathogenic role is however uncertain since they are also found in a minority of patients with other neurological disorders and in some healthy subjects [86].

5.2 Experimental autoimmune neuritis (EAN)

EAN resembles many of the clinical, electrophysiological and immunological aspects of human GBS. Hence, it has been widely used as a model to investigate disease mechanisms in acute inflammatory demyelinating disease of the PNS. Moreover, EAN, besides other autoimmune models like experimental autoimmune encephalomyelitis (EAE), experimental autoimmune uveitis, etc., may be used as a tool to study autoimmunity and tolerance induction towards tissue specific antigens.

Histologically, acute EAN is characterized by infiltration in the nerve roots and peripheral nerves with macrophages and lymphocytes, and by primary demyelination associated with some degree of axonal damage (Fig. 4). EAN can be actively induced in susceptible rat strains by immunization with purified PNS myelin, bovine P2-protein, recombinant human

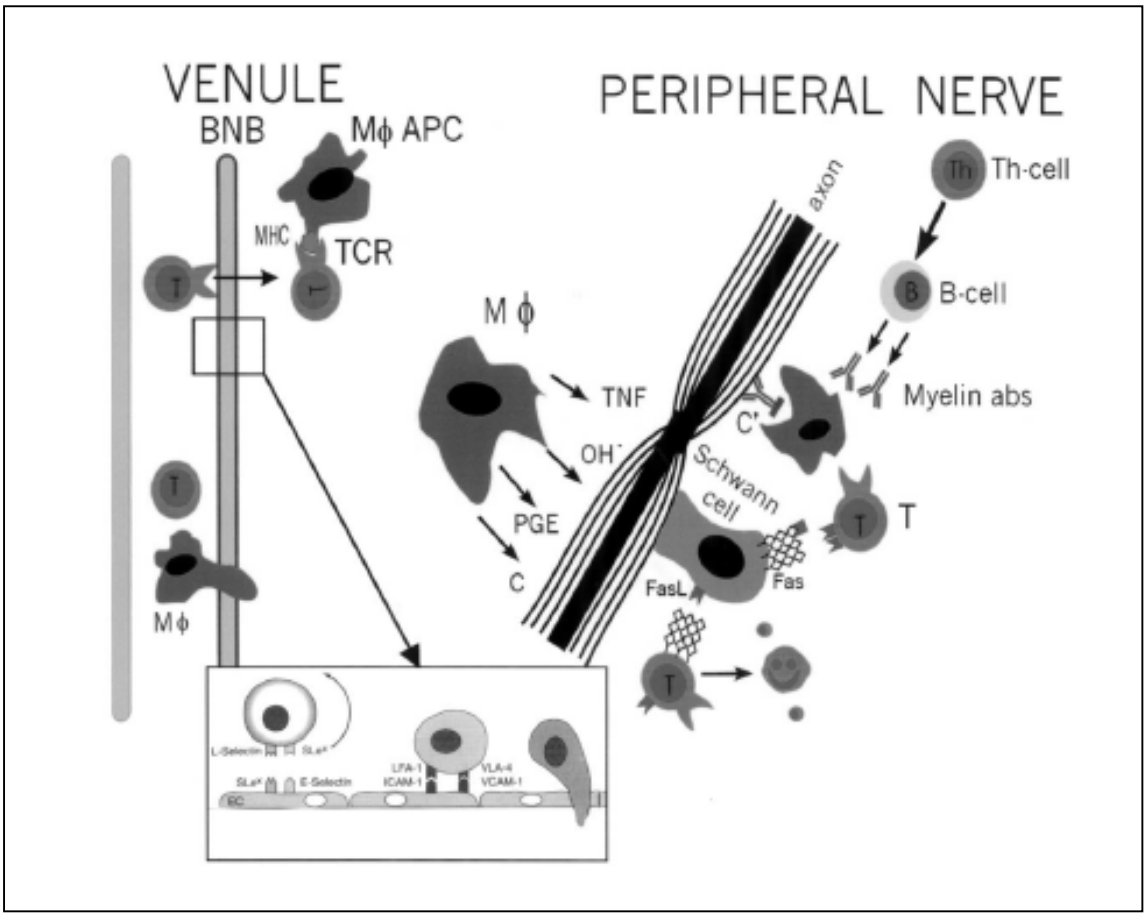

Figure 4. Simplified scheme depicting the hypothetic sequence of major immune mechanisms in EAN. Th, T-helper cell; TCR, T-cell receptor; MHC, major histocompatibility complex; APC, antigen presenting cell; C, complement components, IFN-γ, interferon gamma; MMP, matrix metalloproteinases; OH, hydroxyl radicals; PGE, prostaglandin E; MØ, macrophage; TNF, tumor necrosis factor. The insert shows the transendothelial migration of activated T cells across the blood nerve barrier, which is mediated by adhesion molecules. Through the action of selectins, T-cells establish a reversible contact with endothelial cells via exposed sugar residues (SLex). This initial rolling is followed by a firm reversible adhesion mediated by the integrins VLA-4 and LFA-1 and their immunoglobulin-like counter receptors ICAM-1 and VCAM-1. The subsequent transendothelial migration of T-cells through the BNB is thought to be mediated mainly by the interaction between VLA-4 and VCAM-1.

P2 protein, or with a peptide spanning the neuritogenic epitope (amino acid 53–78) of P2 protein [26] in the presence of complete Freund's adjuvant (CFA). Further autoantigens that have been identified in EAN models in rats and mice are P0 protein [48], myelin basic protein (MBP) [1], peripheral myelin protein 22 (PMP22) [21], and myelin-associated glycoprotein (MAG) [87]. Theoretically, it is conceivable that a variety of other antigens of the PNS also become targets of the immune attack, similar to the situation in the central nervous system (CNS) [70]. The central role of T-cells in the pathogenesis of EAN was shown by adoptive transfer (AT-EAN) of a T-cell line specific for P2 protein capable of inducing EAN in Lewis rats [47] and also in BN rats which are resistant to active disease. Subsequently T-cell lines specific for P2 peptides or P0 and P0 peptides have been shown to transfer disease.

The central role of T-cells is further supported by the inability of T-cell deficient rats to develop EAN upon active immunization [11] and by the prevention of EAN by treatment with antibodies directed at the α/β-Tcell receptor [38], or the IL-2 receptor [30]. Early invasion of the PNS by T-lymphocytes has been documented immunocytochemically [34], and the degree of cellular infiltration subsequently correlates well with the degree of nerve function deficit [29]. Recent studies confirmed that T-cell infiltration is the first pathological sign in AT-EAN, and that this infiltration is accompanied by a rapid increase in permeability of the blood–nerve barrier [49].

After activation, neuroantigen specific T-cells invade the peripheral nerve and migrate intraneurally and damage nerve fibres by cellular cytotoxic effects (NK cells, CD8 T-cells and antibody-dependent cellular cytotoxic effects), through cytotoxic proinflamatory mediators and through B-cell activation leading to antibody production and complement attack on nerve fibres.

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Aim of the study

In this study, we examined the contribution of endogenous myelin protein P0 to repertoire selection of T-cells specific for this self-antigen in wt and P0 homozygous deficient mice. Based on the finding that intrathymic expression of tissue-specific antigens is a common occurrence, we tested whether this "promiscuous" expression holds true also for myelin protein P0.

In addition, using bone marrow chimeras we investigated the capacity of BM derived cells versus thymic epithelial cells to induce tolerance towards P0. We reconstituted lethally irradiated wt mice with BM cells from P0 deficient mice and vice versa and tested the reactivity of P0 in the chimeric animals.

As yet, no reliable model for experimental autoimmune neuritis in C57BL/6 mice, the founder strain for most transgenic mice, has been established. Using synthetic peptides spanning the immunodominant region of P0, we looked for its ability, in conjunction with adjuvants and various immunomodulatory molecules, to trigger an autoimmune response against myelin proteins and to induce EAN.

Materials and Methods

1. Mouse strains

- **Wild type C57BL/6 (H-2^b) mice** were purchased from Charles River Breeding Laboratories (Sulzfeld, Germany) or derived from intercrosses between P0$^{+/-}$ mice on this genetic background and bred at in-house animal facilities under specific pathogen-free conditions. Note that cell-surface expression of H2-E^b MHC class II molecules is absent in C57BL/6 mice.
- **P0 knockout mice** deficient in the gene for protein zero were generated as described previously [24] and backcrossed with C57BL/6 mice for at least 10 generations. Homozygous P0 deficient mice show striking abnormalities, such as severe hypomyelination and decompaction of myelin. Behavioural phenotype manifests through deficiency in motor coordination and tremors. With increasing age, this behaviour becomes more pronounced. Homozygous P0 deficient mice have almost the same life expectancy as wt mice. Heterozygous P0 mice display a normal behaviour phenotype.

All mice employed in the experiments were 6 to 12 weeks old except when specified otherwise in the text.

2. Immunization

Under general anaesthesia, mice were immunized by subcutaneous injection at the base of the tail and/or in a single footpad with an inoculum containing a saline solution/precipitate of either P0 peptide or whole myelin homogenate emulsified in CFA. Occasionally mice received pertussis toxin (PT; Sigma-Aldrich, Taufkirchen, Germany) intravenously (i.v.) or intraperitoneally (i.p.) as specified in the text. CFA was prepared mixing pistilled powder of *Mycobacterium tuberculosis* H37 RA with incomplete Freund's adjuvant (IFA) both from Difco Laboratories, Detroit, USA. Each mouse received a volume of 50-100 µl of emulsion containing 50-100 µg peptide or 2-4 mg myelin homogenate and 50-200 µg mycobacteria. In all immunization protocols, except for the EAN experiments, mice received no more than

50 µg mycobacteria per individual. Permission for animal experimentation was obtained from local state authorities.

To evaluate the severity of clinical signs of EAN, mice were weighed and inspected every other day employing a scale from 0 to 5 as follows:

0 - no illness

1 - flaccid tail

2 – moderate paraparesis

3 – severe paraparesis

4 – tetraparesis

5 – death.

3. Isolation of mononuclear cells from lymph nodes and spleens

Inguinal and popliteal lymph nodes (LN) as well as spleen were aseptically removed from mice euthanized in CO_2 and collected in Hepes 2.5 mM (Gibco BRL, Karlsruhe, Germany) buffer. Single cell suspensions of mononuclear cells (MNC) were obtained by gentle passing the tissue through a cell strainer (BD Labware Europe, Le Pont De Claix, France). Cells were washed two times in Hepes buffer before being resuspended in culture medium to the desired concentration.

4. Cell culture and proliferation assay

4.1 Cell culture

For antigen specific activation of primed spleen or LN cells they were cultured in RPMI 1640 medium (GibcoBRL) supplemented with 3% fetal calf serum (FCS; PAA Laboratories, Cölbe, Germany), 5 x 10^{-5} M 2-mercaptoethanol (Sigma), penicillin 100 IU/ml (Biochrom, Berlin, Germany), streptomycin 100µg/ml (Biochrom), sodium-pyruvat MEM 0.01 mM (Gibco), glutamine 2 mM (Gibco) and the respective antigen. After activation, the medium was supplemented in addition with 10% rat Concanavalin A (Con A; Amersham Pharmacia Biotech, Freiburg, Germany) supernatant as source of T cell

growth factors or 2.5 ng/ml mouse recombinant interleukin-2 (IL-2; Sigma). FCS concentration was increased to 10%.

4.2 Proliferation assay

For proliferation assays, cells were cultured in 96-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark) at a density of 2.5 x 10^5 cells per well in the presence of selected concentrations of synthetic peptides or myelin homogenate. Each well contained 100 µl of medium. Cultures were set up in triplicate. T-cells were pulse-labelled with [^{3}H]-thymidine (0.2 µCi/well) during the last 16-18 h of culture. After 72 h, cells were harvested with a Betaplate 96-well harvester (Pharmacia) and incorporation of radioactivity was detected using a Betaplate liquid scintillation counter (Pharmacia). The results representing counts per minute (CPM) were expressed as the mean of triplicate cultures ± SD.

4.3 Generation of permanent antigen-specific T-cell lines

In order to establish T-cell lines, lymph node cells (5 x 10^6/ml) from C57BL/6 mice previously immunized with P0 synthetic peptides were stimulated 'in vitro' with corresponding peptides for three days. Following activation, viable cells were purified by means of density gradient centrifugation using a high-density solution (Lymphoprep, Axis-Shield, Oslo, Norway). After washing two times, blast cells were plated at an initial density of 0.8 x 10^6 cells/ml and expanded for 7-8 days in 6-well plates. Dividing cells were split when necessary. T-cells were then restimulated with peptide and irradiated syngeneic splenic APCs at a ratio of 0.6 x 10^6 T cells: 4 x 10^6 APCs/ml and the cycle was repeated 3-4 times until the T-cell lines were stable. Specificity of the lines was confirmed in proliferation assays.

5. Bone marrow chimeras

5.1 Bone marrow preparation

Femur and tibia were removed from wild type (wt) and P0$^{-/-}$ mice by cutting and separating the bulk of muscles and tendons around the bones. The rest of muscle tissue and tendons

were wiped off with a paper towel with much care to preserve the integrity of the bones. The next steps were done in aseptic conditions. Using sterile forceps the bones were bathed one time in ethanol 70% and two times in RPMI to prevent contamination of the bone marrow (BM) cells. Next, one end of the bone was cut away and the BM was removed in a Petri dish with RPMI 1640 squeezed into the lumen of the bone through a needle attached to a 1 ml syringe. BM cells were then centrifuge at 1000 rot/min, 4° C for 10 min and resuspended in PBS at 10^7 BM cells/ml.

5.2 Bone marrow chimeras

15 wt and $P0^{-/-}$ mice were lethally irradiated with 1000 rad dose from a cesium source administrated in a single dose approved by animal experimentation Bavarian state authorities. Immediately after irradiation, each mouse received intravenously injection with 100 µl PBS containing 10^6 wt or $P0^{-/-}$ BM cells. Thus, four groups resulted:

1. irradiated wt mice reconstituted with wt BM (wt → wt), as control;
2. irradiated $P0^{-/-}$ mice reconstituted with $P0^{-/-}$ BM (ko → ko), as control;
3. irradiated wt mice reconstituted with $P0^{-/-}$ BM (ko → wt) and
4. irradiated $P0^{-/-}$ mice reconstituted with wt BM (wt → ko).

2-3 days before and until 3 weeks after irradiation, the mice were maintained on aqueous antibiotics by supplementing the water source with 2 mg/ml neomycin sulphate (Biochrom). To prevent nasopharyngeal colonization with *Pseudomonas aeruginosa*, the drinking water was acidified to pH 2.5-3. By three weeks after irradiation and reconstitution the animals were turned to normal conditions with regard to housing and other facility requirements. The survival of animals during the whole experiment was 100%.

6. DNA extraction

Genomic DNA was extracted with the DNeasy Tissue Kit (Qiagen GmbH, Hilden, Germany) from tail tips cut at a maximum length of 1.2 cm. Purified DNA from one tail tip was finally collected in 200 µl elution buffer from which 1 µl was used in PCR reaction.

7. Polymerase chain reaction (PCR)

PCR is usually used to directly amplify rare specific DNA sequences in a complex mixture when the ends of the sequence are known. To genotype P0-mutants, conventional PCR was performed using the following oligonucleotide primers (MWG Biotech, Ebersberg, Germany):

5'–CAGTTCCTTGTCCCCCGCTCTC–3',
5'-GGCTGCAGGGTCGCTCGGTGTTC–3'and
5'-ACTTGTCTCTTCTGGGTAATCAA – 3'

These combination of primers lead to a 334 base-pair (bp) product for the P0 null mutation and a 500 bp products for the wild type allele. PCR was performed in 20 µl of reaction containing:

1x PCR buffer
200 µM dNTP (Roche, Mannheim, Germany)
25 U/ml Taq polymerase (Roche)
1 µl DNA
primers for a final concentrations of 100 nM.

DNA was amplified during 40 cycles with the following steps:

- initial denaturation: 60 sec at 94º
- denaturation of cycle: 15 sec at 94º
- annealing: 30 sec at 55º
- elongation: 30 sec at 72º
- final elongation: 7 min at 72º.

PCR products were mixed with loading buffer at a final concentration 1:10 and loaded on agarose gel (1.5%). Electrophoresis was performed with the running parameters at following values: 100 V, 490 mA and 30 min for a 40 ml 2% agarose gel and 130 V, 1000 mA and 1 h for a 150 ml 1.5% agarose gel. Gels were visualised by using 4 µl ethidium bromide (10 mg/ml) per 100 ml of gel.

The PCR technique was used for genotyping the P0 alleles of the offspring of our breeding colony. Thus, we could sort out the wt and $P0^{+/-}$ mice, which display the same phenotype. PCR was performed on a termocycler from Perkin Elmer, Norwalk, USA.

8. RNA extraction

- **Homogenization.** Total RNA was prepared from homogenized thymus, thymic stromal tissue or thymocytes (cells which remained left after separating the stroma) with Trizol (Life Technologies, Gaithersburg, USA), a ready-to-use reagent for the isolation of total RNA from cells and tissues. To isolate thymic stromal tissue, thymus was gently mashed through a cell strainer in PBS on a Petri dish for few minutes until the solid tissue remained free of thymocytes.

The tissue samples in 1 ml Trizol reagent per 50-100 mg of tissue were homogenized using a rotor-stator homogenizer Art-Miccra-D8 (Art moderne Labortechnik, Müllheim, Germany).

- **Phase separation.** The homogenized samples were incubated for 5 min at RT to permit the complete dissociation of nucleoprotein complexes. 0.2 ml of chloroform per 1 ml Trizol reagent was added, the samples were capped securely, shaken vigorously by hand for 15 sec and incubated at RT for 2 to 3 min. Then samples were centrifuged at 12,000-x g for 15 min at 4° C. Following centrifugation, the mixtures separated into a lower red, phenol-chloroform phase, an interphase and a colourless upper aqueous phase. RNA remains exclusively in the aqueous phase.

- **RNA precipitation and further processing**. The aqueous phase was transferred to a new tube and the RNA precipitated from the aqueous phase by mixing with 0.5 ml isopropyl alcohol per 1 ml Trizol used for the initial homogenization. Samples were incubated at room temperature (RT) for 10 min and thereafter centrifuged at 12,000-x g for 10 min at 4° C. The RNA precipitate formed a gel-like pellet on the side and bottom of the tube.

After precipitation, the supernatant was removed and the pellet was washed once with 1ml of 75% ethanol per 1 ml Trizol used for the initial homogenization. Prior to centrifugation at 7,500-x g for 5 min at 4° C, samples were mixed by vortexing.

After discarding the ethanol, the RNA pellet was briefly dried (air-dry for 5-10 min) and dissolved in RNase-free water by passing the solution a few times through a pipette tip and incubating for 10 min at 55°-60° C.

9. ELISpot

ELISpot detects cytokine secretion at the single cell level based on to monoclonal antibodies, which capture and detect cytokines produced by individual cells. The second antibody is biotinylated and thus readily detected by an enzyme coupled with streptavidine. The enzyme catalyses a colorimetric reaction, which visualizes the cytokine secreting cells. A blue-black coloured precipitate forms at the sites of cytokine localization and appear as spots, each one representing an individual cytokine secreting cell (Fig.5).

- **Buffers**

0.1 M PBS (phosphate buffer saline), pH 7.4	- 0.1 M Na_2 HPO_4	810 ml
	- 0.1 M NaH_2PO_4	190 ml
0.1 M CBCB (carbonat-bicarbonat buffer), pH 9.6	- 4.24 g Na_2CO_3	
	- 5.04 g $NaHCO_3$	
	- H_2O up to 1000 ml	

- **Assay procedure**

All reagents were used at room temperature except the detection antibody solution, which was used at 4° C. All samples and controls were assayed at least in triplicates.

1) Wells in a 96-well microplate (Millipore, Bedford, USA) were filled with 50 µl of CBCB containing 4 µg/ml of coating antibody (Pharmingen, San Diego, USA) and incubated over night, at 4° C or for 3 h at room temperature.
2) After coating, the plates were washed three times with 150 µl PBS using a multi-pipette. PBS was discarded by flicking the plates. 100 µl of HL-1 medium (BioWhittaker Europe, Verviers, Belgium) was added until the cells were ready to be plated.
3) After removing the medium, the cells were plated at the desired concentration in 100 µl of HL-1 medium and stimulated with antigens. Plates were incubated for 20 h in a humidified incubator set up at 37° C and 5% CO_2. The cells were not disturbed during this period.

The first three steps were performed under sterile conditions.

The following steps were performed under non-sterile conditions.

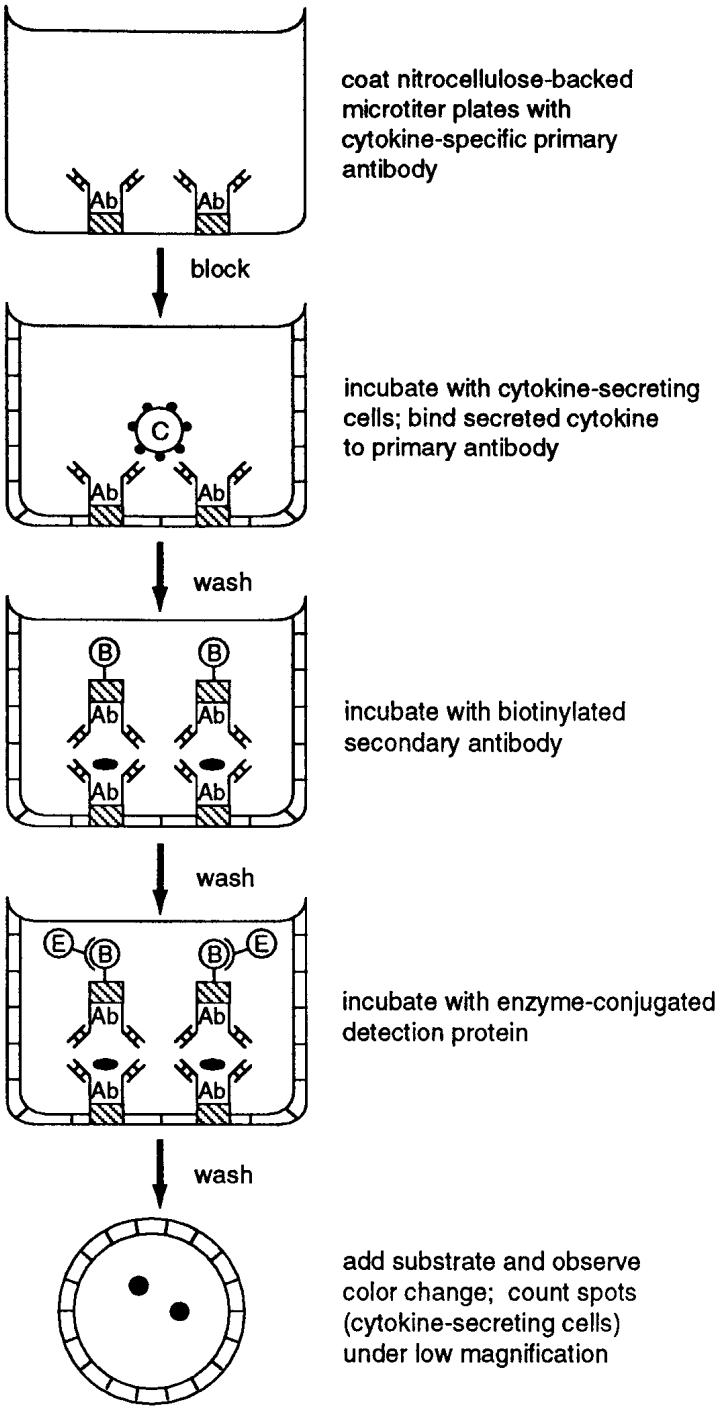


Figure 5. Schematical representation of the ELISpot principle

4) Cells were discarded and the plates were rinsed three times with PBS/Tween 0.05% using an Elisa washer (Nunc). The plates were dried by tapping against clean paper towels and then washed again three times with PBS without Tween with a squirt bottle and finally tapped dry. Complete removal of liquid at each step is essential to good performance.
5) 50 µl of PBS/BSA 1% containing 2 µg/ml of biotinilated antibody (Pharmingen) was added into each well and incubated for 2 h at 37° C.
6) Step 4 was repeated.
7) 50 µl of PBS/BSA 1% containing streptavidine alkaline-phosphatase (Dako, Hamburg, Germany) diluted at 1:1000 was added and incubated for 1 h at 37° C.
8) Step 4 was repeated.
9) 50 µl of BCIP/NBT (Bio-Rad Laboratories, Hercules, USA) development solution was laid into each well and incubated in the dark for 5-10 min until bluish spots developed.
10) Chromogen solution was discarded and the plates were rinsed two times with distilled water. The flexible plastic support from the bottom of the microplate was removed and the plates were tapped against paper towels. After air-drying, the plates were analyzed using an automated ELISpot reader (CTL Europe, Leinfelden-Echterdingen, Germany) and ImmunoSpot software (CTL, Cleveland, USA).

10. Western Blot Analyses

10.1 Materials

- Washing buffer – PBS, 0.1 % Tween 20
- Blocking buffer – PBS, 0.1 % Tween, 20.5% milk powder
- Laemmli's buffer 5x
  - 25 ml TRIS 1.5 M, pH 6,8
  - 12 g SDS
  - 60 ml glycerol
  - 13 ml distilled water
  - 2 ml 0.2% bromphenol
  - 37.3 ml β-mercaptoethanol

- Running buffer 5x - 100 g glycin
  - 15 g TRIS
  - 5 g SDS
- Blotting buffer -10 ml of 10x buffer (144 g glycin, 30.3 g TRIS, H_20 up to 1 l)
  - 20 ml methanol
  - 70 ml bidistilled water

All reagents were purchased from Merck, Darmstadt, Germany.

10.2 SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE under denaturing conditions (0.1% SDS) separates protein based on molecular size as they move through a polyacrylamide gel matrix toward the anode. The polyacrylamide gel is cast as a separating gel topped by a stacking gel and secured in an electrophoresis apparatus (BioRad).

The final acrylamide concentration in the stacking gel is 5%, while the acrylamide concentration in the separating gel has to be adjusted according to the protein size- 15% gel for myelin P0 (29 kDa).

For protein extraction, thymic stromal tissue was homogenized in Laemmli buffer [45] at a volume ratio of 1: 4 and boiled for 5 min. 10 µl of the lysate were loaded on the gel together with a high range 9.1-184.5 kDa molecular weight marker (Invitrogen, Groningen, The Netherlands) and a low range 14.4-97 kDa molecular weight marker (Pharmacia). In parallel, 11 µg of mouse myelin loaded onto the gel served as positive control. The electrophoresis was performed for 60 min using a current of 20 mA per gel in the presence of running buffer.

10.3 Immunoblotting

The blotting to the nitrocellulose membrane (Protran - Schleicher & Schuell, Dassel, Germany) was performed using a semi-dry blot system (BioRad). The membrane was stained by anti P0 monoclonal antibody - mouse IgG1 specific for the extracellular domain of P0 [6]. Coomassie staining was performed in order to assure whether there are protein bands with the expected size and to estimate the protein content.

After disassembling the polyacrylamid gel (Roth, Karlsruhe, Germany) and discarding the stacking gel, the separating gel was equilibrated for several minutes in blotting buffer. The

nitrocellulose membrane and Whatman filters (A. Hartenstein, Würzburg, Germany) were prewet and equilibrated for a few minutes in blotting buffer.

The transfer stack was assembled as follows:

Cathode electrode plate

3 sheets of Whatman 3 MM filter paper

Gel

Transfer membrane

3 sheet of Whatman 3 MM filter paper

Anode electrode plate

The proteins were transferred under constant current (100 mA per SDS-gel) for 45 min.

10.4 Immunodetection

Immunodetection was performed in the following steps:

- *Staining* the transfer membrane in Ponceau-S (Sigma) solution for 5 min on an orbital shaker to visualize the proteins and thereafter washing in distilled water. The membrane was cut into strips and the markers were taken away and dried between Whatman papers;
- *Blocking* of the strips for 1½ -2 h at RT with blocking buffer on an orbital shaker;
- *Incubation with the primary antibody:* the strips were incubated over night at 4° C on an orbital shaker with the primary antibody diluted in blocking buffer. Anti-P0 monoclonal antibody [6] was diluted to 1:100, 1:1000 and 1:10000;
- *Washing of the strips:* 3x 10 min with washing buffer;
- *Incubation with the second antibody:* the strips were incubated for 1h at RT on an orbital shaker with the secondary antibody – sheep anti-mouse IgG antibody (Pharmacia) - diluted 1:3000 in blocking buffer;
- *Washing of the membrane:* 3x 10 min with washing buffer;
- *Visualisation of proteins* using ECL Plus Detection Kit (Pharmacia): Strips were incubated for 1 min in equal volumes of reagent 1 and reagent 2. The detection reagent was drained off and the blots wrapped in plastic foil. The ECL hyperfilm (Pharmacia) was put on top of the blot and exposed for between 1 sec and 10 min.

11. RT-PCR

The reaction was performed using Titan One Tube RT-PCR System (Roche) a sensitive technique that allows detection of rare RNA messages in one step reaction.

a) Primers

The following combinations of primers (MWG) for P0 were used:

- Whole P0 protein: P01 total FWR 5'-ATT GTG GTT TAC ACG GAC AGG-3'
  P01 total REV 5'-TTC TTA TCC TTG CGA GAC TCC-3'

- Extracellular domain: P01 total FWR 5'-ATT GTG GTT TAC ACG GAC AGG-3'
  P02 ex REV 5'-CCT AGT GGG CAC TTT TTC AAA G-3'

- Transmembrane domain: P03 trans FWR 5'-TGG TGT TGG GAG CAG TGA T-3'
  P03 trans REV 5'-TCA GGT AGA AGA GCA ACA GC-3'

- Cytoplasmic domain: P04 cyt FWR 5'-TGC AGA GAA GGC TCA GTG C-3'
  P04 cyt REV 5'-TCT TAT CCT TGC GAG ACT CCC-3'

- Middle segment of P0: P05 middle FWR 5'-AAG GAT GGC TCC ATT GTC AT-3'
  P05 middle REV 5'-CAG CCA GCA GTA CCG AAT CA-3'

b) Reaction components (provided by Roche) were:

Master Mix 1 (total of 21 µl)

1 µl dNTP (10 mM each)
3 µl RNA template (± 0.15 µg/µl)
2.5 µl DDT (100 mM)
0.25 µl RNase Inhibitor
14.25 µl H_2O

Master Mix 2 (total of 25 µl)

10 µl 5x RT-PCR Buffer
1 µl Enzyme mix
14 µl H_2O

Master Mix (MM) 1, Master Mix 2 and the primers were brought together in one 0.5 ml Eppendorf tube and subjected to the following RT-PCR reaction (using a Perkin Elmer thermocycler).

Total (50 µl)	Ex (50 µl)	Trans (50 µl)
21 µl MM 1	21 µl MM 1	21 µl MM 1
2 µl P01 total FWR (58°)	2 µl P01 total FWR (58°)	2 µl P03 trans FWR (57°)
2 µl P01 total REV (58°)	2 µl P02 ex REV (58.5°)	2 µl P03 trans REV (57.3°)
2 5µl MM 2	25 µl MM 2	25 µl MM 2

Cyt (50 µl)	Middle (50 µl)
21 µl MM 1	21 µl MM 1
2 µl P04 cyt FWR (58.8°)	2 µl P05 middle FWR (55.3°)
2 µl P04 cyt REV (59.8°)	2 µl P05 middle REV (59.4°)
25 µl MM 2	25 µl MM 2

c) RT-PCR conditions

1 x	Incubation:	50° C,	30 min
	Denaturation:	94° C,	2 min
10x	Denaturation:	94° C,	15 sec
	Annealing:	59° C,	30 sec
	Elongation:	68° C,	45 sec
40x	Denaturation:	94° C,	15 sec
	Annealing:	59° C,	30 sec
	Elongation:	68° C,	45 sec + 5 sec. more at each cycle
1x	Elongation:	68° C,	7 min

RT-PCR products were visualized by electrophoresis on a 1.5% agarose gel using a 50 bp ladder (Pharmacia) as marker.

In order to determine the specificity of the RT-PCR products, they were cloned and sequenced as follows:

d) Nucleic acid cleaning and purification

RT-PCR products were subjected to agarose gel 1.5% electrophoresis using a 50 bp ladder (Pharmacia) as marker. Bands of the expected size were cut from the gel and extracted using MinElute™ Gel Extraction Kit (Qiagen).

e) Subcloning of PCR products

Purified RT-PCR products were cloned using the Zero Blunt™ PCR Cloning Kit (Invitrogen). This kit is designed to clone blunt PCR fragments (or any blunt DNA fragment) with a low background of recombinants. Ligation of the insert into the linearized pCR-Zero Blunt vector and transformation into the One Shot™ Top 10 competent cells was done according to manufacturer's instructions. Transformed bacteria were plated on LB agar plate containing kanamycin as a selection marker and incubated at 37° C over night.

f) Plasmid extraction

Plasmid extraction from transformed bacteria was performed using Wizard^R Plus SV Minipreps DNA Purification Systems (Promega, Madison, USA).

g) DNA Digestion with restriction enzymes

EcoRI DNA digestion was performed in order to analyse the insertion of the DNA fragments into the pCR-Zero Blunt vector.

Reaction components:

1 µg plasmid DNA

2 µl 10x restriction enzyme buffer

5 U EcoRI restriction enzyme (New England Biolabs, Frankfurt am Main, Germany)

H_2O up to 20 µl

The enzymatic reaction was carried on at 37° C for 1 h.

Digestion fragments were visualized by electrophoresis on a 1% agarose gel.

h) Sequencing reaction

Sequencing (Applied Biosytem sequencer, Foster City, USA) of the pCR-Zero Blunt plasmids that contained an insert was used to prove the identity of the DNA fragment.

1. The following sequencing primers (Gibco) were used:

M13F (universal primer) – 5' –GTA AAA CGA CGG CCA G-3'

M13R (universal primer) – 5'-CAG GAA ACA GCT ATG AC-3'

Preparation of the sequencing reaction was done using the following protocol:

2. Reaction components (for a total of 20 µl):
   4 µl ABI Prism®BigDye™ Terminator (Applied Biosystems)
   0.5 µl forward or reverse primer (10 nmol/µl)
   500 ng DNA
   H_2O up to 20 µl
3. PCR conditions:

1 cycle-	denaturation	3 min at 95° C
25 cycles -	denaturation	30 sec at 94° C,
	annealing	1 min at 55° C
	elongation	3 min 60° C
1 cycle -	elongation	5 min at 72° C

4. DNA precipitation – 20 µl PCR product were precipitated with 80 µl isopropanol 75%, vortexed and incubated for 15 min at room RT, followed by centrifugation at top speed at RT for 20 min. After the removal of the supernatant, the DNA pellet was washed with 250 µl isopropanol 75%, mixed gently and centrifuged for another 5 min, top speed, at RT. The pellet was left to dry for 10-15 min at RT.
5. Resuspension of DNA – in 18 µl template suppression reagent (Applied Biosystems) by pipetting up and down to ensure proper solution of the DNA
6. Denaturation of DNA - 3 min at 95° C

12. Immunohistochemistry

12.1 Paraffin embedding

The nerves were washed several times with 0.1 M PBS to completely remove the paraformaldehyde (PFA) and left in this buffer at 4° C until further processing. The paraffin embedding was done automatically using Citadel 1000 (Thermo Shadon, Pittsburgh, USA). After an immersion fixation, the tissue was dehydrated by an alcohol series (50%, 70%, 80%, 96%, and 2 x 100% ethanol). A 1:1 mixture of 100% ethanol/chloroform was used for the delipidization (2 x 30 min), followed by incubation in pure chloroform (2 x 30 min). Subsequently, the tissue was incubated in paraffin (1 x 50 min, 1 x 30 min) and embedded in plastic moulds using a tissue block system (TBS 88; Mechte Medizintechnik, Germany).

12.2 Immunohistochemistry

Paraffin embedded nerves were cut in 4 µm-thick sections and transferred to distilled water, adhered on polyL-lysine coated slides and dried at 37° C, overnight. Tissue sections were deparaffinized by incubating them two times, 15 min in 100% xylene, followed by dehydration with a descending alcohol series (2 min 100% ethanol, 2 min 96% ethanol, 2 min 70% ethanol). After dehydration, the sections were washed three times with TBS.

Unspecific binding sites were blocked with 10% BSA for 30 min. Primary antibodies F4/80 IgG2b rat anti-mouse (Serotec, Eching, Germany) against macrophages and CD3 IgG1 rat anti-human (Serotec) against T-cells were diluted in PBS 10% BSA at 20 µg/ml and 5 µg/ml respectively and applied on the sections over night at 4° C. To quench endogenous peroxidases, sections were incubated in 100% methanol per 0.25 M sodium azid per 3% H_2O_2 for 10 min.

After rinsing the slides three times for 5 min with TBS, detection of the primary antibody was achieved using a biotinylated rabbit anti-rat IgG antibody (Vector, Burlingame, USA) 2.5 µg/ml applied on the sections for 45 min. After washing three times with TBS, the sections were incubated 30 min with streptavidin-HRP (-**h**orse**r**adish **p**eroxidase), which was diluted at 1:100.

3,3' Diaminobenzidine (DAB; Kem-En-Tec, Copenhagen, Denmark) was used as chromogen substrate for the horseradish peroxidase. A DAB tablet (10 mg) was dissolved in 10 ml 0.05 M Tris-HCl, pH 7.5. 0.015% H_2O_2 was added, the solution filtered and applied to the sections. The colour reaction was stopped after approximately 10 min by rinsing the section in distilled water. Additionally, the sections were counterstained with hematoxylin for 20 sec, rinsed with distilled water, dehydrated (2 x 2 min 70% ethanol, 2 min 90% ethanol, 2 x 2 min 96% ethanol, 3 x 2 min 100% ethanol and 10 min Xylene) and mounted with VitroClud (R. Langenbrinck, Emmendingen, Germany).

All incubation steps were performed in a humid chamber at room temperature.

13. Mouse peripheral myelin (MPM) preparation

MPM was prepared after the method established by Norton and Poduslo, 1973 [59]. Briefly, 5 g of sciatic nerves were collected from approximate 300 mice derived from our retired colony and stored at -80° C until the purification procedure started.

- **Sucrose gradient purification**

Nerve tissue from -80° C was brought to room temperature and homogenized in 30 ml sucrose 0.32 M with a tissue homogenizer. The homogenate was placed in two ultracentrifuge tubes and filled up to 40 ml with sucrose 0.32 M. 12ml of sucrose 0.8 M was gently laid at the bottom of each tube in order to prevent mingling of the two phases. The tubes were centrifuged at 30,000 rpm for 45 min (using a centrifuge from Kontron Instruments, Neufahrn, Germany). The deposit at the interface, which represents the myelin, was collected and washed with 200 ml distilled water by centrifuging at 30,000 rpm for 45 min. After discarding the supernatant, the myelin was mixed with 20 ml distilled water and frozen at -80° C followed by lyophilization.

14. Fetal thymic organ culture (FTOC)

Fetal thymi were dissected from embryos at day 15 and cultured on Millipore filters (8mm pore size) in 12-well plates containing RPMI 1640 medium supplemented with 10% FCS. FTOC was treated with deoxyguanosine 1.35 mM for the duration of the culture. At three days after culture set-up, medium and supplements were renewed. After six days, the culture was terminated and prepared for RT-PCR analyses. This procedure allows epithelium to grow while depleting FTOC of thymocytes [36, 37].

Results

1. WT and P0 ko C57BL/6 mice are both fully immunocompetent

In order to see whether both subtypes are fully immunocompetent we immunized wt and P0 ko mice with PBS emulsified in CFA, which contain inactivated mycobacteria, as foreign antigen. We performed cytokine ELISpot assays to measure the frequency of antigen-specific T-cells in freshly primed cell populations (Fig.6B). The magnitude of the IFN-γ recall response to this control antigen was comparable in wt and P0 ko mice despite the fact that the P0 ko mice show a strikingly different phenotype (small size, impaired movements,

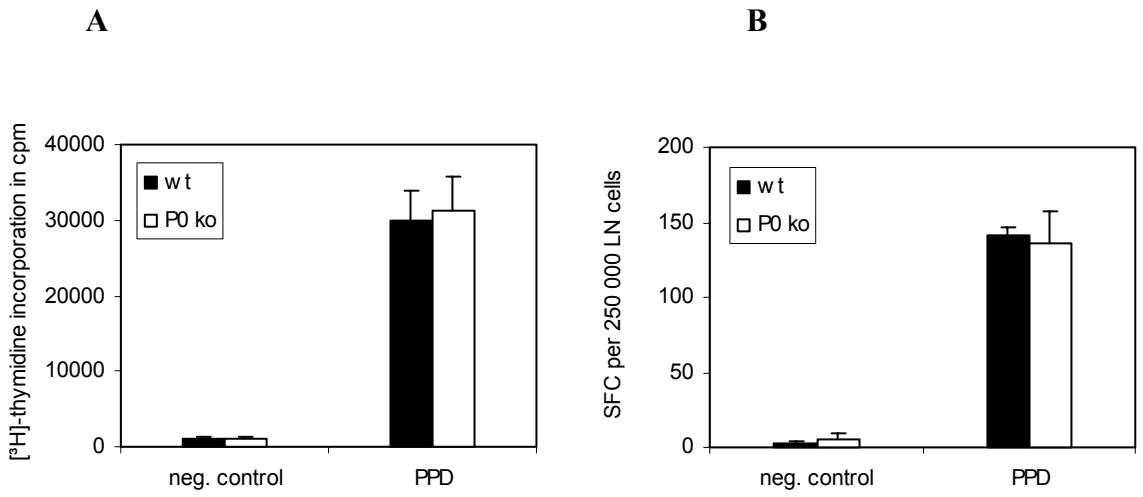


Figure 6. Immunoreactivity status of wt and P0 ko mice. Three wt and three P0 ko mice were immunized with PBS in CFA. After 10 days of in vivo priming, LN cells from each group of mice were pooled and tested in proliferation assays and IFN-γ ELISpot assay at a concentration of 250,000 LN cells per well. Mean + SD from triplicate wells. The experiment was reproduced three times. **(A)** – proliferation assay. Y axis represents [³H]-thymidine incorporation in counts per minute (cpm). **(B)** – ELISpot assay. Y axis represents numbers of spot forming cells (SFC) per 250,000 LN cells. X axis in both assays shows the antigens used. PPD was used as recall antigen at a final concentration of 10 µg/ml.

tremors) which could also affect their physiology through deficient innervation of the immune organs. Despite their neurological defect, the P0 ko mice were fully immunocompetent. This result was confirmed also in proliferation assays (Fig.6A).
The IL-2 recall response to the same antigen PPD showed similar frequencies of specific memory T-cells. No detectable antigen specific IL-4 and IL-5 production were observed, showing that wt and P0 ko C57BL/6 mice mount a type-1 immune response when immunized with CFA (not shown).

2. P0 peptide 5 contains the T-cell immunodominant determinant in P0 ko mice

Our hypothesis was that due to the presence of P0 during ontogeny wt mice are tolerized towards P0, while P0 ko mice which lack the protein should be reactive. Because of the unavailability of the purified soluble myelin protein P0 we used overlapping synthetic peptides designed according to the P0 amino acid sequence.

2.1 P0 peptide pool array

In order to find the immunodominant peptide in P0, we designed 21 twenty-mer peptides overlapping by 10 amino acids and spanning the entire sequence of P0 (Fig.7). These peptides were grouped in 10 different pools [82] such that overlapping peptides were not present in the same pool and every two pools contained a common peptide. The pools were arranged into an array with 5 ‘column’ pools and 5 ‘row’ pools (Fig.8).
For each reactive peptide, there must be a ‘row’ pool and a ‘column’ pool that induce a positive reaction in the ELISpot assay. By finding the intersection of these two reactive pools in the array, the specific reactive 20-mer peptide can be identified.

Peptide number	Peptide sequence	Localization in the amino acid sequence of P0
1.	I VVYTDREIYGAVGSQVTLH	1-20
2.	GAVGSQVTLHCSFWSSEWVS	11-30
3.	CSFWSSEWVSDDISFTWRYQ	21-40
4.	DDISFTWRYQPEGGRDAISI	31-50
5.	PEGGRDAISIFHYAKGQPYI	41-60
6.	FHYAKGQPYIDEVGAFKERI	51-70
7.	DEVGAFKERIQWVGDPRWKD	61-80
8.	QWVGDPRWKDGSIVIHNLDY	71-90
9.	GSIVIHNLDYSDNGTFTCDV	81-100
10.	SDNGTFTCDVKNPPDIVGKT	91-110
11.	KNPPDIVGKTSQVTLYVFEK	101-120
12.	SQVTLYVFEKVPTRYGVVLG	111-130
13.	VPTRYGVVLGAVIGGILGVV	121-140
14.	AVIGGILGVVLLLLLLFYLI	131-150
15.	LLLLLLFYLIRYCWLRRQAA	140-160
16.	RYCWLRRQAA LQRRLSAMEK	151-170
17.	LQRRLSAMEKGRFHKSSKDS	160-180
18.	GRFHKSSKDSSKR	171-183
19.	SSKRGRQTPVLYAMLDHSRS	180-199
20.	YAMLDHSRSTKAASEKKSKG	191-210
21.	KAASEKKSKGLGESRKDKK	201-219

Figure 7. Overlapping peptides covering the entire P0 amino acid sequence. Note that peptide 18 (with 13 amino acid only) could not be synthesized at full length. All peptides were obtained from Biotrend, Köln, Germany.

Pools	A	B	C	D	E
I	1	3	5	7	20
II	9	11	13	15	
III	17	19	21	2	
IV	4	6	8	10	
V	12	14	16	18	

Figure 8. P0 peptide pool array. Row (left) and column (top) pools of 4 or 5 P0 peptides each (pool E contains only one peptide) were then constructed as shown with the numbers in the array corresponding to the individual peptides with peptide 1 from amino acids 1–20, peptide 2 from 11–30, etc., according to the complete sequence of the P0 protein. Pools were constructed so that overlapping peptides e.g., peptides 1 and 2 are not contained in the same pool. Pools were used to challenge peripheral myelin primed T cells. At the intersection of the responding pools, the reactive peptide is found, e.g. a positive response in pools B and IV points to peptide 6. Using this approach, the screening of the immune response by ELISpot technique is greatly facilitated.

2.2. The P0 peptide pool array identifies peptide 5 as the immunogenic peptide in the P0 amino acid sequence

C57BL/6 wt and P0 ko mice were immunized in one footpad with CFA containing bovine peripheral myelin (BPM) as a source of P0 and pulsed with 400 ng PT i.p. After 10 days, spleen cells were tested in the highly sensitive ELISpot assay using the peptide pool array to stimulate the production of IFN-γ of BPM primed cells. As shown in Fig.9, peptide pools I and C were able to induce IFN-γ spot formation. With regard to the P0 peptide pool array (Fig.8) this result indicates indirectly that P0 peptide 5 contains the immunodominant epitope as this is the only common peptide in these two reactive pools.

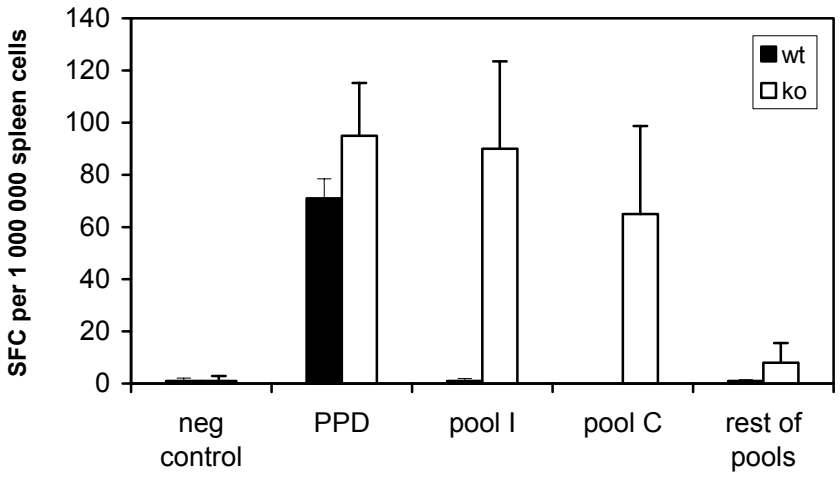


Figure 9. ELISpot responses to P0 peptide pool array. Wt and P0 ko mice were immunized with 2.5 mg BPM emulsified in CFA. Ten days later splenocytes were harvested and stimulated with the indicated P0-derived pools of peptides in IFN-γ ELISpot assay. Y axis represents number of spot forming cells per 1,000,000 spleen cells. X axis shows the recall antigens used in culture. Because no significant spot formation was seen with pools II, III, IV, V, A, B, D and E these results are presented in a single bar. Peptides in the pools were used in cell culture at a final concentration of 10 µg/ml each. Mean + SD for three individual mice. The experiment was reproduced twice with similar results.

2.3 Fine specificity mapping with overlapping 20-mers confirms P0 peptide 5 as strongly immunogenic and peptide 6 as less immunogenic

In order to directly confirm the finding of the P0 immunogenic peptide it is necessary to prime the animals with species-specific P0, in our case mouse P0. The immune cells are challenged in vitro with individual overlapping P0 peptides instead of pools of peptides. As source of P0, mouse peripheral myelin (which contains about 60% P0) was purified from mouse sciatic nerves and used in the immunization protocol. Ten days after immunization with MPM, wt and $P0^{-/-}$ mice were sacrificed and the cytokine production and proliferative response of the LN cells were tested. As expected, the response recalled with peptide 5 was by far the strongest and followed by the overlapping peptide 6 (Fig.10). Some P0 ko mice showed a low degree of reactivity also towards peptide 4 (not shown). None of the peptides was stimulatory in wt mice. Peptides 4, 5 and 6 are overlapping peptides comprising the amino-acid sequence 31-70 located within the extracellular domain of P0. The fact that peptide 6 and occasionally peptide 4 were also immunogenic in P0 ko mice

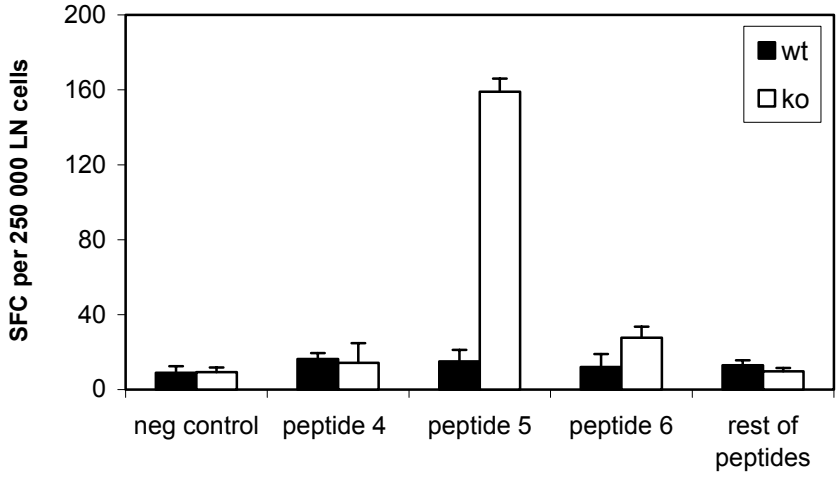


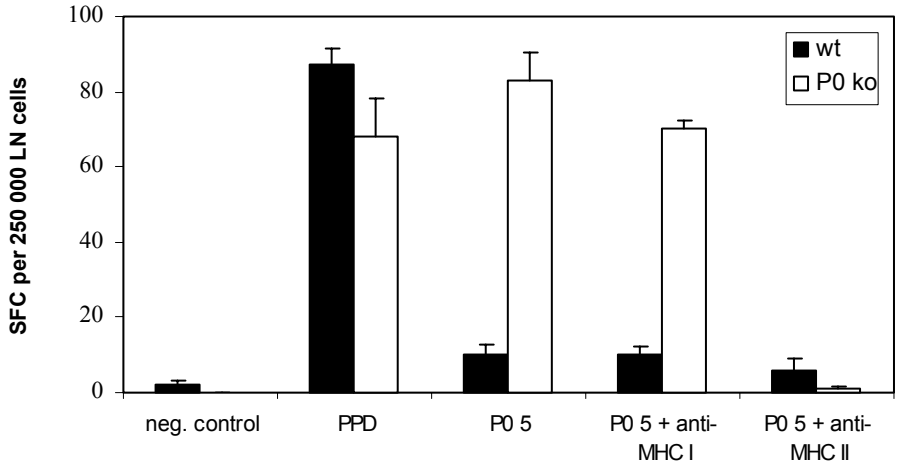
Figure 10. Mapping of the P0 immune response in wt and P0 ko mice. Six mice, three for each genotype, were immunized with 2.5 mg MPM emulsified in CFA and injected i.p. with 400 ng PT. After 10 days, LN cells were pooled and tested in ELISpot assays for specific IFN-γ production following peptide challenge. Because no significant spot formation was seen with peptides 2, 3 and 7-21, these results are presented in a single bar. Y axis represents number of spot forming cells per 250,000 LN cells. X axis shows the recall antigens used in culture. Mean + SD for triplicate wells. The experiment was reproduced three times with similar results.

suggests that the amino acid sequence P0 31-70 constitutes an imunodominant region where most potent T-cell epitope(s) reside(s) in the core P0 peptide 5.

3. The response to P0 peptide 5 under the CFA polarizing conditions is MHC II restricted and of the Th1 type

In order to characterize the immune response to P0 peptide 5 under the polarizing conditions imposed by peptide/CFA immunization, different cytokines representative for Th1 and Th2 types of immunity were tested. In addition, the activity of MHC I and MHC II molecules was blocked alternatively in order to test the MHC restriction of the immune response to peptide 5. CFA is the most widely used adjuvant to induce a type-1 immunity, which is reflected mostly by the secretion of cytokines IFN-γ and IL-2. Mice were immunized with P0 peptide 5 emulsified in CFA and the antigen-specific recall response was tested in draining LN cells by ELISpot assay.

A



B

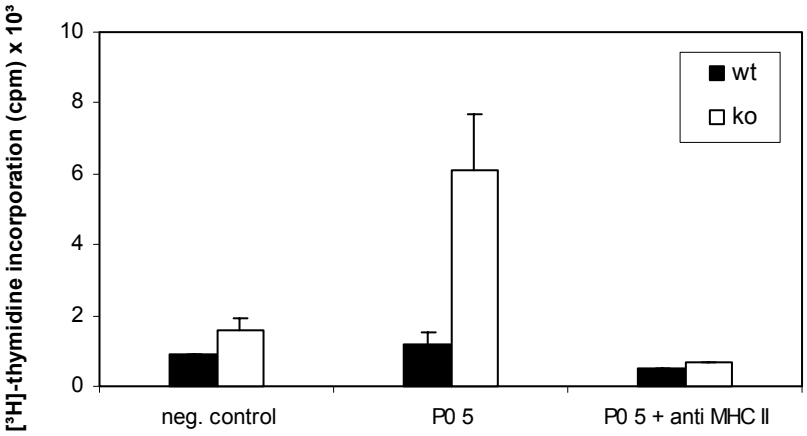


Figure 11. Characterization of the immune response driven by peptide 5/CFA immunization. Mice were immunized in foot pads each with 40 µg P0 5 emulsified in CFA. **(A)** Specific reactivity of IFN-gamma secreting LN cells was tested in response to peptide 5 alone or in combination with anti-mouse H-2D^b/H-2K^b (MHC I) or anti mouse I-A (MHC II) antibodies. Mean + SD for three mice per group. Experiment was reproduced twice with similar results. **(B)** P0 5 primed LN cells from three mice per group were pooled and tested in proliferation assays. Mean + SD for triplicate wells. X axis on both graphs shows the recall antigens used in culture.

We found that the number of P0 5 specific IFN-γ secreting cells equalled that of IL-2 secreting cells (not shown). No specific IL-4 and IL-5 secreting cells as indicators of type-2 immunity was found (not shown), proving that the immune response to P0 peptide 5 is of type-1. When LN cells were co-cultured together with an anti-MHC II antibody, the immune response to peptide 5 dropped almost to the level of the negative control (Fig.11A). Blocking the activity of MHC I molecules did not influence the response, suggesting that the immune response to peptide 5 is restricted by MHC II molecules. Results were also confirmed in proliferation assays (Fig.11B).

4. The P0 peptide 5 specific T-cell repertoire is inactivated in wt

It is expected that some forms of tolerogenic mechanisms should operate to prevent the immune system from autoimmune attack. That means T-cells with high or intermediate specificities for P0 must be either removed from mature T-cell repertoire or suppressed. To a certain extent both mechanisms might operate. On the other hand, in homozygous P0 deficient mice, P0 protein may behave as a foreign antigen since they develop and reach maturity in the absence of this protein.

In order to detect P0 specific cells, we immunized wt and homozygous P0 deficient mice with 40 µg peptide 5 emulsified in CFA. After 10 days, LN cells were challenged with the same peptide in a dose dependent manner.

As shown in Fig.12, in ko mice the peptide 5 response could be recalled with doses as low as 0.1 µg/ml and followed an ascending curve until 100 µg/ml, the highest dose used to re-stimulate LN cells. Upon recall with 100 µg/ml, 100 IFN-γ producing cells per 250,000 LN cells were found in ko mice, five times as much as in wt mice. In wt mice, the lowest amount of peptide to recall a specific response was 10 µg/ml which stimulated about 7 LN cells out of a quarter of million. In P0 ko mice the same degree of reactivity was obtained with 0.1 µg/ml of peptide 5. There was a 2 Log unit shift to the left in the response of wt P0 specific T-cells as compared with ko mice suggesting that ko mice have a P0 specific TC repertoire with a much higher avidity than that of wt mice.

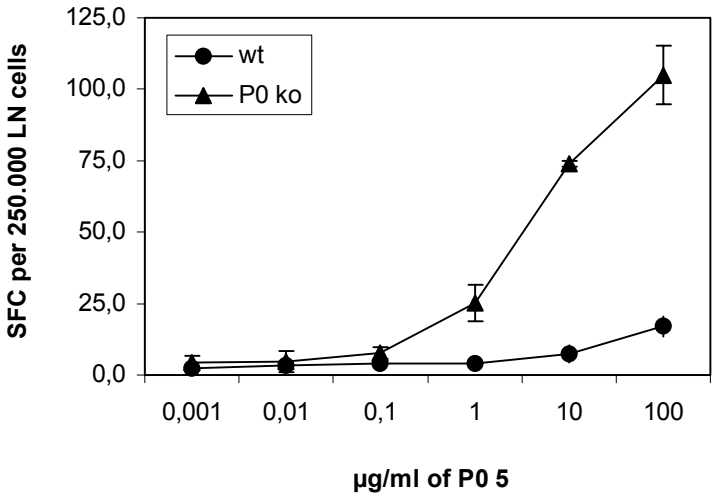


Figure 12. Peptide 5 dose dependent recall response in wt versus P0 ko mice. Wt and P0 ko mice were immunized with 40 µg of peptide 5 and tested for reactivity to the peptide in a dose dependent manner. The data show IFN-γ response of LN cells that were pooled from three mice for each subtype. Mean ± SD for triplicate wells. Experiment was reproduced twice with similar results.

5. Induction of tolerance in P0 heterozygous mice is not dependent on gene dosage

Next, we wanted to investigate whether tolerance/reactivity towards P0 was affected in heterozygous P0 deficient mice. $P0^{+/-}$ mice have one allele of the P0 gene in contrast to wt mice (two alleles) and $P0^{-/-}$ mice (zero alleles). Therefore there is a uniform increase in the gene dosage of P0 from $P0^{-/-}$ to $P0^{+/-}$ and $P0^{+/+}$. If tolerance induction for a particular self-antigen would be directly dependent on the gene dosage accounting for that antigen, a different degree of tolerance should be expected when only half of the gene is transcribed. In order to investigate this possibility, we immunized all three genotypes ($P0^{-/-}$, $P0^{+/-}$ and $P0^{+/+}$ mice) with P0 peptide 5. IFN-γ production at the single cell level and proliferation activity was measured in recall cultures after 10 days.

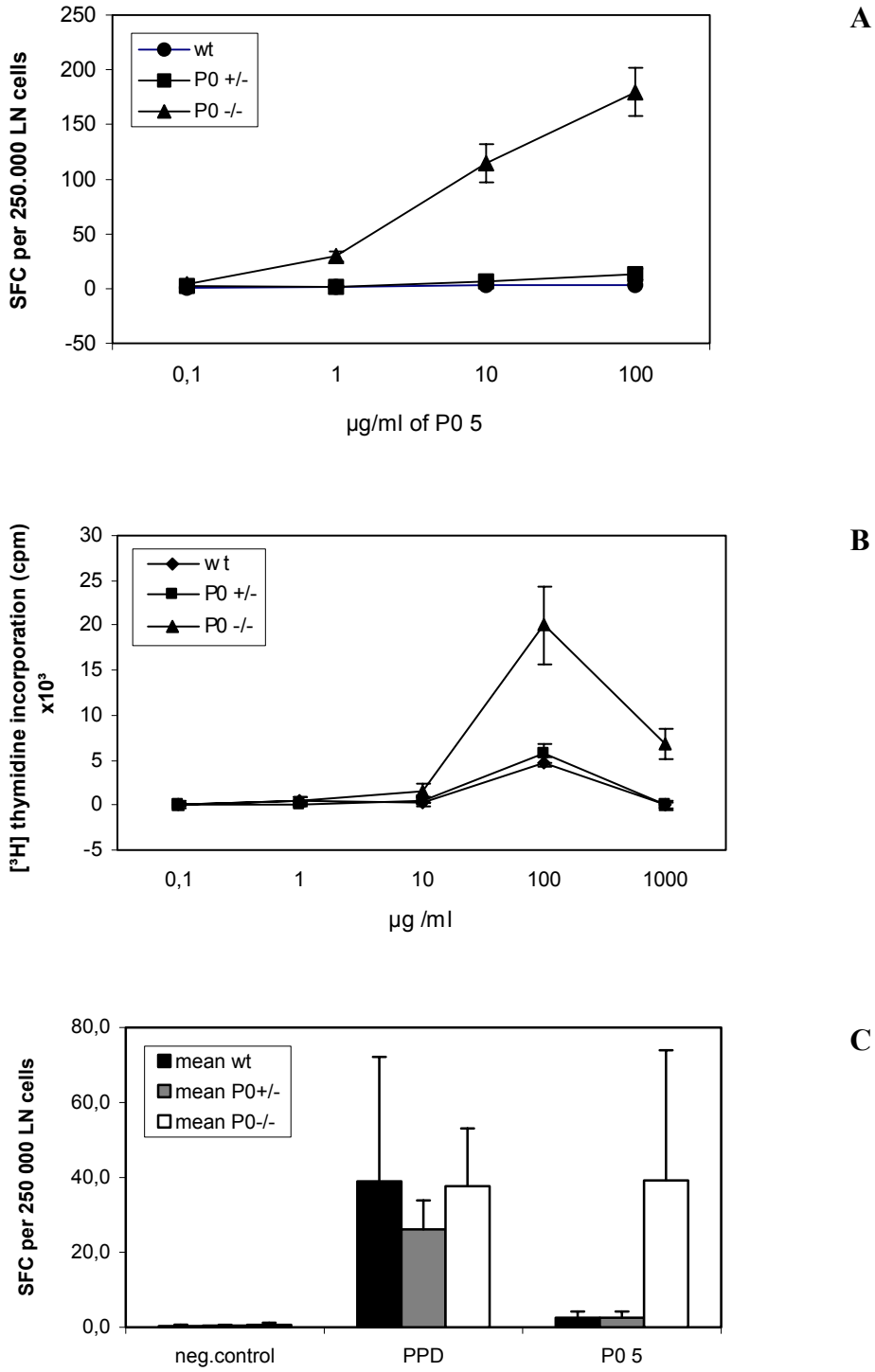


Figure 13. Response to peptide 5 depending on P0 gene dosage. Wt, $P0^{+/-}$ and $P0^{-/-}$ were immunized with 50 µg P0 peptide 5 and the reactivity was tested in IFN-γ ELISpot assay **(A)** and proliferation assay **(B).** The response was recalled with increasing doses of peptide. Mean ± SD from groups of three mice. **(C)** The response was recalled with peptide 5 at a concentration of 10 µg/ml. In addition, positive and negative controls for all subtypes are included. Mean + SD for three mice tested individually.

We found that the recall response to P0 measured by both proliferation assay and Elispot was very low and with similar magnitude in wt and $P0^{+/-}$ deficient mice. In contrast, it was much higher in $P0^{-/-}$ mice (Fig. 13, A, B and C). These results reveal that tolerance towards P0 is fully established in heterozygous P0 deficient mice as well.

It was previously reported that $P0^{+/-}$ mice display immune cell infiltration in the nerves at a higher level then their wt counterparts when they are older then 6 months [69] while no difference was observed in mice at 4 months of age. Concomitantly, the infiltration of immune cells in $P0^{+/-}$ associates with demyelination and myelin degeneration of the peripheral nerves. These two findings would suggest that autoimmune responses against myelin proteins occur later in life in $P0^{+/-}$ and cause demyelination. In order to investigate whether the demyelination is antigen dependent, we tested freshly isolated naïve spleen cells from wt and $P0^{+/-}$ mice with ages ranging from 6 to 20 months in ELISpot and proliferation assays.

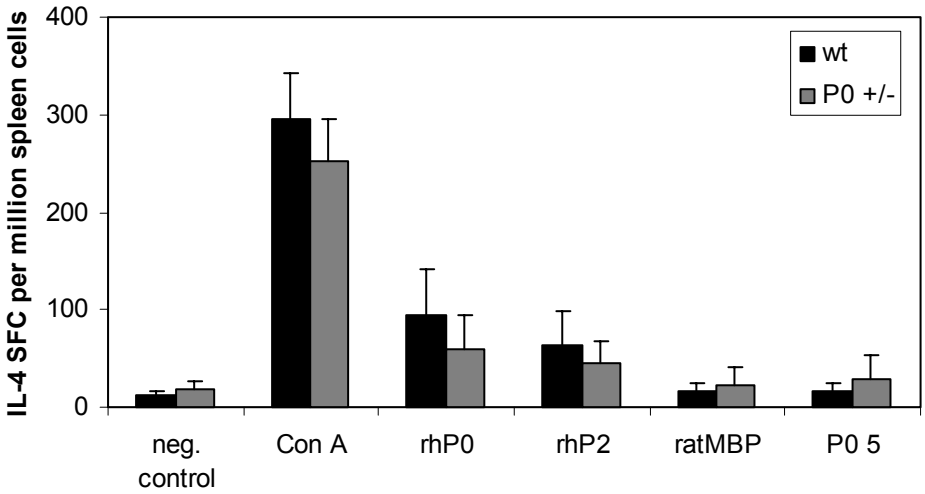


Figure 14. Naive spleen cell response to peripheral myelin antigens. Freshly isolated naïve spleen cells were cultured together with different myelin antigens: recombinant human P0 (rhP0), recombinant human P2 (rhP2), rat myelin basic protein (rat MBP) and P0 peptide 5, and specific cytokine production and proliferative response were tested. Shown here it is an IL-4 ELISpot assay. Mean + SD of four experiments. Each experiment includes 3-4 mice per group.

There was no difference between wt and $P0^{+/-}$ in response to different peripheral myelin antigens when the ability of naïve spleen cells to secrete IL-4 was tested (Fig.14). Moreover, the overall proliferative response to myelin antigens showed the same pattern (data not shown). With regard to this approach, it is suggested that the demyelination seen in $P0^{+/-}$ mice is not antigen dependent. There was no antigen specific IFN-γ response in both wt and $P0^{+/-}$ (data not shown).

6. Myelin P0 protein is expressed in thymic stromal cells but not in thymocytes

In order to learn more about the site of tolerogenic mechanism that accounts for P0 tolerance we looked first for the expression of P0 in thymus. We investigated the presence of P0 in whole thymus, in stroma tissue, thymocytes and FTOC. This was done by RT-PCR and Western Blot analyses. Under the term of thymocytes we encompass the typical thymocytes (precursors of T-cells), mature T-cells ready to leave the thymus, dendritic cells and macrophages, all of hematopoietic origin.

6.1 Detection of P0 in thymus at the RNA level

Total RNA was extracted from whole adult thymus, thymocytes and from thymic stroma - which contains largely epithelial cells - and used in an RT-PCR reaction. Five pairs of RT-PCR primers were designed in order to detect the total length of the P0 message as well as different fragments of RNA message corresponding to the extracellular, transmembrane and cytoplasmic domains of P0. A last pair of primers would amplify a fragment located in the middle of the P0 RNA message, corresponding to a 110-190 amino acids sequence in the P0 protein. We chose this strategy in order to ensure the detection of the P0 message, in case only parts of P0 and not the whole transcript are present in the thymus.
The sequence of the five pairs of primers is described in Materials and Methods. The expected size of the products was 652 bp for the total P0, 368 bp for the extracellular fragment, 69 bp for the transmembrane fragment, 155 bp for the cytoplasmic fragment and 231 bp for the middle fragment.

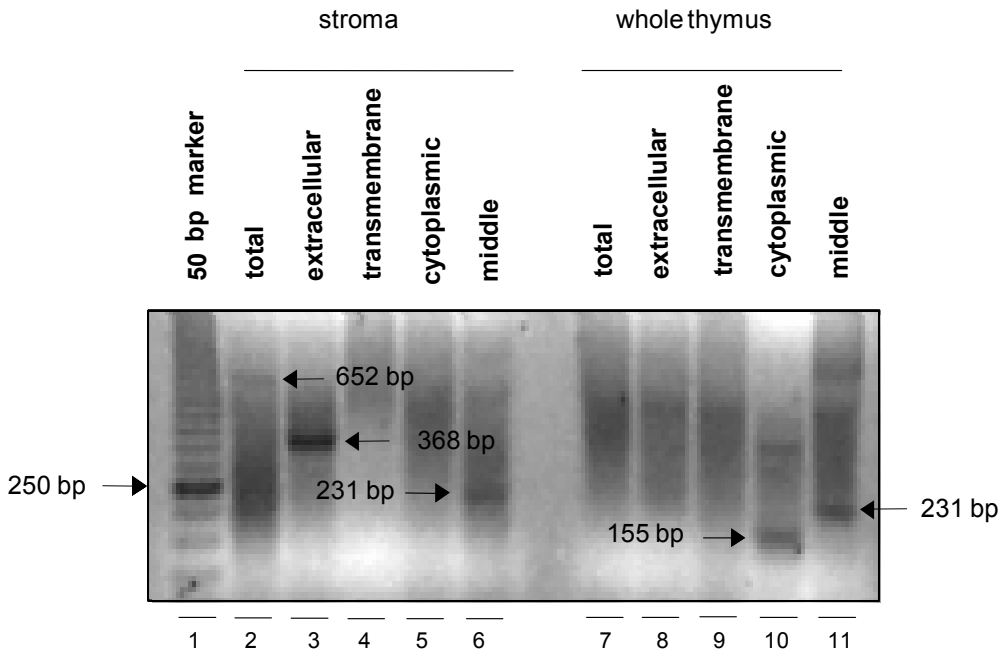


Figure 15. Detection of P0 transcripts in the thymus by RT-PCR. 1 µg of total RNA extracted from stroma (lanes 2-6) and whole thymus (lanes 7-11) was used as template in an RT-PCR reaction. Four different combinations of primers were used to amplify the **total** length of the RNA message (lanes 2,7) as well as the three cellular domains- **extracellular, transmembrane** and **cytoplasmic** (lanes 5, 10). An additional pair of primers amplified a fragment located in the **middle** of the molecule (lanes 6, 11). Positive bands were found on lanes 2, 3, 6, 10 and 11. Lane 1 - 50 bp DNA ladder.

Bands of the expected size were obtained for the total, extracellular and middle fragments in the stroma extract (Fig. 15, lanes 2, 3, 6) and for the cytoplasmic and middle fragments in the whole thymus extract (Fig. 15, lanes 10, 11). The specificity of these bands was confirmed by sequencing (not shown).

6.2 Detection of P0 transcripts in fetal thymic organ culture at the RNA level

To confirm that P0 is expressed in stromal cells, we isolated thymic lobes from embryos on day 15 and set up fetal thymic organ culture treated with deoxyguanosine [36]. This procedure depletes FTOC of thymocytes but allows thymic epithelium to grow.

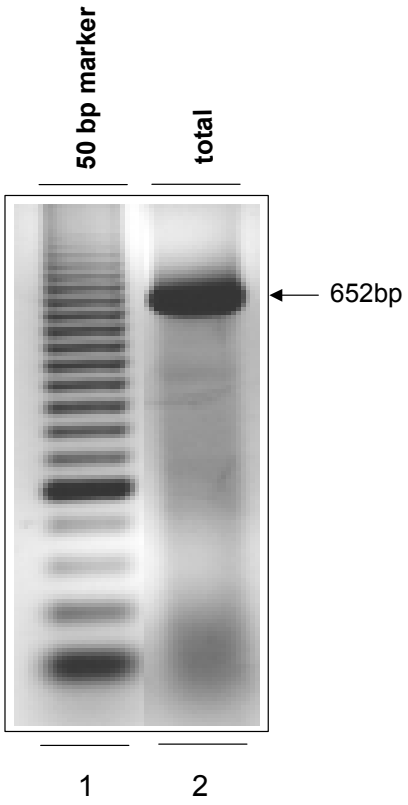


Figure 16. Detection of P0 transcripts in FTOC by RT-PCR. 1 µg of total RNA extracted from FTOC was used as template in an RT-PCR reaction. Previously decribed combination of primers was used to amplify the **total** length of the RNA message (lane 2). Lane 1 - 50 bp DNA ladder.

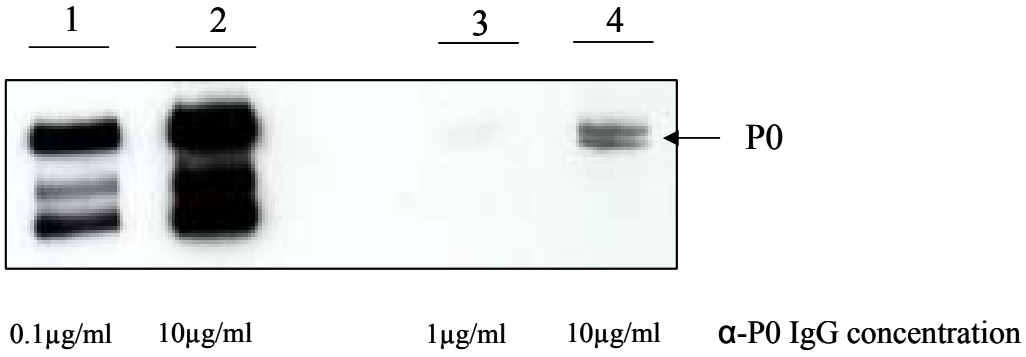


Figure 17. Detection of P0 protein in the thymus by Western Blot analyses. P0 protein was detected in a thymic epithelial tissue protein extract (lanes 3, 4) by using different concentrations of α-P0 antibody as indicated. Mouse myelin was used as a positive control (lanes 1, 2).

After six days of culture, thymic rudiments were tested for the presence of P0 mRNA. A strong signal corresponding to the total length of the P0 mRNA was detected by RT-PCR. The specificity of the signal was confirmed by sequencing.

6.3 Detection of P0 in thymus at the protein level

To confirm the presence of P0 in thymus observed by RT-PCR, proteins were extracted from thymic stromal cells and Western Blot analyses were performed. The proteins stained specifically with the monoclonal anti-P0 antibody as indicated by the dark bands in lanes 1, 2 and 4. Lanes 1 and 2 represent specific P0 staining on mouse myelin extract serving as positive control. Lane 4 shows specific staining of proteins from thymic stromal tissue. The presence of a specific band confirms the expression of P0 in thymus (Fig.17). A very faint but specific band can be seen also in lane 3. Double bands that appear in all positive lanes represent degradation products of P0.

7. Wild type bone marrow derived cells are able to induce partial tolerance in lethally irradiated P0 knockout recipients

To evaluate the influence of hematopoietic cells on immune reactivity to P0 we constructed BM chimeras. Wt and P0 ko mice received lethal irradiation to ablate their immune system and then were reconstituted with syngeneic $P0^{-/-}$ BM cells and wt BM respectively. This approach would ensure that wt BM cells transferred to irradiated $P0^{-/-}$ mice would give rise to a T-cell repertoire which has developed in the absence of P0 expression. Similarly, $P0^{-/-}$ BM cells in irradiated wt recipients would generate a T-cell repertoire educated in the presence of P0.

To confirm the functionality of chimerism, we also generated wt → wt and ko → ko chimeras as controls. The pattern of their P0 5 specific response mirrors that of wt and P0 ko mice respectively (Fig.18). Ko → wt chimeras show little reactivity towards P0 which is comparable with wt → wt chimeras, suggesting that the presence of P0 gene in the hematopoietic system is not necessary for tolerance induction (Fig.18).

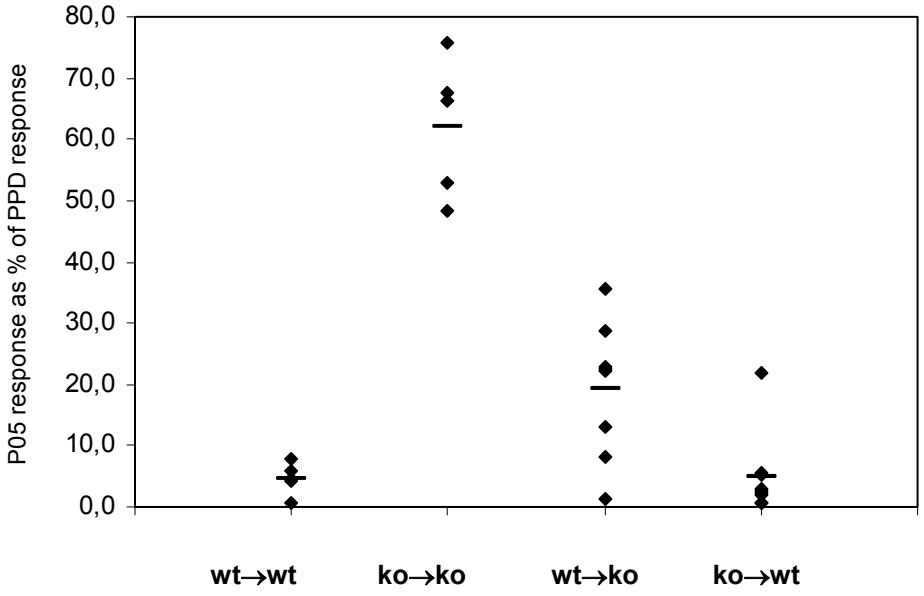


Figure 18. P0 tolerance status in BM chimeras. At two months after bone marrow reconstitution, chimeric mice were immunized with 50 µg of P0 peptide 5. After 9-11 days, LN cells were harvested and their proliferative response to P0 5 was tested. Wt → wt and ko → ko served as negative respectively positive controls. X axis indicates the type of chimeras. On Y axis the numbers designate the magnitude of P0 5 response as percentage of PPD response. Diamonds represent individual chimeras; for wt → wt, n = 5; ko → ko, n = 5; wt → ko, n = 8; ko → wt, n = 9.

Also, chimeric mice with functional P0 gene only in the hematopoietic system show reduced P0 reactivity when compared with ko → ko ($P<0.05$) although their distribution encompasses a variable range of values. This suggests that hemopoietic cells are able to lower the reactivity towards P0 even when the expression of P0 gene is confined to cells of BM origin. Nevertheless, the immune reactivity to P0 was slightly but significantly higher ($P<0.05$) as opposed to wt → wt chimeras suggesting that for complete tolerance, expression of P0 gene on nonhematopoietic tissues, supposedly on thymic stroma, is needed. Altogether, this experiment argues for redundancy of the P0 tolerogenic mechanisms.

8. Peptide 8 is a cryptic epitope in both wt and P0 ko mice of C57 BL/6 background

8.1 Immunization with a pool of 21 overlapping P0 peptides reveals six immunogenic peptides in P0 ko mice and four immunogenic peptides in wt mice

To identify the presence of putative cryptic epitopes in the amino-acid sequence of P0, mice were immunized with a mixture of all 21 overlapping peptides. Each mouse received 40µg of each peptide and after 10 days of in vivo priming, LN cells were challenged with the individual peptides.

The pattern of reactivity was to a certain extent similar in both subtypes. Peptides 5, 6 and 8 were immunogenic in both wt and P0 ko mice although in different ranges. For instance, the response to peptides 5 and 6 was lower in wt compared to P0 ko mice while P0 peptide 8 gave a slightly higher response in wt mice.

On the other hand, qualitative differences exist between the two subtypes regarding the

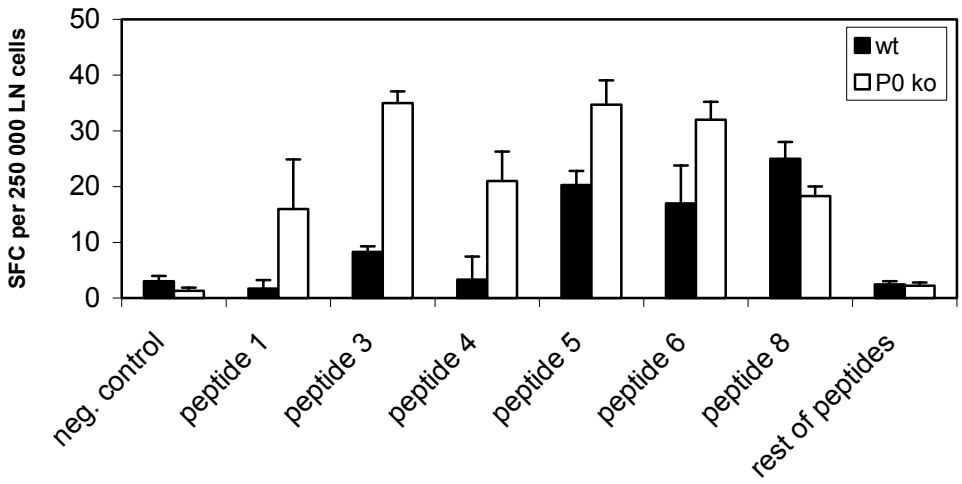


Figure 19. Mapping the T-cell response to P0 peptides. Wt and P0 ko mice were immunized with a mixture comprising all 21 overlapping P0 peptides. The amount of each peptide injected was about 40 µg per mouse. 10 days later the draining LN of three mice in each group were pooled and challenged with individual peptides at 10 µg/ml in IFN-γ ELISpot assays. Because no significant response was seen with peptides 2, 7 and 9 to 21, they were represented in a single bar for clarity. Mean + SD for triplicate wells. The results shown here were reproduced twice.

reactivity of peptides 1, 3 and 4. Thus, none of these three peptides was stimulatory in wt mice but gave consistent responses in P0 ko mice. Upon immunization with the native source of P0 (MPM) none of the peptides 1, 3 and 8 was able to recall a response (Fig.19). Since they are immunogenic when injected in peptide form, these results might suggest that their T-cell epitopes are not efficiently presented by APCs after natural processing of the native protein. Thus peptides 1, 3 and 8 may represent cryptic self-epitopes.

8.2 Peptide 8 represents an 'absolute' cryptic epitope

In order to investigate whether peptides 1, 3 and 8 are indeed cryptic epitopes wt mice were immunized with one of these peptides and the response was recalled with MPM (Fig.20). If they were cryptic epitopes, MPM would not recall any response. The immunization with peptide 5 of wt and P0 ko mice served as negative and positive control respectively. The peptide 5, as dominant determinant in P0 amino-acid sequence, produced a powerful

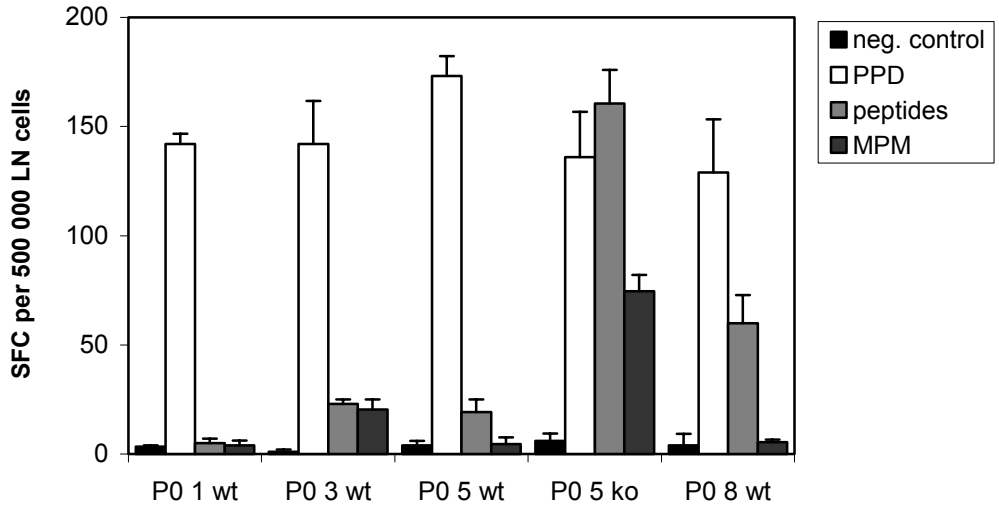


Figure 20. MPM recognition upon immunization with peptides 1, 3, 5 and 8. Wt and P0 ko mice were immunized with individual peptides as indicated on the X axis. The amount of peptide received by each mouse was 70 µg. Ten days later, the LN cells from three mice per group were pooled and challenged with MPM or the same peptide as the one used for immunization. Negative controls (medium) and positive controls (PPD) were included. Mean + SD for triplicate wells. Experiment was reproduced twice with similar results.

immune response in P0 ko mice, which could be consistently recalled with MPM. By contrast, the response in wt mice was of low magnitude for both antigens. Immunization with peptide 1 did not produce any immune response as can be seen in the recall with peptide 1 and MPM. Immunization with peptide 3 did produce a small immune response that could be recalled by both the peptide itself and MPM in contrast to P0 8 immunization, which drove a significant response recalled by peptide 8 but not MPM (Fig.20). Since they are reactive in knockout but not in wild type mice, peptide 1 and peptide 3 might represent subdominant epitopes. This result clearly demonstrates that P0 8 is a cryptic epitope. It also shows that the response to this cryptic epitope does not depend on the P0 genotype of the animals tested.

9. Wt and heterozygous P0 deficient mice do not develop clinical signs of EAN

In order to induce disease in BL/6 mice we tried both active and adoptive transfer EAN (Fig.21). For active EAN we immunized mice with different P0 peptides and also MPM or BPM emulsified in CFA. Concomitantly, we modulated the response in various immunomodulatory approaches as shown in Table1.

None of these immunization protocols could trigger autoimmune pathology as reflected by the classical clinical signs of EAN. Neither wt nor $P0^{+/-}$ mice displayed clinical EAN. When we looked for histological signs of disease by immunohistochemistry we did find macrophage (F4/80 staining) and T-cell (CD3 staining) infiltration into the sciatic nerves in two out of five wt mice following the immunization with P0 peptide 3 (P0 21-40) under the experimental conditions outlined in the Table1 at position 12 (Fig.22).

The adoptive transfer approach also did not induce clinical EAN symptoms. We tried two different protocols. In one of them, we injected 5 wt mice i.v. with 20 million of peptide 5 primed LN cells which were expended in primary culture for three days. In the other one, we transfered naïve spleen cells from P0 ko mice into wt mice followed by peptide 5 immunization of the recipients (in vivo expansion). In both cases mice were given PT i.p. on days 0 and 2 after transfer (not shown). The mice developed no clinical signs of disease.

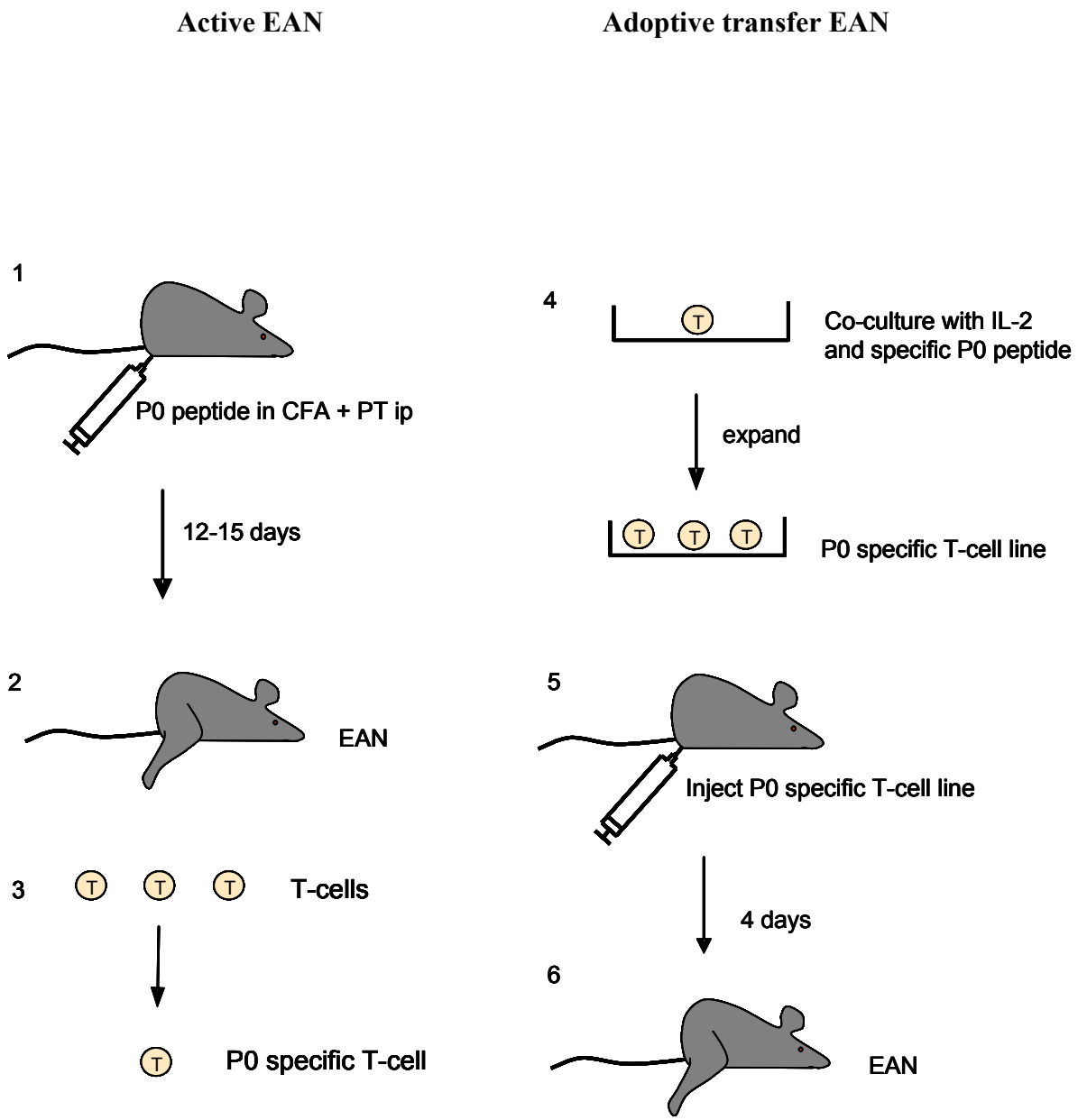


Figure 21. Active and adoptive transfer EAN. In order to induce active EAN, mice are immunized with PNS antigens emulsified in CFA in the presence of PT administered i.p. as an adjuvant to break the blood-nerve barrier. Usually after 12-15 days susceptible mice get the first clinical signs of EAN. For adoptive transfer EAN, PNS antigen primed T cells are restimulated in vitro for several cycles and then the T-cell line is injected i.v. or i.p. together with PT. PNS specific T-cell line should transfer symptoms of EAN into naive recipients in a much shorter time.

No	Immunization	Amount	Mycobac-terium	α-CTLA-4	CpG	IL-6 (ng)	PT (ng)	Boost	Clinical score
		µg / mouse							
1	P0 152-166	100	100	-	-	-	400§	no	0
2	P0 152-166	100	200	50	-	-	400§	no	0
3	P0 180-199	100	200	50	50	-	400§	no	0
4	P0 180-199	70	200	-	-	-	800§	yes	0
5	P0 180-199	70	200	-	-	-	800§	yes	0
6	P0 188-202	100	200	50	-	-	400§	no	0
7	P0 41-60	100	200	-	-	-	1000‡	yes	0
8	P0 41-60*	100	200	-	-	-	1000‡	yes	0
9	P0 41-60	150	200	50	30	-	200◊	yes	0
10	P0 41-60	200	200	-	-	-	400◊	yes	0
11	P0 41-60	150	200	50	30	90	200◊	yes	0
12	P0 21-40	150	200	50	30	90	200◊	yes	0
13	P0 71-90	150	200	50	30	90	200◊	yes	0
14	MPM	3500	200	-	-	-	400◊	yes	0
15	BPM	3500	200	-	-	-	400◊	yes	0

Table 1. Active EAN. Mice were immunized subcutaneously into the flanks, as specified in the table, with various amounts of P0 peptides or MPM/BPM emulsified in CFA. The dose of mycobacterium used is shown in the third column. When indicated, booster immunizations were done subcutaneously in the abdominal region after six days. The same amount of antigen was used as for the initial immunization. Anti-CTLA-4 antibody was injected i.p. every other day for two weeks at the dose indicated in the table. CpG was injected i.p. at the time of immunization. IL-6 was administrated i.p. on days -1, 0, 2 and 6 after immunization. For each immunization, five mice per group were used. For the negative control groups, the same set-up scheme was applied but without antigens.

§ Total amount received by a mouse in two i.p. injections on days 0 and 2.

‡ Total amount received by a mouse in four i.p. injections on days -1, 0, 1 and 3.

◊ Total amount received by a mouse in one i.p. injection on day 0.

* The recipients of this immunization were $P0^{+/-}$ mice.

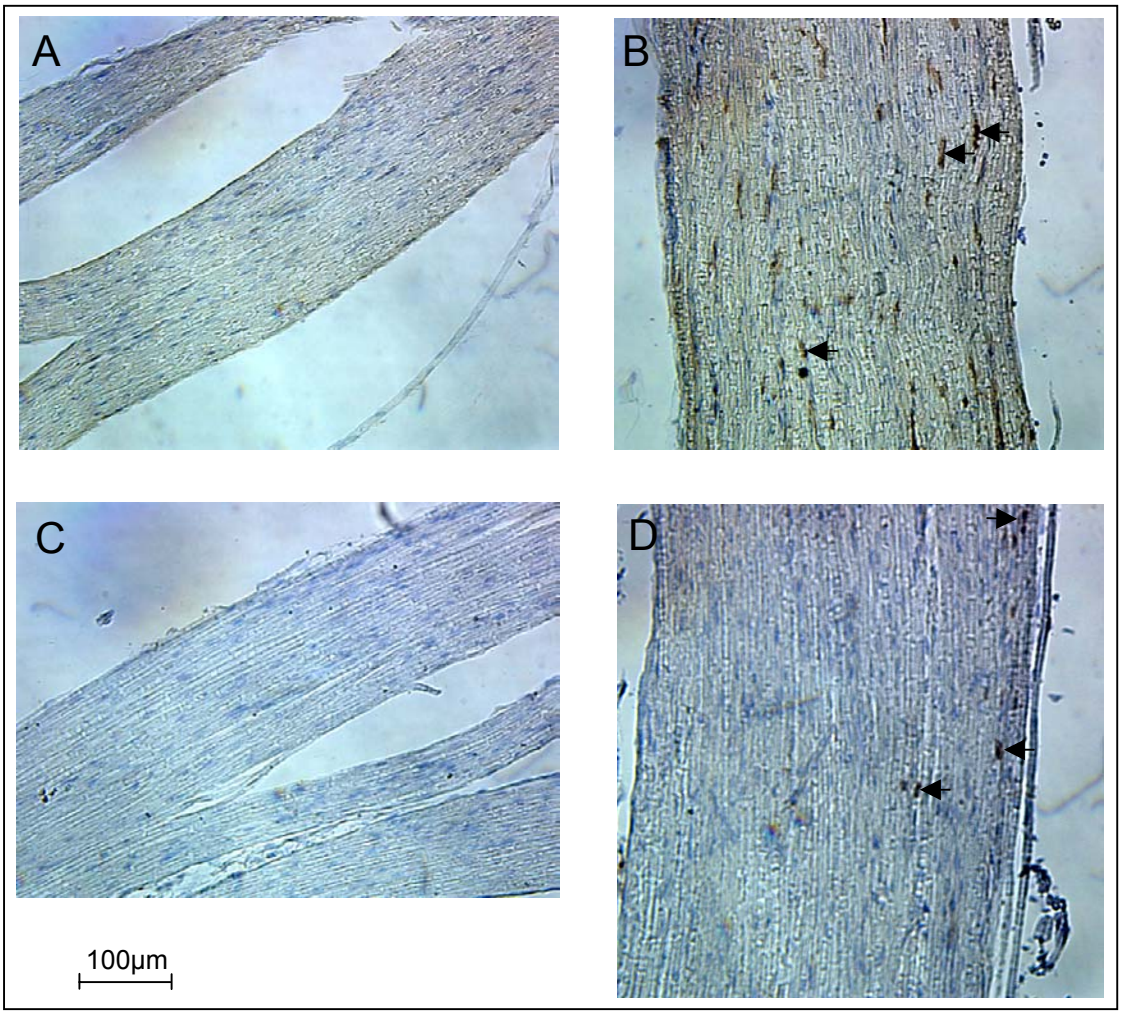


Figure 22. Semithin sections through sciatic nerves taken from wt mice at day 20 after P0 3 (B, D) and control immunizations (A, C). Nerve sections on A and B were stained for F4/80; C, D – CD3 staining. Arrows indicate positive cells as brown dots. Hematoxylin (blue colour) stains all nucleated cells.

We failed in our attempts to raise a P0 specific T-cell line and therefore we could not investigate the possibility of EAN induced by adoptive transfer of a P0 specific T-cell line. Related to our inability to raise a T-cell line, we noticed that the ratio between P0 5 and PPD recall responses in ELISpot assays is different from that seen in proliferation assays. We found that proliferative response to P0 peptide 5 was much reduced comparing with the IFN-γ P0 5 response when PPD response was taken as baseline both in ELISpot and proliferation assays (Fig.23). In IFN-γ ELISpot assays, the peptide 5 response was even slightly higher than PPD response, while in proliferation assays the reactivity to peptide 5 was three times lower than that to PPD.

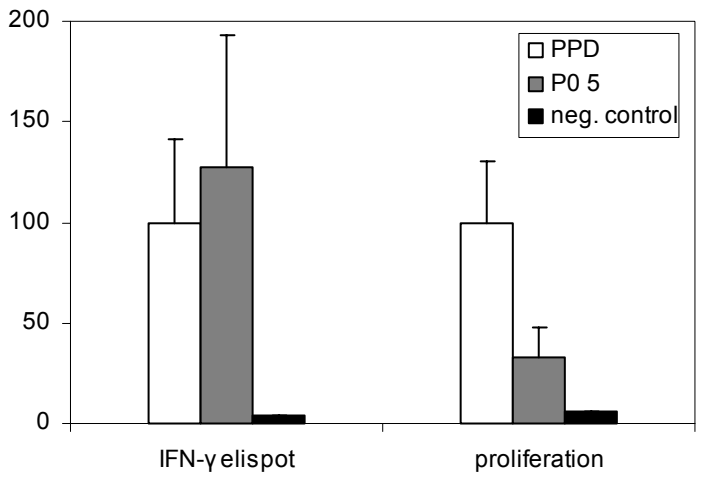


Figure 23. Proliferative and IFN-γ specific response to P0 peptide 5. LN cells from $P0^{-/-}$ mice immunized with 50 µg P0 peptide 5 were tested in both proliferation and ELISpot assays. The response to PPD was considered 100% and the response to P0 5 was calculated as percentage of PPD response. The numbers on the Y axis represent percentages from PPD response. On the X axis the type of assay is described. Mean + SD for five independent experiments of which three experiments included one mouse per group and two experiments included three mice per group.

Discussion

In the present study, we show that the T-cell response to myelin protein P0 is tolerized in wt mice but not in P0 ko mice. We newly describe that the immune response to P0 in C57BL/6 mice is mainly directed against the amino acid sequence 41-60 (P0 peptide 5) which contains the core immunodominant determinant in myelin P0 (Fig.9-10). We found that this T-cell epitope is restricted to H2-IA^b molecules (Fig.11).

P0 is a typical tissue specific antigen whose expression was thought to be limited to myelinating Schwann cells. We show here that P0 is expressed also in thymus at both RNA and protein level (Fig.15, 16 and 17). P0 was detected in thymic stroma but not in thymocytes, which comprise cells with bone marrow origin. Traditionally the thymus was regarded as being the main site for T-cell tolerance induction for ubiquitous proteins and for abundant blood-borne antigens that could get easily access to thymus via circulation. By contrast, various peripheral mechanisms would account for tolerance towards tissue-specific antigens, which are secluded from the mainstream circulation and consequently from the immune system.

This view has changed during the last years as an increasing body of data showed that tissue specific antigens and other antigens alike are actually expressed also in thymus besides their original tissue [16, 31, 44]. Thus, we provide here another piece of evidence that tissue specific antigens are ectopically expressed in the thymus. Antigen expression in the thymus is sufficient to induce a state of self tolerance while lack of expression of tissue-restricted antigens on TEC is associated with susceptibility for autoimmune diseases in both wt animals [43] and manipulated chimeras [44]. Furthermore, very recent work revealed that the transcription factor AIRE (autoimmune regulator) prevents autoimmunity by promoting ectopic expression of most tissue-specific antigens on medullary epithelial cells of the thymus [5]. Overall, these data suggest that tissue-specific antigens primarily tolerize T-cells in the thymus although this does not exclude complementary or redundant mechanisms that function in the periphery. Thus, the idea of tissue-restricted antigens with their seclusion from central tolerance induction can no longer be maintained in the light of the recently accumulated evidence.

It seems likely that P0 expression in the thymus originates from TEC, as these are the major component of thymic stroma. However, thymus is an innervated organ and the P0

signal that we detected in Western Blot and RT-PCR could originate from myelinated nerves or from Schwann cells respectively. We think this is not the case since thymus contains unmyelinated nerve fibres [9] and Schwann cells associated with unmyelinated axons do not express P0 [46, 56, 64, 95]. Moreover, it was shown that Schwann cells in the absence of neuronal bodies down regulate their P0 mRNA to basal level which is negligible compared to the level in vivo [81, 56]. Nevertheless, we detected strong P0 mRNA signal in deoxyguanosine treated FTOC, which is free of neuronal bodies (Fig.16). These data suggest that P0 expression comes from thymic stromal cells.

In a wt versus P0 ko approach we showed that P0 ko mice retain a high frequency of P0 reactive T-cells in their T-cell receptor repertoire, while in wt mice only a low frequency can be observed. The P0 ko mice showed a strong immune response towards peptide 5 upon immunization with both MPM or peptide itself (Fig. 9-10). There is always a higher reactivity for foreign antigens then for self antigens. P0 is a foreign antigen in P0 ko mice and such an immune response is in principle desirable. Our data show that endogenous P0 induces a profound inactivation of the P0 specific immune response in wt mice. The abrogation of the P0 immune response in wt mice is reflected not only in the low frequency of the responsive cells, but also in their low avidity for P0. To get the same degree of LN cells stimulation, a hundred fold higher concentration of peptide 5 was needed in wt comparing with P0 ko mice (Fig.12). Even more, the IL-2 and IFN-γ production of the responsive wt LN cells was reduced as reflected by the average spot size (data not shown).
Similar results were also obtained in other autoantigen deficient mice and their wt counterparts [42, 80, 89]. These results demonstrate that the presence of a certain antigen in the thymus is not required for the selection of its specific T-cell receptor repertoire. In contrast, as immature thymocytes have a receptor-repertoire with specificities for any potential self or nonself antigens our data confirm that expression of antigens on self tissues is required in fact for inactivation of their specific T-cells.

It is clear that positive selection is promoted by weak interactions between T-cells and self MHC:self peptides expressed on various thymic APCs [7, 88]. Therefore, mature T-cells virtually specific for foreign antigens will retain also low specificities for the selecting self peptides. We did find T-cells with low reactivity towards P0 in wt mice. Upon immunization with MPM as a source of native P0 protein, peptide 5 could not recall a specific response suggesting that wt mice were thoroughly tolerized towards self P0

(Fig.10). However, when used as immunogen, peptide 5 recalled a low immune response in wt mice (Fig.11, 12) confirming also other similar findings [80], which were nevertheless differently interpreted. This low immunogenicity was not due to some cryptic features of peptide 5 as this is a dominant epitope in P0 ko mice upon immunization with MPM (Fig.10). Moreover, P0 ko mice have basically the same MHC genotype as wt mice. On the contrary, it seems most likely that the reactivity to peptide 5 in wt, although very low, is due to stimulation of those T-cells positively selected upon interaction with peptide 5 or other P0 peptides that share the same immunodominant epitope. In adition, peptide 5 primed $P0^{-/-}$ LN cells could be activated with MPM in recall culture. As both wt and P0 ko are on the same genetic background there is no reason to believe that peptide 5 behaves differently in wt mice in regard to its processing and presentation, events which determine the crypticity. Furthermore, it was shown that crypticity is MHC associated and not a structural attribute of the determinant. For instance, the pattern of response of C57BL/6 mice to a cryptic epitope correlated precisely to that of BALB.B mice, which have the same MHC haplotype but otherwise a different non-MHC genetic background [57]. On the contrary, there was no similarity in the pattern of response to the same epitope in BALB.B and BALB/c mice, which have the same genetic background but different MHC genes.

To find the dominant determinant we used bovine peripheral myelin as source of P0 since this protein is highly conserved in the phylogeny. In the 219 amino-acid sequence of P0 there are only 16 substitutions in the bovine P0 sequence when compared with mouse P0. Therefore, the probability that T-cells with specificities for P0 from one species to cross-react with P0 from another species is very high. More importantly, in most of these substitutions one amino acid is replaced by another from the same category (conservative substitution), e.g. isoleucine from position 33 is replaced with leucine in bovine and both are hydrophobic amino acids. In addition, the amino acid sequence 41-60 corresponding to the immunogenic peptide 5 is 100% identical in both species.
Nevertheless, to make sure that peptide 5 is also a dominant determinant upon processing and presentation of native mouse P0, we immunized the mice with MPM and recalled the response with individual peptides instead of using the peptide pool array. The fine specificity mapping of the immune response to P0 confirmed that peptide 5 is the core immunodominant determinant in P0 ko mice (Fig.10). These results also demonstrate that there is a high degree of T-cell cross-reactivity for mouse and bovine P0.

We found a cryptic epitope in the amino acid sequence of P0 contained by peptide 8. This is a cryptic epitope because it did not recall a response upon immunization with MPM (Fig.10) but when used as immunogen could recall a strong response with the peptide (Fig.19, 20) but not with MPM (Fig.20). The fact that peptide 8 induce a high response even in wt mice means that T-cells with specificities for this peptide were not clonally deleted. Since P0 is a nonself antigen it is expectable that P0 ko mice would retain full specificities for it. However, P0 is a self antigen in wt mice and highly self-reactive T-cells should be removed. For peptide 8 specific T-cells this was not the case (Fig.20), suggesting that this peptide is not available for MHC presentation on thymic APCs to mediate negative selection of self-reactive T-cells.

Regarding peptides 1, 3 and 4 definitive conclusions cannot be drawn. It is profitable to think that there could be a gradual series of determinants from a dominant to an 'absolute' cryptic epitope. Intermediate epitopes would include subdominant and subcryptic epitopes. Peptide 4, as part of the immunodominant region P0 31-70, might be a weakly immunogenic peptide in P0 ko mice since was occasionally stimulatory also on MPM primed cells (not shown). The fact that peptides 1 and 3 induced responses in P0 ko mice when injected in the form of the 21 peptide mixture but not with MPM would argue that they are cryptic. However, they were not immunogenic in wt mice (Fig.19, 20), which means they are available for MHC presentations and thus for tolerance induction. This would contradict the fact that they are cryptic epitopes. Thus, peptides 1 and 3 may represent subdominant epitopes. It is such determinants that may be targeted for autoaggression. In certain situations, their presentation may be upregulated or T-cells with specificity for this epitopes expanded by means of cross-reactivity or molecular mimicry and thus they might become targets for autoimmune pathology.

The behavior of T-cell determinants seems to be dependent on the way of handling. The fact that the 21 overlapping P0 peptides were injected in one single mixture had at least two major consequences. On the one hand, there is an extensive peptide competition for MHC molecules [73]. This could explain why the difference between the reactivity to peptide 5 and that to peptides 4 or 6 is not as high as when tested on MPM primed cells (Fig.10). It seems that P0 amino acid sequence 31-70 (overlapping peptides 4, 5 and 6) contains more agretopes, which compete with each other when injected together, thus limiting the exposure of peptide 5 to its specific T-cells.

On the other hand, the peptide mixture will activate a large pool of T-cells with a receptor repertoire of broad specificities, given the number of potential antigens. Some of these T-

cells may have overlapping specificities and cross-react with other peptides than those they were selected for. In this line of argumentation, after immunization with the peptide mixture, the response to P0 5 was high also in wt (and comparable with that in P0 ko mice) because T-cells primed by the flanking peptides cross-reacted with peptide 5 and thus the overall response to this peptide was boosted (Fig.19). The same scenario would apply also for peptide 6. By contrast, in many other experiments along this work we showed that immunization with peptide 5, injected as individual peptide, produced a high immune response in P0 ko mice but a limited one in wt mice.

Previous studies showed that tolerance induction to self antigens is dependent on the dosage of the genes responsible for those antigens [13, 33, 72]. While a homozygous genotype would ensure self tolerance, a knockout genotype would be the premise for full reactivity. Heterozygous animals would display a tolerance statue between reactivity and tolerance in a linear correlation with the gene copy numbers. At least one study [4] showed that the tolerance to a self antigen was not disrupted in the respective heterozygous deficient mouse. Our data reveal that $P0^{+/-}$ mice underwent the same degree of tolerance as wt mice suggesting that one copy of P0 gene translate sufficient amounts of protein to mediate complete tolerance (Fig.13). It was reported by Martini and colleagues that heterozygous P0 deficient but not wt mice show signs of demyelination after four months of age supposedly because of autoimmunity to myelin proteins [69]. When we tested the reactivity of wt and $P0^{+/-}$ mice (exceeding 4 months of age) to myelin proteins in ELISpot and proliferation assays we did not find any significant difference between the two subtypes, suggesting that the demyelination occurring in $P0^{+/-}$ at later ages is not antigen dependent (Fig.14). Different and more relevant approaches should be employed in order to clarify these contradicting results. In particular, a direct comparison of the split-well assay used by Martini's group and the ELISpot method is required.

We found that a P0 ko immune system grafted onto wt mice exhibit profound tolerance towards P0, comparable to wt → wt chimeras (Fig.18), suggesting that P0 expression on hematopoietic cells is not necessary for P0 tolerance induction. As P0 is expressed on thymic stromal cells, tolerance to P0 could be established at this level. However, we did not investigate whether peripheral mechanisms would also account for P0 tolerance.

Unexpectedly, we found that wt BM derived cells were able to acquire some degree of tolerance in P0 ko recipient mice although not at the wt level. This suggests that for complete T-cell tolerance, expression of the P0 gene on nonhemopoietic tissues, supposedly on thymic stroma, is also needed. As BM derived cells do not express P0 this raises a question mark about the source of P0 needed for self tolerization. It is possible that trace amounts of P0 are still produced by BM cells but remain undetected with conventional methods. MBP, the most studied autoantigen and hitherto regarded as a tissue specific antigen, was found to be 'promiscuously' expressed both in thymic epithelial cells (TEC) [53] and in differentiated blood cell lineages and hematopoietic progenitors [52]. Another possibility is that wt BM cells, which include the most primitive to the most committed stem cells, were signalled by demyelinated nerves of P0 deficient mice to differentiate in remyelinating Schwann cells that produce P0. This is an appealing hypothesis as it was recently shown that BM contains a population of stem-like cells, which can give rise to neurons and glial cells such as Schwann cells [17, 39; 68]. Transplantation of these cells into demyelinated lesions of the animal results in remyelination. Moreover, green fluorescent protein (GFP)-expressing BM cells were shown to colocalize with P0-positive cellular elements in the remyelinated region [3]. Thus, such wt BM derived Schwann cells may be a source of tolerogenic amounts of P0 in P0 ko mice.

Another reason that has driven this work was to create a reliable EAN model in C57BL/6 mice. This is the most common inbreed strain of mice used in experimental research and concomitantly the founder strain for most transgenic mice. Therefore, establishing EAN in BL/6 mice would provide a powerful tool, which can benefit from all relevant genetically modified mice with this genetic background.

We found that neither C57BL/6 wt nor $P0^{+/-}$ deficient mice developed clinical signs of EAN when using protocols that cause disease in susceptible strains. In various protocols, we tried different immunomodulatory molecules, which were reported to help induce or worsen the disease (Table.1). PT is commonly used as adjuvant to increase the incidence of the disease and we used it in all protocols [12, 96]. CTLA-4 blockade was shown to exacerbate the clinical disease in both active EAE [32, 65] and EAN models [91]. None of these molecules helped to induce clinical symptoms.

It was proven that IL-6 deficient mice are resistant to EAE induction [54, 61] revealing that IL-6 has an important role in regulation of the immune response. In addition, DCs treated with IL-6 were shown to present determinants that were previously cryptic [18]. Since T-

cells recognizing self cryptic epitopes escape tolerance mechanisms, they may cause immunopathology when such epitopes become available for T-cell recognition. Despite that, mice immunized with the cryptic peptide 8 or subdominant peptide 3 and treated for several days with IL-6 did not reveal clinical signs of disease. However, macrophage and T-cell infiltration was observed in mice immunized and boosted with P0 peptide 3 suggesting that they may develop sub-clinical forms of EAN.

In our hands, P0 peptide 180-199 [2] could not induce EAN even when used in protocols claimed by other groups to induce clinical signs of disease [91, 92, 93, 96]. In addition, peptide 180-199 did not recall a response upon immunization with either MPM or peptide mixture (Fig.10 and 19). This held true for both wt and P0 ko mice.

We conclude that tolerance towards P0 is fully established in wt C57BL/6 mice and not possible to break it and induce EAN with the dominant P0 peptide 5 given the shortage and the low avidity of its specific T-cells. The reactivity to peptide 5 in wt (Fig.11, 12), which is anyway very low, reflects an artificial situation and is a consequence of the high doses of peptide used for immunization and recall culture. These high doses of antigen are unlikely to accumulate in the body. Thus, such low self-reactive T-cells are not likely to have an aggressive phenotype and mediate autoimmune pathology in animal models and even less likely in a naturally occurring situation.

On the other hand, the fact that also adoptive transfer of P0 primed $P0^{-/-}$ LN cells or adoptive transfer of naïve $P0^{-/-}$ spleen cells combined with peptide 5 immunization (in vivo expansion of P0 ko cells) did not induce EAN may support the idea that C57BL/6 mice are genetically not prone to develop pathologic autoimmunity in the PNS. Alternatively, it is possible that tolerance to P0 also involves regulatory T-cells that may specifically prevent autoaggression.

Nevertheless, adoptive transfer of a stable P0 ko T-cell line with high reactivity towards P0 may be a more appropriate approach to address the susceptibility to EAN in C57BL/6 mice. We could not investigate this possibility because of difficulties in raising a permanent P0 peptide 5 specific T-cell line. We link this incapacity with the poor proliferative response induced by peptide 5 (Fig.23). It seems that proliferation and cytokine secretion are dissociated processes in response to an antigenic stimulus [58] since peptide 5 is highly reactive as emerged from cytokine measurements but a rather poor inducer of cell proliferation (Fig.23). In order to restore the proliferative response to the determinant contained by peptide 5 it may be useful to further map the immune response to peptide 5 towards overlapping peptide 6 as this was also to some degree stimulatory.

Such high reactivity to P0 as a foreign antigen in P0 ko mice might be a drawback when seen from gene therapy perspective. Some forms of Charcot-Marie-Tooth disease and Dejerine-Sotas neuropathy are caused by mutations in the P0 gene that lead to either complete loss, or gain of function induced clinical symptoms. Such monogenic diseases are good candidates for gene therapy [85]. However, even when considerable obstacles such as gene delivery, targeting the desired cell type, short persistence of the transgene or immune responses against delivering viral vectors are surpassed we may still have to get around this specific problem. The immune system of the transgene recipient will be confronted with the new or corrected protein perceived now as foreign, especially when the corrected genetic defect embrace also the immunodominant region of the corresponding protein. A straightforward approach would be to target the corrected gene also to thymic tissue as an attempt to override the immunoreactivity of the new protein through the generation of antigen specific suppressor T-cells. These suppressor cells may tolerize the actual repertoire of the P0 specific T-cells already present in the periphery. Extensive studies of gene therapy on animal models for mutated P0 must be employed before proceeding to human clinical trials.

Summary

Myelin protein zero (P0) is a key myelin component in maintaining the integrity and functionality of the peripheral nervous system. Mutated variants are the cause for several disabilitating peripheral neuropathies such as Charcot-Marie-Tooth disease or Dejerine – Sotas syndrome. Using P0 knockout mice - a mouse model for these diseases - together with their wt counterparts on C57BL/6 background we studied the shaping of the T-cell repertoire specific for P0 in the presence and in the absence of this protein during the ontogeny of T-cells. Our approach was to use a series of overlapping 20-mer peptides covering the entire amino acid sequence of P0. This series of P0 peptides was employed for epitope mapping of the H2-A^b restricted T cell response. Thus, P0 peptide 5 (P0 41-60) in the extracellular domain of P0 was identified as the main immunogenic peptide.

The immunogenic peptide containing the core immunodominant determinant in the P0 sequence was employed in studies of tolerance, revealing a highly reactive P0 specific T-cell repertoire in P0 ko mice while in wt mice the high avidity repertoire was inactivated in order to ensure self tolerance. In wild type and heterozygous P0 mice tolerance is not dependent on gene dosage.

P0 is a tissue specific antigen whose expression is limited to myelinating Schwann cells. The classical view on tolerance to tissue specific antigens attributed this role to peripheral mechanisms. Driven by the finding that intrathymic expression of tissue-specific antigens is a common occurrence, we confirmed that “promiscuous” expression on thymic stroma holds true also for myelin P0. In addition, using bone marrow chimeras we investigated the capacity of bone marrow derived cells versus nonhematopoietic cells to induce tolerance towards P0. Our findings show that bone marrow derived cells although tolerogenic to some degree are not sufficient to mediate complete tolerance. P0 expression on cells with origin other than bone marrow showed to be sufficient and necessary to induce sound tolerance.

We identified one cryptic (P0 peptide 8) and two subdominant epitopes (P0 petides 1, and 3). P0 peptide 8 was reactive in both wt and P0 ko mice. Peptides 1 and 3 were

immunogenic in P0 ko but not in wt mice. Several P0 peptides including the immunogenic peptide 5 were involved in direct and adoptive transfer EAN studies. None of them induced clinical signs of EAN. Immunization with P0 peptide 3 did induce inflammation of the peripheral nerves reflected by the infiltration of macrophages and CD3 positive cells. More studies involving highly P0 specific T-cell lines are needed to characterize the P0 induced EAN.

Our findings may have direct implications for secondary autoimmunity and inflammation in peripheral nerves developing after correcting the P0 genetic defect by gene therapy in aforementioned diseases.

Zusammenfassung

Das Myelinprotein P0 stellt eine zentrale Komponente für die Stabilität und Funktionalität der Myelinscheiden des peripheren Nervensystems dar. Mutationen des P0-Proteins führen zu verschiedenen, schwer behindernden peripheren Neuropathien wie der Charcot-Marie-Tooth- oder der Dejerine-Sotas-Erkrankung. Wir haben das Tiermodell der P0-Knock-Out-Mäuse verwendet, um im Vergleich zu den C57BL/6-Wildtyp-Tieren Selektionsmechanismen des P0-spezifischen T-Zell-Repertoires zu untersuchen. Dazu wurde eine Reihe von überlappenden 20-mer-Peptiden benutzt, die die gesamte Aminosäuresequenz von P0 abdeckten. Mit Hilfe dieser Peptide wurde ein sog. „Epitop-Mapping“ der H2-A^b-restringierten T-Zell-Antwort durchgeführt. Auf diese Weise konnte das P0-Peptid 5 (Aminosäure 41-60) in der extrazellulären P0-Domäne als immunogene Determinante identifiziert werden.

Dieses immunogene Peptid wurde dann für Untersuchungen der Toleranzmechanismen verwendet und zeigte, dass in P0-Knock-Out-Mäusen ein hochreaktives P0-spezifisches T-Zell-Repertoire vorliegt, während es in Wildtyp-Tieren inaktiviert ist und so Selbsttoleranz erzeugt wird. Die Toleranzerzeugung in Wildtyp- und heterozygoten P0 +/- Mäusen hängt nicht von der Gen-Dosis ab.

P0 ist ein gewebespezifisches Antigen, dessen Expression normalerweise auf myelinisierende Schwann-Zellen beschränkt ist. Die klassischen Vorstellungen zu Toleranzmechanismen gegenüber gewebsspezifischen Antigenen schrieben diese vor allem peripheren Immunmechanismen zu. Durch den erstmaligen Nachweis von intrathymischer Expression gewebsspezifischer Antigene wie P0 konnten wir bestätigen, dass für P0 offensichtlich die Expression deutlich weiter verbreitet ist, insbesondere auch auf Thymus-Stroma-Zellen. Unter Verwendung von Knochenmarkschimären haben wir weitere Untersuchungen durchgeführt, wie Knochenmarks-abstammende Zellen im Vergleich zu nicht-hämatopoetischen Zellen Toleranz gegenüber P0 erzeugen können. Unsere Befunde zeigen, dass Knochenmarks-abhängige Zellen nicht ausreichen, um völlige Toleranz zu erzeugen. Zusätzlich wurde eine P0-Expression auf anderen Geweben wie dem Thymus benötigt, um komplette Toleranz zu erhalten.

Wir identifizierten ein kryptisches P0-Peptid 8 und zwei subdominante P0-Peptide 1 und 3. Während das Peptid 8 sowohl in Wildtyp- als auch Knock-Out-Mäusen erkannt wurde, wurden die Peptide 1 und 3 in Wildtyp-Mäusen nicht als Immunogen erkannt. Die genannten Peptide wurden verwendet, um eine experimentelle autoimmune Neuritis (EAN) zu erzeugen. Mit keinem der experimentellen Ansätze konnten wir klinische Zeichen einer EAN generieren, allerdings mit dem Peptid 3 doch Entzündung im peripheren Nerven beobachten. Es werden zukünftig weitere Untersuchungen benötigt, um P0-spezifische T-Zell-Linien zu etablieren und so mit höherer Effizienz eine EAN zu erzeugen.

Unsere Untersuchungen sprechen dafür, dass bei gentherapeutischen Ansätzen bei erblichen Neuropathien vorsichtig und schrittweise vorgegangen werden muss, da mit sekundärer Autoimmunität und damit Inflammation im peripheren Nerven zu rechnen ist.

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Abbreviations

APCs	antigen presenting cells
AT-EAN	adoptive transfer EAN
BCIP/NBT	5-bromo,4-chloro,3-indolylphosphate/nitroblue tetrazolium
BM	bone marrow
BMdC	bone marrow derived cells
BN (rats)	Brown Norway rats
BNB	blood-nerve barrier
bp	base pairs
BPM	bovine peripheral myelin
BSA	bovine serum albumin
C	complement components
CBCB	carbonat-bicarbonat buffer
CFA	complete Freund's adjuvant
CMT 1B	Charcot-Marie-Tooth 1B
CNS	central nervous system
Con A	concanavalin A
CpG	cytosine linked to guanine by a phosphate residue; oligodeoxynucleotides containing this motif
cTECs	cortical thymic epithelial cells
CTLA-4	cytotoxic T lymphocyte-associated antigen 4
DAB	3,3' diaminobenzidine
DCs	dendritic cells
DDT	dithiothreinol
DP	double-positive
DNA	deoxyribonucleic acid
dNTP	deooxynucleoside triphosphate
EAE	experimental autoimmune encephalomyelitis
EAN	experimental autoimmune neuritis
FCS	fetal calf serum
FTOC	fetal thymic organ culture

FWR	forward primer
GBS	Guillain–Barré syndrome
ICAM-1	intercellular adhesion molecule 1
IFN-γ	interferon-γ
IgG	immunoglobulin G
IL-2/ IL-4/ IL-5/ IL-6	interleukin-2/ 4/ 5/ 6
i.p	intraperitoneal
i.v.	intravenously
IFA	incomplete Freund's adjuvant
ko	knockout
LB	luria broth
LFA-1	lymphocyte-function-associated antigen-1
LN	lymph nodes
MAG	myelin-associated glycoprotein
MØ	macrophage
MBP	myelin basic protein
MHC	major histocompatibility complex
MM	master mix
MMP	matrix metalloproteinases
MNC	mononuclear cells
MOG	myelin oligodendrocyte glycoprotein
MPM	mouse peripheral myelin
mTECs	medullary thymic epithelial cells
NK	natural killer cells
OH	hydroxyl radicals
P0	myelin protein zero
$P0^{+/+}$	wild type genotype of Po
$P0^{+/-}$	heterozygous P0 deficient mouse
$P0^{-/-}$	P0 knockout mouse
P2	peripheral myelin protein 2
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PGE	prostaglandin E

PMP22	peripheral myelin protein 22
PNS	peripheral nervous system
PLP	proteolipid protein
PPD	purified protein derivative of mycobacterium
PT	pertussis toxin
RAG-1	recombination-activating gene 1
REV	reverse primer
RNA	ribonucleic acid
RNase	ribonuclease
rhP0	recombinant human P0
rhP2	recombinant human P2
rpm	rotations per minute
RT	room temperature
RT-PCR	reverse-transcriptase polymerase chain reaction
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-Polyacrylamide Gel Electrophoresis
SFC	spot forming cells
SP	single-positive
SLex	exposed sugar residues
TBS	TRIS buffer solution
TC	T-cell
TCR	T-cell receptor
TECs	thymic epithelial cells
TNF	tumor necrosis factor
Th	T helper cell
TRIS	tris(hydroxymethyl)aminomethane
VCAM-1	vascular cell adhesion molecule 1
VLA-4	very late antigen-4
wt	wild type

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- **Visan L.** *T-cell autoimmunity against myelin proteins in wild-type versus P0 knockout mice.* Joint Retreat of the Graduate Colleges 520 and 592, "Immunomodulation meets Lymphocyte Activation" 7/2001, Zeilitzheim
- **Visan L., Jung S. and Gold R.** *P0 specific T cell repertoire in wild-type and P0 deficient mice.* Joint Retreat of the Graduate Colleges 520 and 592, "Immunomodulation meets Lymphocyte Activation" 5/2002, Zeilitzheim
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Eidesstattliche Erklärungen

Hiermit erkläre ich ehrenwörtlich, dass die vorliegende Dissertation „***P0 specific T-cell repertoire in wild-type and P0 deficient mice***“ selbständig an der Neurologischen Klinik der Universität Würzburg angefertigt wurde und dass ich keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe.

Würzburg, 25 Mars 2003
(Dipl. Biol. Lucian Visan)

Hiermit erkläre ich ehrenwörtlich, dass die vorliegende Dissertation „***P0 specific T-cell repertoire in wild-type and P0 deficient mice***“ in gleicher oder ähnlicher Form noch nicht in einem anderen Prüfungsverfahren vorgelegen hat.

Würzburg, 25 Mars 2003
(Dipl. Biol. Lucian Visan)

Hiermit erkläre ich ehrenwörtlich, dass ich bisher noch keine akademische Grade erworben oder zu erwerben versucht habe.

Würzburg, 25 Mars 2003
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