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Tolerance Induction by Intrathymic Expression of P0¹

Lucian Visan,* Ioana A. Visan,* Andreas Weishaupt,* Harald H. Hofstetter,* Klaus V. Toyka,* Thomas Hüning,† and Ralf Gold²*  

Genetic deficiency or instability of myelin protein zero (P0) results in hereditary motor sensory neuropathy. In view of recent advances in gene therapy, substitution of the molecular defect may become realistic in the near future. Here we investigate the impact of genetic deficiency of P0 on selection of the autoreactive T cell repertoire in the corresponding mouse model. We show that P0 mRNA transcripts are expressed in thymic stroma, similar to other myelin proteins and that expression of intact P0 protein can be detected by Western blot. Using a library of overlapping 20mer peptides spanning the entire length of P0 and applying the ELISPOT technique, we detected a strong immune response toward P0 extracellular domain peptide aa 41–60 in P0⁻/⁻ knockout mice, but not in heterozygous P0⁺/⁻ or wild-type (wt) mice. In addition, one cryptic epitope and two subdominant epitopes of P0 were identified. Using P0⁻/⁻ into wt bone marrow (BM) chimeras we found that P0 expression in the host suffices for full tolerance induction, which is in line with its presence in thymic stroma. However, repopulation of P0⁻/⁻ mice with wt BM led to partial induction of tolerance, suggesting that BM derived cells can also express this protein. Our findings may have implications for secondary autoimmune neuritis in susceptible animals, because the repaired protein will then represent a foreign, nontolerized Ag. The Journal of Immunology, 2004, 172: 1364–1370.

Inherited peripheral neuropathies are incurable disorders of the peripheral nervous system leading to severe neurological disability. One of the major causes is represented by mutations in the myelin protein zero (P0)¹ gene locus, which leads to Charcot-Marie-Tooth type 1B disorder or Dejerine-Sottas syndrome (1). Heterozygous and homozygous P0-deficient mice serve as animal models for these disorders. Studies in these P0-deficient mice revealed the potential of P0 to act as an immune target (2). Moreover, Ab responses to P0 have been detected in patients’ sera with chronic polynuerritis (3) and immunization with P0 peptides and adoptive transfer of peptide-specific T cells induce experimental autoimmune neuritis in susceptible animals (4, 5).

The peripheral nervous system was counted among the immunoprivileged sites. It was thought that “ignorance” due either to sequestration of the autoantigens from patrolling T cells or too tiny amounts of Ag “invisible” for T cells, or other peripheral mechanisms such as anergy, would account for tissue-specific tolerance. In contrast, negative selection in the thymus (central tolerance) was shown to be the prime tolerogenic mechanism for abundant blood Ags or proteins derived from housekeeping genes (6). Recently, there is growing evidence of organ-specific Ag expression on thymic stromal cells (7), which attributes a direct role of the thymus in achieving tolerance to this type of autoantigens. This was elegantly demonstrated for proteolipid protein (8, 9).

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3 Abbreviations used in this paper: P0, myelin protein zero; wt, wild type; BM, bone marrow; BPM, bovine peripheral myelin; MPM, mouse peripheral myelin; ko, knockout; LN, lymph node; FTOC, fetal thymic organ culture; PPD, purified protein derivative of mycobacterium.
chimeras was obtained from Bavarian local state authorities for animal experimentation.

**BM chimeras**

Recipient mice were lethally irradiated (10 Gy) to ablate their immune system and reconstituted with BM cells (1 \times 10^7 cells) immediately thereafter. Grafted mice were immunized after another 8–9 wk.

**Tritium-thymidine proliferation assay**

Single cell suspensions from spleen or lymph nodes (LNs) were prepared. Cells were cultured in 96-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark) at a density of 2.5 \times 10^3 cells per 100 \mu l well in the presence of selected concentrations of synthetic peptides or myelin homogenate. Cultures were set up in triplicate and maintained at 37°C and 5% CO_2 in a humidified atmosphere. T cells were pulse-labeled with [\(^{3}H\)]thymidine (Amersham-Buchler, Braunschweig, Germany) during the last 16–18 h of culture. Seventy-two hours after culture was initiated, cells were harvested with a Betaplate 96-well harvester (Pharmacia, Freiburg, Germany) and incorporation of radioactivity was detected using a Betaplate liquid scintillation counter (Pharmacia).

**Cytokine ELISPOT assays**

ELISPOT assays were performed as described (13). Briefly, ImmunoSpot M200 plates (Cellular Technology, Cleveland, OH) were coated overnight at 4°C with the capture Ab in sterile carbonate-bicarbonate buffer. R46A2 (BD PharMingen, San Diego, CA), at 4 \mu g/ml was used for IFN-\(\gamma\). The plates were washed three times with sterile PBS and once with HL-1 medium. Spleen or LN cells (2.5 \times 10^3 or 5 \times 10^3 per well) were plated in 100 \mu l HL-1 medium (BioWhittaker, Walkersville, MD) together with and without Ags in triplicate cultures each. Subsequently plates were incubated at 37°C, 5% CO_2 for 20 h. After washing with PBS 0.05% Tween followed by PBS, detection Ab, XMG1.2 (BD PharMingen) for IFN-\(\gamma\) was incubated for 2 h at 37°C. The cytokine secreting cells were then visualized by adding streptavidin-alkaline phosphatase (DAKO, Hamburg, Germany) and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate (Bio-Rad, Munich, Germany). Image analysis of ELISPOT assays was performed with the ImmunoSpot Analysis Software after scanning the plates with an ImmuNoSpot Analyzer (Cellular Technology, Cleveland, OH).

**Statistical analysis**

For statistical analysis, Graph Pad Prism 3.0 (Graph Pad Software, San Diego, CA) was used. Values of \(p\) were calculated using the Mann-Whitney U test.

**Western blot tests**

To prepare thymic stroma tissue, thymi from three young adult wt mice (4 wk old) were collected and gently minced in a cell strainer until only the hard tissue (stroma) remained. The tissue was then passed through several petri dishes containing RPMI medium to wash away the remaining hematopoietic cells. Thymic stroma tissue was homogenized in Laemmli sample buffer (14) at a volume ratio of 1:4 and together with 11 \mu g of MPM serving as positive control was electrophoresed on 15% SDS-polyacrylamide gels. Gels were either stained with Coomassie brilliant blue or transferred to nitrocellulose for immunoblot analysis (15). The nitrocellulose (Schleicher & Schüll, Dassel, Germany) sheet was cut into stripes and washed three times in PBS/0.05% Tween 20. Unspecific binding sites were blocked by incubation for 2 h in PBS containing 10% FCS and 1% BSA. Thereafter, the stripes were incubated with mononclonal mouse Ab recognizing extracellular domain of P0 (16) for 2 h and washed three times in PBS/0.05% Tween 20. Following incubation with sheep anti-mouse IgG Ab coupled to peroxidase, the blot was developed using ECL Plus Detection Kit (Pharmacia).

**RT-PCR**

Total RNA was extracted from thymic stromal tissue or fetal thymic organ culture (FTOC) with TRIzol (Life Technologies, Gaithersburg, MD) according to the manufacturer’s instructions.

The reaction was performed using Titan One Tube RT-PCR System (Roche Diagnostics, Mannheim, Germany). The following combinations of primers (MWG Biotech, Ebersberg, Germany) for P0 amplification were used: Whole P0 protein: P01 total FWR: 5'-ATT GTG GTT TAC ACC GAC AGG-3', P01 total REV: 5'-TTC TTA TCC TCG GAC GAT CCC', Extracellular domain: P01 total FWR: 5'-ATT GTG GTT TAC ACC GAC AGG-3', P02 ex REV: 5'-CCT AGT GGG CAC TTT TTC AAA G-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', Middle segment: P05 middle FWR: 5'-AGG GAT GGC TCC ATT GTC AT-3', P05 middle REV: 5'-CAG CCA GCA GTA CGG AAT CA-3'.

RT-PCR was performed using 10 + 40 cycles of amplification at 38°C annealing temperature and 45-s elongation time. RT-PCR products were visualized by electrophoresis on a 1.5% agarose gel. To determine the specificity of the RT-PCR products, they were cloned and sequenced.

**FTOC**

Fetal thymi were dissected from embryos at day 15 and cultured on Millicell filters (8-\mu m 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 3 days after culture set-up, medium and supplements were renewed. At day 6, the culture was terminated and prepared for RT-PCR analyses. This procedure allows epitope-specific growth while depleting FTOC of thymocytes (17, 18).

**Results**

**P0 expression in the thymus**

Thymic expression of self Ags was shown to be sufficient to impart immunological tolerance (19). We investigated the presence of P0 in blood, thymic stroma, thymocytes, and FTOC. We identified the presence of P0 mRNA in thymic stroma (Fig. 1A) but not in thymocytes or blood (not shown). In RT-PCR the entire length of P0 cDNA derived from stromal cells could be amplified together with shorter cDNAs, which corresponded to different cellular domains of P0. This was confirmed by sequencing the products. The expression of P0 protein in thymic stroma was also shown by Western blot analyses (Fig. 1B). To rule out the possibility that P0 expression is not due to contamination with hematopoietic cells that may express P0, we performed RT-PCR on FTOC treated with deoxyguanosine, which depletes the thymus of thymocytes but spares the epithelial cells. Again, we detected a strong signal corresponding to the entire length of P0 cDNA (Fig. 1C).
FIGURE 1. P0 expression in the thymus at mRNA and protein level. One microgram of total RNA extracted from stroma (A; lanes 2–5) and FTOC (C) was used as template in an RT-PCR. Three different combinations of primers were used to amplify the total length of the RNA message (A; lane 2) as well as two cellular domains: extracellular and cytoplasmic (A; lanes 3–4). An additional pair of primers amplified a fragment located in the middle of the molecule (A; lane 5). The size of the specific bands is indicated. B, P0 protein (28 kDa) was detected by Western blot analyses in a thymic stromal tissue protein extract (lane 1) using α-P0 Ab as indicated. Mouse peripheral myelin was used as a positive control (lane 2) as well as P0 extracellular domain construct (lane 3) containing 107 aa residues (aa 1–102 + 5× His-tag) from extracellular domain of human P0 with an apparent molecular mass of 11 kDa. Note that the bands at ~22 kDa and 33 kDa in lane 3 probably correspond to P0 extracellular domain dimeric and trimeric products (20). The smaller band seen in lane 1 below the P0 signal is most probably a degradation product of P0 protein. C, Previously described combination of primers was used to amplify the total length of the RNA message from FTOC (lane 2).

an array with 5 column pools and 5 row pools. 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 20mer peptide can be identified.

C57BL/6 wt and P0 ko mice were immunized with BPM as a source of P0 because this protein is highly conserved in the phylogeny and very similar to murine P0. In the 219 aa sequence of P0, there are only 16 substitutions between bovine and mouse P0 and most of them are conservative, e.g., isoleucine from position 33 is replaced with leucine in bovine and both are hydrophobic amino acids. After 10 days, spleen cells were tested in the highly sensitive IFN-γ ELISPOT assay using the peptide pool array for Ag-specific recall in splenocytes of BPM primed mice. As shown in Fig. 2B, peptide pools I and C were able to induce IFN-γ spot formation. With regard to the P0 peptide pool array (Fig. 2A) this result indirectly indicates that P0 peptide 5 (Table I) contains the immunodominant epitope as this is the only common peptide in these two reactive pools.

To further confirm the finding of the P0 immunodominant peptide, we also primed animals with murine P0. As source of murine P0 in the immunization protocols we used MPM, which contains ~60% P0. Ten days after, MPM immunization, wt and P0−/− mice were sacrificed and the IFN-γ production of their LN cells was tested in response to individual peptides instead of pools. The response recalled with peptide 5 was again by far the strongest, followed by the overlapping peptide 6 (Fig. 2C). Thus, mapping the immune response to MPM with individual peptides instead of pools confirmed peptide 5 as the core immunodominant determinant also in murine P0 sequence. Note that the amino acid sequence 41–60 corresponding to the immunogenic peptide 5 is 100% identical in both species. Some P0−/− mice showed a low degree of reactivity also toward the overlapping peptide 4 (not

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FIGURE 2. Mapping the immune response to P0. A, P0 peptide pool array. Row (left) and column (top) pools of four or five P0 peptides each (pool E contains only one peptide) were constructed as shown with the numbers in the array corresponding to the individual peptides according to the complete sequence of the P0 protein. Pools were designed 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 spleen cells. At the intersection of the responding pools, the reactive peptide is found. B and C, Wt and P0 ko mice were immunized with 2.5 μg of BPM (B) or MPM (C) emulsified in CFA. Ten days later splenocytes and LN cells were harvested and stimulated with the indicated P0-derived pools of peptides (B) or individual peptides (C) in IFN-γ ELISPOT assay. Because no significant spot formation was seen with pools II, III, IV, V, A, B, D, and E (B) and with peptides 2, 3, and 7–21 (C) these results are summarized in a single bar. B, Mean + SD for six individual mice. C, Data are from pooled cells of three mice per group. Mean + SD for six wells. The experiment was reproduced three times with similar results.
shown). None of the peptides was stimulatory in immunized wt mice. Peptides 4, 5, and 6 are overlapping peptides comprising the amino acid sequence 31–70 located within the extracellular domain of P0.

**Characterization of the immune response to P0 peptide 5**

To distinguish between MHC class I and MHC class II presentation of the dominant epitope we tested the recall response to P0 5 primed wt and ko LN cells in the presence of anti-MHC class II and anti-MHC class I Abs. The results showed that Ab blockade of MHC class II but not MHC class I abrogates the immune response to this peptide in ko mice (Fig. 3, A and B) indicating that recognition of P0 5 is MHC class II restricted. There was only a limited response to P0 5 in wt mice.

ELISPOT for a broader panel of key T cell cytokines revealed that P0 5/CFA immunization induces secretion of IL-2 and IFN-γ cytokines, indicators of Th1 type of immunity, but no IL-4 or IL-5 production (data not shown). Interestingly, the ratio between P0 5 and purified protein derivative of mycobacterium (PPD) recall responses in IFN-γ ELISPOT assays was different from that seen in proliferation assays. The P0 5 response measured in proliferation assays was clearly reduced compared with the IFN-γ ELISPOT response, when the PPD response was taken as baseline in both assays (Fig. 3C). In IFN-γ ELISPOT assays, the P0 5 response was comparable with the PPD response while in proliferation assays the reactivity to P0 5 was three times lower than that to PPD.

**P0 71–90 is an absolute cryptic epitope**

To identify the presence of putative cryptic epitopes in the amino acid sequence of P0, wt or P0 ko mice were immunized with a mixture of all 21 overlapping peptides. After 10 days, LN cells were challenged in vitro with the individual peptides. Besides the overlapping peptides 5 and 6, which were reactive also upon MPM immunization, peptides 1, 3, and 8 gave consistent responses, too (Fig. 4A). The fact that peptides 1, 3, and 8 could not stimulate MPM primed cells (Fig. 2C) but were immunogenic in peptide form suggests that they are cryptic. Nevertheless, responses to peptides 1 and 3 were much lower in wt mice, indicating that they are available for MHC presentation and thus able to tolerize their specific T cells. This argues against the fact that they are cryptic. Alternatively, regulatory T cells to readily available P0 epitopes

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**FIGURE 3.** Characterization of the immune response driven by peptide 5/CFA immunization. Wt and P0 ko mice were immunized with P0 5 emulsified in CFA. A, Specific reactivity of IFN-γ secreting LN cells was tested in response to peptide 5 alone or in combination with anti-mouse H-2D^\*\(d\)/H-2K^\*\(d\) (MHC I) or anti-mouse I-A (MHC II) Abs. Mean + SD for six individual mice. B, P0 5 primed LN cells from three mice per group were pooled and tested in proliferation assays. Mean + SD for triplicate wells. C, P0 5 primed LN cells from P0 ko mice were tested comparatively in both proliferation and ELISPOT assays. The response to P0 5 was considered 100% in both assays and the response to P0 5 was calculated as percentage from PPD response. Mean + SD for five independent experiments of which three experiments included one mouse per group and two experiments included three mice per group.

**FIGURE 4.** Epitope crypticity in P0 amino acid sequence. A, Wt and P0 ko mice were immunized with a mixture comprising all 21 overlapping P0 peptides. The amount of each peptide injected was ~40 μg per mouse. Ten days later the draining LN of 3 mice in each group were pooled and challenged with individual peptides in IFN-γ ELISPOT assays. Because no significant response was seen with peptides 2, 7, and 9–21, they were represented in a single bar for clarity. Mean + SD for triplicate wells. The results shown here were reproduced twice. B, 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 3 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 6 wells. Experiment was reproduced twice with similar results.
could suppress the response to these peptides in trans. This, however, seems unlikely because peptide 8 behaved like a “truly” cryptic epitope to which similarly strong responses were observed in both wt and ko mice. To further assess this possibility mice were immunized with the peptide 8 and after 10 days the response was recalled with different Ags (Fig. 4B). The immunization of wt and P0−/− mice with peptide 5 served as negative and positive control respectively. P0 8 immunization drove a significant immune response recalled by peptide 8 but not by PMP (Fig. 4B). This result clearly demonstrates that P0 8 is a cryptic epitope.

The functional avidity of P0 specific T cells repertoire is higher in P0−/− than in wt mice

To address whether the T cell repertoire in wt mice is completely tolerated or of low avidity, we assessed the recall response of LN cells from P0 peptide 5 immunized wt and P0−/− mice in a dose-dependent manner. As shown in Fig. 5A, in ko mice the peptide 5 response could be recalled with doses as low as 0.1–1 µg/ml and followed an ascending curve until 100 µg/ml, the highest dose used to challenge LN cells. Upon recall with 100 µg/ml, there was a 5-fold increased reactivity in P0−/− mice compared with wt mice. In wt mice, the response was greatly reduced and the lowest amount of peptide evolving a specific recall response was 10 µg/ml. There was a 2 log unit shift to the right in the response to peptide 5 in wt when compared with P0−/− mice suggesting that P0−/− mice have a P0 specific T cell repertoire with a much higher avidity than that of wt mice.

Tolerance induction in wt and P0−/− mice is not dependent on gene dosage

Next, we wanted to assess tolerance toward P0 in heterozygous P0 deficient mice. Because there is a uniform increase in the gene dosage of P0 from P0−/+ to P0−/− and P0−/− mice, it is conceivable that tolerance induction is influenced by the presence of one vs two P0 genes, as was recently proposed (22, 23). To investigate this possibility, we immunized mice of all three genotypes (P0−/−, P0−/−, and P0−/−) with P0 peptide 5. The recall response to P0 5 measured by both proliferation assay and IFN-γ ELISPot (Fig. 5, B and C) was very low and of similar magnitude in wt and P0−/− deficient mice (p > 0.05). In contrast, it was much higher in P0−/− mice. These results reveal that tolerance toward P0 is fully established in heterozygous P0 deficient mice as well.

Tolerance to P0 requires P0 expression on radio-resistant nonhematopoietic cells

To evaluate the contribution of hematopoietic cells as a possible source of P0 Ag to immune tolerance to P0 we constructed BM chimeras. Wt and P0−/− mice received lethal irradiation to ablate their immune system and were then reconstituted with syngeneic P0−/− BM cells or wt BM, respectively. To confirm the functionality of chimerism, we also generated wt→wt and ko→ko BM chimeras as controls. The pattern of the P0 5 specific response in these control chimeras mirrored that of wt and P0 ko mice respectively (Fig. 6). Ko→wt BM chimeras show little reactivity toward P0, which is comparable with wt→wt chimeras, indicating that the presence of the P0 gene in the hematopoietic system is not necessary for tolerance induction (Fig. 6). However, wt→ko BM chimeric mice with a functional P0 gene only in the hematopoietic system also show reduced P0 reactivity when compared with ko→ko (p < 0.001), with an average of ~30% of that of ko chimeras. This suggests that P0 Ag derived from hematopoietic cells can contribute to some degree to tolerance induction.

Discussion

In the present study, we show that the T cell response to P0 is tolerated in wt mice but not in P0 ko mice primarily due to the expression of P0 in thymic stromal cells. The immune response to

FIGURE 5. Peptide 5 response in wt and P0 deficient mice. A, Wt and P0 ko mice were immunized with peptide 5 and the response was recalled in a dose dependent manner as indicated. The data show IFN-γ response of LN cells that were pooled from 3 mice for each subtype. Mean ± SD for triplicate wells. Experiment was reproduced twice with similar results. B and C, Wt, P0+/−, and P0−/− were immunized with 50 µg P0 peptide 5 and the reactivity was tested in IFN-γ ELISPot assay (B) and proliferation assay (C). The response was recalled with peptide 5 at a final concentration of 10 µg/ml (B) or with increasing doses of peptide (C). Positive and negative controls for all subtypes are included. Mean ± SD for 6 mice tested individually (B) and mean ± SD of 8 results representing 5 mice tested individually and 3 groups tested by pooling the cells from 3 to 4 mice per group (C). In C, the difference between wt and P0+/− is not significant (p > 0.05).

FIGURE 6. P0 tolerance status in BM chimeras. At 2 months after BM reconstitution, chimeric mice were immunized with 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 BM chimeras served as negative respectively positive controls. The peptide 5 reactivity was normalized as percentage from PPD response.
P0 in C57BL/6 mice is mainly directed against the amino acid sequence 41–60 (P0 peptide 5) and restricted to H2-IA^b molecules. Also, no gene dosage effect was found in heterozygous P0^+/− mice with regard to tolerance induction. Using BM chimeras, we demonstrate a critical role of P0 expression on thymic stroma cells for the development of complete T cell tolerance.

P0 is a typical tissue-specific Ag whose expression was thought to be limited to myelinating Schwann cells. In agreement with a recent report (10), we show that P0 is expressed also in thymic stroma at the mRNA level (Fig. 1A), and extend this finding to the detection of P0 protein (Fig. 1B). We did not, however, detect P0 transcripts in thymocytes or blood, which comprise cells of BM origin. Thus, we provide another piece of evidence that tissue specific Ags are promiscuously expressed in the thymus (8, 19, 24).

Very recent work revealed that the transcription factor autoimmune regulator prevents autoimmunity by promoting ectopic expression of many tissue-specific Ags on thymic medullary epithelial cells (25), thereby regulating negative selection (26). As yet, we do not know whether this or other promiscuous thymic expression mechanisms account for P0 tolerance. Alternatively, ectopic expression of tissue-specific Ags in the thymus may lead to self tolerance through the generation of regulatory T cells. Collectively, these data suggest that some tissue-specific Ags primarily tolerate 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 Ags with their seclusion from central tolerance induction can no longer be maintained in light of the recently accumulated evidence.

There is a possibility that thymic P0 originates from thymic nerves or Schwann cells in adult mice. However, fetal thymus innervations cannot be detected until embryonic day 17 of 18 and we used fetal thymi from 15-day embryos for thymic culture (27, 28, 29, 30). Moreover, Schwann cells in the absence of neuronal bodies down-regulate their P0 mRNA to basal level, which is negligible compared with the level in vivo (31, 32). Nevertheless, we detected a strong P0 mRNA signal (Fig. 1C) in deoxyguanosine-treated FTOC, which is free of neuronal bodies (33, 34).

In a wt vs P0 ko approach, we showed that P0 ko mice retain a high frequency of P0 reactive T cells in their T cell repertoire, while in wt mice only a low frequency can be observed. Our data confirm previous results obtained in autoantigen-deficient mice and their wt counterparts (8, 35–37). P0 ko but not wt mice showed a strong immune response toward peptide 5 upon immunization with both MPM or peptide itself (Figs. 2C and 3, A and B). These results indicate that endogenous P0 induces a profound inactivation of the P0 specific immune response in wt mice. To achieve the same degree of peripheral T cell stimulation, a hundredfold higher concentration of peptide 5 was needed in wt mice in comparison to P0 ko mice (Fig. 5A).

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. 2C) but when used as an immunogen, it induced a strong response that could be recalled with the peptide (Fig. 4, A and B) but not with MPM (Fig. 4B). Peptides 1 and 3 were not stimulatory upon MPM immunization. However, when injected as peptides they were immunogenic in P0 ko but tolerated by wt mice. Thus, they do not fit the definition of either dominant or cryptic epitopes (38). It thus seems plausible 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 such as peptide 1 and 3. It is such determinants that may be targeted for autoagression. In certain situations, their presentation may be up-regulated (39) or T cells with specificity for these epitopes expanded by means of cross-reactivity or molecular mimicry and thus they might become targets for autoimmune pathology. The consistent response induced by peptides 4, 5, and 6 (Fig. 4A) may suggest that amino acid sequence P0 31–70 represent a larger immunodominant region.

Previous studies showed that tolerance induction to self Ags is dependent on the dosage of the genes responsible for those Ags (22, 23). Although a wt genotype would ensure self-tolerance, a ko genotype would be the premise for full reactivity. At least one study (40) showed that the tolerance to a self Ag was not disrupted in the respective heterozygous deficient mouse. Our data reveal that P0^+/− mice underwent the same degree of tolerance to P0 5 as wt mice (Fig. 5, B and C) suggesting that one copy of P0 gene translates sufficient amounts of protein to mediate complete tolerance. Our results seem contradictory to recent data showing that P0-reactive T cells are eliminated in a gene-dosage dependent manner (10). However, those results were obtained using another peptide, P0 180–199, which was initially described as an immunodominant peptide in Lewis rats (5). Our mapping experiments of the immune response to P0 on BL/6 background prompted us to use the immunodominant determinant of P0 (P0 41–60), which may behave differently compared with the P0 180–199. In our hands P0 180–199 could not recall a specific response in MPM-primed LN cells, however it may be possible that, when injected as individual peptide, it could trigger an immune response. An important issue to address in further studies is the neurotogenicity of immunodominant peptides tested on active or adoptive transfer EAN models in both wt and P0^+/− mice.

We found that a P0 ko immune system grafted onto wt mice exhibit profound tolerance toward P0, comparable to wt→wt BM chimeras (Fig. 6). As P0 is expressed on thymic stromal cells, tolerance to P0 could be established at this level (19, 37, 41). It was shown very recently that wt mice grafted with P0 ko thymus failed to induce tolerance to P0 180–199 peptide (10). Conversely, P0^+/− mice grafted with wt thymus showed tolerance to P0 180–199, indicating that the thymus is the critical site for P0 tolerance induction. Although this result is in line with our previous findings on the expression of P0 protein in thymic stroma and on the full acquisition of tolerance in ko→wt BM chimeras, we also made an unexpected observation suggesting a contribution of P0 derived from hematopoietic origin. Thus, 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. As BM-derived cells are thought not to express P0, this raises a question mark about the source of P0 protein contributing to self tolerization in wt→ko BM chimeras. It is possible that trace amounts of P0 are still produced by BM cells but remain undetected with conventional methods. MBP was found to be “promiscuously” expressed both in thymic epithelial cells (42) and in differentiated blood cell lineages and hematopoietic progenitors (43). Another possibility is that wt BM cells, which include the most primitive to the most committed stem cells, were signaled 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 cells which can give rise to neurons and glial cells such as Schwann cells (44–46). Transplantation of these cells into demyelinated lesions of the animal resulted in remyelination. Moreover, green fluorescent protein-expressing BM cells were shown to colocalize with P0-positive cellular elements in the remyelinated region (47). Thus, such wt BM derived Schwann cells may be a source of tolerogenic amounts of P0 in P0 ko recipients.

The high reactivity to P0 as a foreign Ag in P0 ko mice might be a drawback when seen from the perspective of gene therapy. Some forms of Charcot-Marie-Tooth disease and Dejerine-Sottas...
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 (48). 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, the problem of P0 immunogenicity may persist. 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 embraces also the immunodominant region of the corresponding protein. A straightforward approach would be to target the corrected gene also to thymic tissue at an age where still T cells are exported from the thymus, as an attempt to override the immunoreactivity of the new protein through the generation of Ag specific regulatory T cells. Extensive studies of gene therapy on animal models for mutated P0 must be used before proceeding to human clinical trials.

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