Myelin protein zero is naturally processed in the B cells of monoclonal gammopathy of undetermined significance of immunoglobulin M isotype: aberrant triggering of a patient's T cells

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The Online version of this paper has a Supplementary Appendix.

ABSTRACT

Background
Monoclonal gammopathy of undetermined significance of immunoglobulin M isotype is a condition with clonally expanded B cells, recently suggested to have an infectious origin. This monoclonal gammopathy is frequently associated with polyneuropathy and antibodies against myelin protein zero, whereas the role of the T cells remains largely unknown. We analyzed protein zero-specific B cells, as antigen-presenting cells, and their capacity to activate T helper cells.

Design and Methods
We used a well-characterized monoclonal gammopathy of undetermined significance-derived B-cell line, TJ2, expressing anti-protein zero immunoglobulin M. The ability of TJ2 cells to bind, endocytose, process, and present protein zero was investigated by receptor-clustering and immunofluorescence. The activation of protein zero-specific autologous T cells was studied by measuring interleukin-2 and interferon-γ with flow cytometry, immunobeads, and enzyme-linked immunospot assays.

Results
Surface-receptor clustering and endocytosis of receptor-ligand (immunoglobulin M/protein zero) complexes were pronounced after exposure to protein zero. Naturally processed or synthetic protein zero peptide (194-208)-pulsed TJ2 cells significantly induced interleukin-2 secretion from autologous T cells compared to control antigen-pulsed cells ($P<0.001$). The numbers of interferon-γ-producing T helper cells, including $CD4^+/CD8^+$ cells, were also significantly increased ($P=0.0152$). Affinity-isolated naturally processed myelin peptides were potent interferon-γ stimulators for autologous peripheral blood mononuclear cells, but not for control peripheral blood mononuclear cells.

Conclusions
We show for the first time that myelin protein zero is naturally processed in B cells from monoclonal gammopathy of undetermined significance of immunoglobulin M isotype, acting as aberrant antigen-presenting cells in activation of a patient's T helper cells. Our findings cast new light on the important role of autoreactive protein zero-specific B cells in the induction of the pathogenic T-cell responses found in nerve lesions of patients with monoclonal gammopathy of undetermined significance with peripheral neuropathy.

Key words: monoclonal gammopathy of undetermined significance, MGUS, myelin P0, peripheral neuropathy, $CD5^+$ B cells.

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Introduction

Monoclonal gammopathy of undetermined significance (MGUS) is a premalignant B-cell/plasma cell disorder found in 3.2% of people over 50 years of age, with the prevalence increasing to 5.3% among people over 70 years old.[1] The disease course is different for immunoglobulin (Ig) G or IgA producing MGUS, as compared to MGUS of IgM class (IgM MGUS).[2,3] IgG or IgA MGUS progresses to multiple myeloma at a rate of 1% per year,[4] whereas IgM MGUS progresses, if it does, to Waldenström's macroglobulinemia or chronic lymphocytic leukemia (CLL) and rarely to other neoplasms.[4,5] Recent data reveal a significantly increased risk of MGUS after respiratory infections,[6] and an association with certain bacterial infections,[7] which has raised the question of an initial microbial trigger followed by cross-reactivity to self-antigens. Peripheral neuropathy is found in 8% to 36% of MGUS patients[8,9] and in 50% of patients with IgM-MGUS.[10-12] These patients show a slowly progressive, sensory/sensory motor demyelinating neuropathy[10] with antibodies[13] and T-cell infiltrates in the nerve lesions.[14] The etiology and detailed mechanisms of peripheral neuropathy in MGUS (PN-MGUS) are, however, still elusive.

Detailed structural analysis of IgM binding specificity would be valuable in understanding the pathogenesis of IgM MGUS. The antibodies previously described in PN-MGUS frequently target a sulfated trisaccharide epitope, termed HNK-1, present on surface membrane molecules of peripheral nerve Schwann cells, including myelin protein zero (P0), a 28 kDa glycoprotein and member of the Ig super gene family with adhesion molecule function mediating compaction of peripheral nerve myelin.[15-17] The HNK-1 oligosaccharide epitope is also found on myelin associated glycoprotein (MAG),[9,18-21] gangliosides[22,23] and sulfate-3-glucoronyl paragloboside.[24,25] Biochemical structural data have shown that mycobacterium bind to P0,[26,27] which is of special interest in view of the recently described association between MGUS and mycobacterial infections.[7]

PN-MGUS nerve lesion biopsies show infiltrating T cells,[14] besides the presence of IgM antibodies. Circulating $CD4^+$ and $CD8^+$ T cells in these patients have an activated phenotype,[28] and increased systemic levels of soluble interleukin (IL)-2-receptors have been observed.[29] There is also an association with HLA-DR haplotypes carrying a non-polar tryptophan residue at position 9 in the DRβ chain.[30] B cells secreting anti-MAG antibody are subject to T-cell regulation *in vitro*[31] and a T-helper 1 (Th1)-like response, with interferon (IFN)-γ secretion in response to peptides from myelin proteins, has been observed in PN-MGUS patients.[16]

In this study, we investigated whether IgM MGUS B cells are efficient antigen-presenting cells (APC) for activation of memory helper T cells. Normal B cells are well known for their APC function, but there has been some controversy regarding the ability of neoplastic B cells to stimulate T-cell responses. Recently, APC function was shown at least in some B-cell lymphomas *i.e.* CLL, representing a monoclonal $CD5^+$ B-cell expansion.[32,33] The CLL cells, however, present antigens aberrantly to T helper (Th) cells, which could explain an autoimmune trigger.[32] Although the expanded B-cell clone in MGUS, which it is worth noting is $CD5^+$ most of the time, could have a similar role in the development of autoimmune polyneuropathy,[15,16] its role in antigen presentation and T-cell activation has not previously been investigated. We followed the processing of biotin-labeled P0 or native myelin in an established anti-P0-specific IgM-MGUS B-cell line, TJ2, and found that naturally processed myelin P0 peptides aberrantly triggered autologous T cells to release IL2 and IFNγ.

Design and Methods

Antibody reagents and antigens

Antibodies and antibody-conjugates to HLA-DP, HLA-DQ, HLA-DR, IgM, LAMP-2, mouse IgG, IFNγ, CD3, CD4, CD8, CD19, CD23, CD46, CD56, and CD69 are detailed in the *Online Supplementary Design and Methods*.

Native myelin and P0 full length protein were purified from bovine peripheral nerves as described elsewhere[34,35] with some modifications.[36] Synthetic P0 peptides representing amino acids 194-208 (P0 194-208) were synthesized based on the human P0 sequence as described previously.[16] This peptide was shown to be immunogenic in PN-MGUS patients including the patient in the current study.[16] Keyhole limpet hemocyanin (KLH) was purchased from Calbiochem, (Lab Kemi, Stockholm, Sweden) and phytohemagglutinin (PHA) from Sigma-Aldrich, (Stockholm, Sweden).

Patient's data and patient-derived cells

Blood samples were collected from a 70-year old female MGUS patient with chronic progressive sensory-motor polyneuropathy. At the time of blood sample collection, the patient was not receiving any therapy and her clinical status was stable. She gave informed consent, under the guidelines from the Linköping University Hospital ethics committee, in compliance with the Declaration of Helsinki. The patient's peripheral blood mononuclear cells (PBMC) were isolated from whole blood using Lymphoprep (Nycomed Pharma A/S, Oslo, Norway). Freshly isolated PBMC were used in the IFNγ enzyme-linked immunosorbent spot (ELISPOT) assay and thawed PBMC were used in the intracellular IFNγ assay.

The Epstein-Barr virus (EBV)-transformed cell line, TJ2, was established previously in our laboratory.[36] The patient's cells expressed HLA-DR4, -DQ8, -DR7, -DQ2.[30] Serum antibodies from this patient were of IgM isotype and showed reactivity against myelin P0, MAG, and the LK-1 gycolipid, all expressing the HNK-epitope. It was concluded that TJ2 IgM had HNK-1 oligosaccharide epitope specificity. The TJ2 cell line produces IgM, λ monoclonal antibodies of mutated Ig heavy chain variable 3-15*07 (IGHV3-15*07) genotype. Phenotypic characterization of the cell line revealed a high expression of B-cell markers CD19, CD20, CD22 and CD23. CD80 and HLA-DR, two receptors necessary for antigen-presentation, were also expressed at high levels. CD5 could be found on the TJ2 cells at the initial stage of culture, but expression decreased over time. Neither the T-cell marker CD3 nor the macrophage marker CD14 was expressed by the TJ2 cells. TJ2 cells were cultured in Iscove's modified Dulbecco's medium (IMDM) mixed with Opti-MEM medium (50:50), supplemented with 10% fetal calf serum (FCS), 1% glutamine and 0.5% penicillin-streptomycin (Invitrogen Ltd., Paisley, UK).

Major histocompatibility complex class I and class II typing and immunoglobulin heavy chain gene sequencing

MHC polymerase chain reaction (PCR)-based typing using

commercial kits was performed according to the manufacturer's instructions. *IGHV* gene sequencing was carried out as previously described[36] and detailed in the *Online Supplementary Design and Methods*.

Receptor clustering and co-localization

Receptor clustering was used to investigate whether surface IgM or MHC class II (MHCII), on TJ2 cells, would bind myelin and P0. The clustering/co-capping method has been described previously[37] and is detailed in the *Online Supplementary Design and Methods*.

B-cell receptor mediated endocytosis of protein zero

The B-cell receptor (BCR) mediated uptake of biotinylated P0 in the endosomal compartment of TJ2 cells was investigated. TJ2 cells, at a concentration of 1×10^6 cells/mL, were pulsed with 10 μg/mL biotinylated P0 for 30 min on ice, after which any unbound antigen was washed away and the cells incubated at 37°C for 0 min, 20 min, 40 min, 60 min, 4 h or 24 h at a cell concentration of 10×10^6 cells/mL to allow endocytosis of the antigen-receptor complex. The cells were fixed on microscope slides using 4% paraformaldehyde and permeabilized in a balanced salt solution, 1% HEPES, 0.1% saponin buffer and stained for intracellular antigen and LAMP-2, a late endosomal marker, as described previously.[38] The primary antibodies used were Alexa488-conjugated streptavidin (2 μg/mL), mouse anti-human LAMP-2 (1:100 dilution) and Alexa594-conjugated goat anti-mouse IgG antibody (4 μg/mL). Three hundred cells were analyzed for co-localization of P0 and LAMP-2 on a Nikon-C1 laser scanning confocal unit attached to a Nikon Eclipse E600 fluorescence microscope (Nikon Instruments Inc., Melville, NY, USA).

Lysosomal staining

TJ2 cells were incubated at 37°C for 24 h in the presence of 40 μg/mL biotinylated myelin and 40 μg/mL Texas Red-conjugated dextran (Molecular Probes). The cells were washed three times with cold PBS, fixed on microscope slides using 4% paraformaldehyde and permeabilized in a balanced salt solution, 1% HEPES, and 0.1% saponin buffer. Intracellular myelin was stained with Alexa488-conjugated streptavidin and co-localization of dextran and myelin was investigated on a Carl Zeiss Axiovert 200M fluorescence microscope (Carl Zeiss).

Purification of HLA-DR, HLA-DP, and HLA-DQ peptides

We isolated naturally processed myelin peptides presented in TJ2 cell MHCII receptors. TJ2 cells were grown and expanded to 1×10^9 cells and incubated with crude myelin (550 μg per 10^9 cells) for 24 h. The cells were then solubilized for 30 min on ice in RIPA-buffer containing protease inhibitors, and the lysate was affinity-purified on a mouse anti-human HLA-DP,-DQ,-DR protein G Sepharose column (GE Healthcare, Uppsala, Sweden) using a cross-linker, disuccinimidyl suberate (DSS), for covalent immobilization of the monoclonal antibody. MHCII-peptide complexes bound to the affinity column were eluted by 10 mM Tris-HCl buffer, pH 2.0. The peak OD fractions were desalted on a Sepharose G-25 column (GE Healthcare), followed by separation on a 3 mL reversed-phase chromatography resource column (GE Healthcare) by fast protein liquid chromatography (FPLC) using an acetonitril gradient (0-100%) in 0.1% triflouroacetic acid. Eluted peptide fractions were freeze-dried.

Interferon-γ enzyme-linked immunosorbent spot assay

In order to investigate whether the purified myelin peptide fractions could activate autologous T cells, an IFNγ ELISPOT assay was performed. Autologous PBMC from a PN-MGUS patient and control PBMC from a healthy donor were prepared and the IFNγ ELISPOT assay was performed as previously described in detail.[39]

T-cell activation assay

The capacity of the P0-specific PN-MGUS derived B-cell line TJ2 to act as APC and activate autologous T cells was investigated by measuring intracellular IFNγ in T cells after 6 h in culture with antigen-primed TJ2 cells. In addition, secreted IL2 was measured in culture supernatants after 7 days. We chose to analyze IL2 in the supernatants, since it was previously shown by us and others that EBV-transformed B-cell lines can produce IFNγ.[40] In addition it was recently shown that potent T-cell and NK-cell reactivity with IFNγ production exist in EBV-seropositive patients against autologous EBV transformed lymphoblastoid cell lines.[41] Considering that the TJ2 cell line was EBV-transformed, we first carefully eliminated the anti-EBV reactive T cells and NK cells by co-culturing autologous PBMC with TJ2 cells for 15 h. B cells ($CD19^+$), NK cells ($CD56^+$) and activated (presumably by EBV-related antigens), IFNγ-secreting cells were then depleted according to the manufacturer's description using CD19/CD56 immunomagnetic MACS microbeads and an IFNγ secretion assay (Miletenyi Biotec). The purity of negatively selected PBMC was determined by flow cytometry on a FACSCanto flow cytometer (BD Biosciences). The depleted PBMC contained less than 4.8% NK cells, 9.9% B cells and 0.21% IFNγ-secreting T cells.

Freshly propagated TJ2 cell cultures were then antigen-primed by incubation with bovine P0, KLH (control antigen) or P0 peptide 194-208. Full-length protein antigens were used at a final concentration of 10 μg/mL for 16 h at 37°C. Sixteen hours was established as the optimal time for antigen presentation in the receptor co-capping studies. The P0 synthetic peptides were incubated with the TJ2 cells for 1 h at a final concentration of 1 μg/mL. The antigen-primed TJ2 cells were subsequently co-cultured in duplicate with autologous PBMC depleted of NK cells, B cells and IFNγ-secreting cells. The cells were either used for flow cytometric intracellular IFNγ staining, or measurement of secreted IL2. PHA stimulation (10 μg/mL) of PBMC was used as a positive control.

Intracellular interferon-γ staining

Antigen-primed TJ2 cells and autologous PBMC were co-cultured for 6 h at 37°C in the presence of 20 μg/mL brefeldin A. The cells were washed twice in cold PBS, 0.1% BSA and stained with anti-CD3 PerCP, anti-CD4-FITC, anti-CD8-APC Cy7, anti-CD19-PE-Cy7, and anti-CD56 APC monoclonal antibodies for 30 min on ice. The cells were washed twice, fixed in cold 4% paraformaldehyde, washed twice and permeabilized in PBS, 0.1% BSA, and 0.1% saponin. Intracellular IFNγ was stained by incubating the cells with anti-IFNγ PE monoclonal antibodies for 30 min on ice. The cells were then washed twice, resuspended at 1×10^6 cells/mL and analyzed on a FACSCanto flow cytometer (BD Biosciences). At least 30,000 cells were collected and IFNγ expression was analyzed both for $CD3^+$ T lymphocytes and $CD3^+$ T lymphoblasts. The percentages of IFNγ-positive cells after antigen stimulation (P0, KLH or P0 peptides 194-208) were calculated and the various T-cell populations were compared using Fisher's exact test.

Interleukin-2 immunoassay

Supernatants from the 7 day co-cultures of antigen-incubated TJ2 cells (P0, KLH or P0 peptides 194-208) with autologous PBMC were collected and analyzed in six replicate experiments for the presence of the T-cell specific cytokine IL2 using a

Milliplex human cytokine/chemokine immunoassay (Millipore, Billerica, MA, USA). The assay was performed according to the manufacturer's description except that one additional standard reference point of 0.64 pg/mL was added to the standard curve. The samples were analyzed on a Luminex 100 instrument (Luminex Corporation, Austin, USA) with STarStation software (V1.1, Applied Cytometry Systems, Sheffield, UK). Mean values were compared using one way ANOVA and Tukey's post hoc test.

Bioinformatic prediction of major histocompatibility complex-binding protein zero peptides

The prediction tools used were PROPRED,[42] SVMHC[43] IEDB-AR[44-47] and HLA-pred. Details of the procedures are given in the *Online Supplementary Design and Methods*.

Results

The aim of this study was to assess the potential role of the expanded CD5+ B-cell clone in PN-MGUS as APC. For this purpose, we used the P0-specific B cell line, TJ2, previously established in our laboratory.[36] We typed and sequenced the MHC class I (MHCI), MHCII and IGHV genes respectively. High resolution MHCI typing of HLA-A and HLA-B was performed for the TJ2 cell line and revealed genotype HLA-A*0201, *3101; HLA-B*1302, *4001. High resolution MHCII typing of HLA-DRB1, HLA-DQA1 and HLA-DQB1 for the TJ2 cell line showed genotype HLA-DRB1*0403, *0701; DQA1*0201, *0301; DQB1*0202, *0302. PCR amplification and sequencing of the IGHV gene from the TJ2 cell line was performed. TJ2 cells express IGHV3-15*07 gene with 90.39% homology to germline gene and a very short HCDR3 with only eight amino acids (ATGGLVGA). Codon 118 is mutated, resulting in the lack of a W-anchor (W is replaced by V), however, the W(F)-G-X-G motif is conserved and in frame. Figure 1 gives an overview of the experimental design and strategy of this study. In general, experiments involving nerve antigens were performed both with purified native myelin and pure P0.

Docking of protein zero ligand to the B-cell recpetor

First, we showed that myelin and P0 bound specifically to surface IgM of TJ2 cells, using two-color immunofluorescence and receptor clustering. The cells were pulsed with biotinylated myelin for 30 min followed by incubation with unbiotinylated antigen and microscopy reading at several time points. P0 binding to IgM was investigated at two time points. Receptor clustering of surface IgM was induced and the frequency of myelin to IgM co-localization on myelin-positive cells was analyzed. Surface IgM clustering (red) induced co-capping of myelin (green) in the majority of TJ2 cells at all time points investigated (Figure 2A-B). At 16 h, the ratio of cells displaying co-localization of surface IgM and myelin decreased from 89% to 50%, followed by an increase to 75% at 24 h. Parallel observations on P0 showed 98-100% co-localization with surface IgM (Figure 2C). We concluded that myelin and P0 specifically bound to the surface IgM BCR of TJ2 cells.

Protein zero uptake and processing

BCR-mediated receptor internalization and intracellular trafficking of P0 was studied by investigating the co-localization of antigen with late endosomal/lysosomal marker LAMP-2 or lysosomal marker dextran conjugated with Texas Red. To follow the P0 trafficking, TJ2 cells were pulsed with biotinylated P0 for 30 min, at 0°C, washed to remove unbound antigen, and then analyzed at various time points (0 min, 20 min, 40 min, 60 min, 4 h, and 24 h

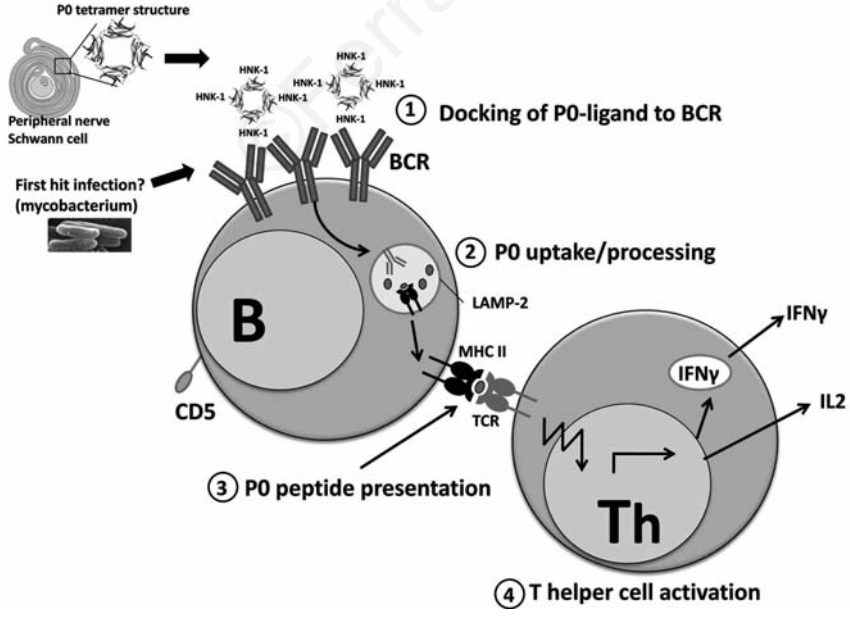

Figure 1. Proposed model and experimental design of the current study. Myelin protein zero (P0) is a glycoprotein present in the membrane of Schwann cells in the peripheral nervous system. The crystal structure of the extracellular domain shown is from rat[61] and is reprinted with kind permission from Dr Petri Kursula (Oulu, Finland).[48] We hypothesize that P0 is exposed to the CD5+ P0 specific MGUS B cells, perhaps due to a nerve injury. (1) P0 binds to and induces clustering of BCR on the myelin-reactive MGUS B cells. (2) The BCR-antigen complex is endocytosed, processed and P0 peptides are loaded onto MHCII molecules. (3) P0 peptides are presented to and activate T cells. (4) The activated T cell starts producing IFNγ and IL2. The numbers shown in the Figure also correspond to the different experiments presented in the Results section. (1) Receptor clustering of surface IgM and co-localization of P0. (2) Co-localization of P0 and late endosomal/lysosomal marker LAMP-2. (3) Receptor clustering of MHCII and co-localization of P0. (4) Activation of autologous T cells by P0 incubated MGUS B cells as measured by intracellular IFNγ and secreted IL2. The ability of naturally processed P0 peptides to activate autologous T cells with the secretion of IFNγ was also investigated.

at 37°C). At the onset (time 0) 87% of TJ2 cells showed surface-location of P0, not co-localized with LAMP-2, as expected (Figure 2D). Already at 20 min, P0 co-localized with the endosomal LAMP-2 marker in 75% of P0-positive cells. At 4 h and 24 h an endosomal accumulation of P0 was found in 32% and 45%, respectively, of P0-positive TJ2 cell (Figure 2E). Confocal images of P0 trafficking in TJ2 cells at the various time points investigated revealed an accumulation in late endosomes (Figure 2D). We confirmed the processing and trafficking of P0 by using native myelin and tracked its location to the late endosomal/lysosomal compartment with the Texas Red-dextran conjugate. At 24 h, distinct 0.5 μm large endosomal vesicles containing both myelin and dextran were observed (Figure 2F).

Myelin peptide presentation in major histocompatibility complex class II

We then followed the trafficking of biotinylated myelin after endocytosis and entry into the endosomal/lysosomal (LAMP-2$^+$) compartment by analyzing its association with MHCII. Approximately 50% of cells showed co-localization of P0 and LAMP-2 to a large extent already at 1 h, as detailed above. Non-endocytosed myelin P0, however, remaining at the surface was always associated with IgM (Figure 2A-C). We, therefore, assumed that the biotinylated myelin that was bound to MHCII was present as processed peptides and not as native protein. The physical association of myelin peptides with MHCII and the kinetics of processed myelin peptides appearing at the surface membrane was investigated by tracking biotinylated myelin or biotinylated P0 followed by dual color staining after receptor-clustering. Biotinylated myelin was associated with MHCII in 13-16% of cells after 2.5 h to 5 h. At 16-24 h 52% of TJ2 cells displayed myelin peptide-MHCII complexes (Figure 2G-H). The kinetics of the biotinylated P0 peptide-MHCII physical association revealed a similar profile to that of native myelin processing and peptide

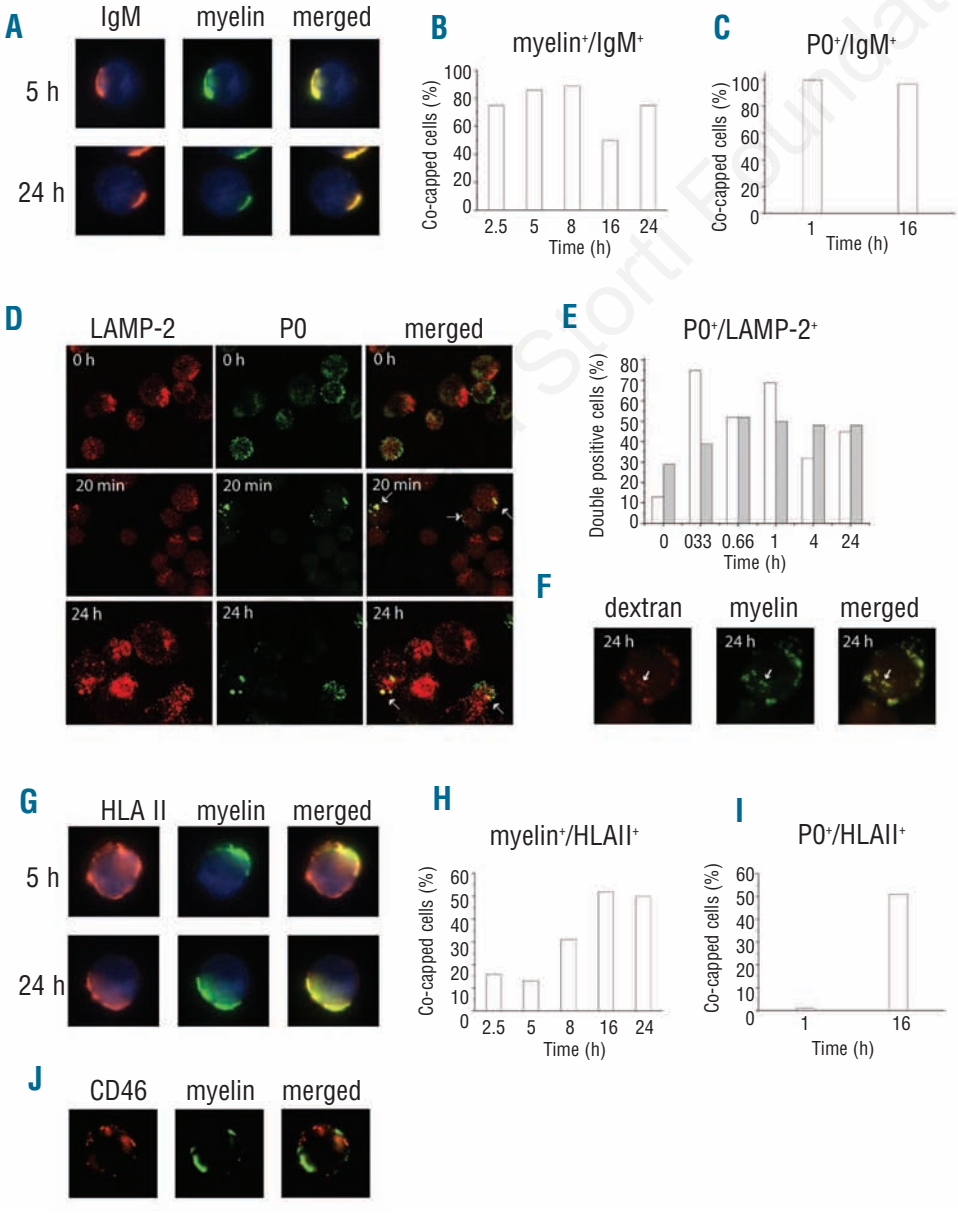

Figure 2. Myelin/P0 binding to surface IgM, presence of P0 in the late endosome/lysosome compartment and presentation of myelin/P0 peptides in MHCII on the P0 specific MGUS B cell line TJ2. (A) Immunofluorescence images of myelin (green) co-clustered with surface IgM (red) on TJ2 cells after 5 h and 24 h. Yellow regions in the overlay images indicate co-localization. (B) Myelin binding to surface IgM was seen on a majority of TJ2 cells at all investigated time points (2.5 h, 5 h, 8 h, 16 h and 24 h). (C) Similar results were seen for P0 and surface IgM. (D) Images show uptake of P0 (green) in TJ2 cells after 0 h, 20 min and 24 h. Endosomal marker, LAMP-2, is shown in red. Yellow regions in the overlay images indicate co-localization, also shown by arrows. (E) Kinetic analysis of BCR-mediated P0 uptake in TJ2 cells. Percent P0-positive and LAMP-2- positive cells are shown after 0 h, 20 min, 40 min, 60 min, 4 h, and 24 h. Results from two independent experiments are shown (white and gray bars). For each time point, at least 300 LAMP-2 positive TJ2 cells were analyzed for the presence of P0. (F) The simultaneous uptake of P0 (green) and dextran (red) in the lysosomal compartment of TJ2 cells was investigated after 24 h. Yellow regions in the overlay images indicate co-localization. (G) Immunofluorescence images of myelin (green) co-clustered with HLAII (red) after 5 h and 24 h respectively. Yellow regions in the overlay images indicate co-localization. (H) Percentage of TJ2 cells exhibiting co-clustering of myelin with HLAII is shown for 2.5 h, 5 h, 8 h, 16 h, and 24 h. (I) Receptor clustering of MHCII and P0. (J) Negative control showing clustering of myelin, but no co-localization with control protein CD46.

presentation (Figure 2I). Figure 2J shows a negative control experiment, in which an irrelevant surface antigen, CD46, was shown not to co-cluster with myelin. The fluorescence patterns of the two proteins do not overlap at all.

T helper cell activation by naturally processed myelin peptides

Biochemical isolation of myelin peptides

The myelin peptides that were generated upon processing in TJ2 cells were isolated by affinity chromatography using an anti-HLA-DR, -DP, -DQ column, followed by purification by reverse phase chromatography on FPLC. All FPLC fractions were screened for biological activity by analyzing IFNγ release from T cells. Freshly isolated autologous and control PBMC were pulsed with FPLC peptide fractions and surveyed in an IFNγ ELISPOT assay. Triplicate samples were analyzed and the mean value was used for each test. Peptides were eluted in fraction 24-28, as shown by the OD_{280} nm peak (Figure 3). These very fractions also induced IFNγ secretion in autologous PBMC (Figure 3A), but not in control cells (Figure 3B). Fraction 28 generated the maximal IFNγ response with a mean value of 72 spots.

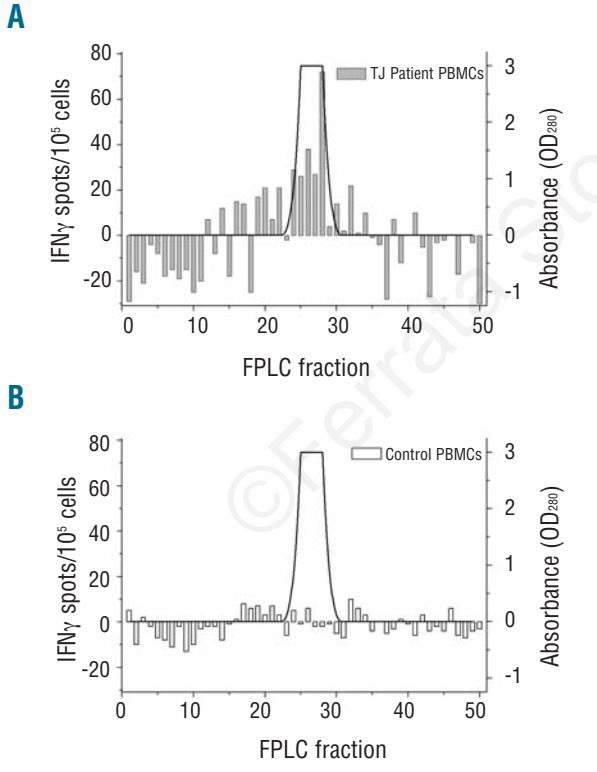

Figure 3. Naturally processed myelin peptides induce IFNγ secretion from PN-MGUS patient's PBMC, but not from control PBMC. Affinity purified and FPLC separated myelin peptide fractions # 1 to 50 were analyzed by ELISPOT for the ability to stimulate IFNγ secretion in (A) autologous PBMC from a PN-MGUS patient and (B) control PBMC from a healthy donor. The spontaneous background was subtracted and mean values from triplicate samples are shown (left axis). Peptide fraction # 24-28 induced an increased secretion of IFNγ in autologous PBMC, but not in control PBMC, and fraction # 28 generated the highest IFNγ response. Peptide fraction # 24-28 coincides with the OD_{280} absorbance peak from the FPLC separation (line) shown on the right axis.

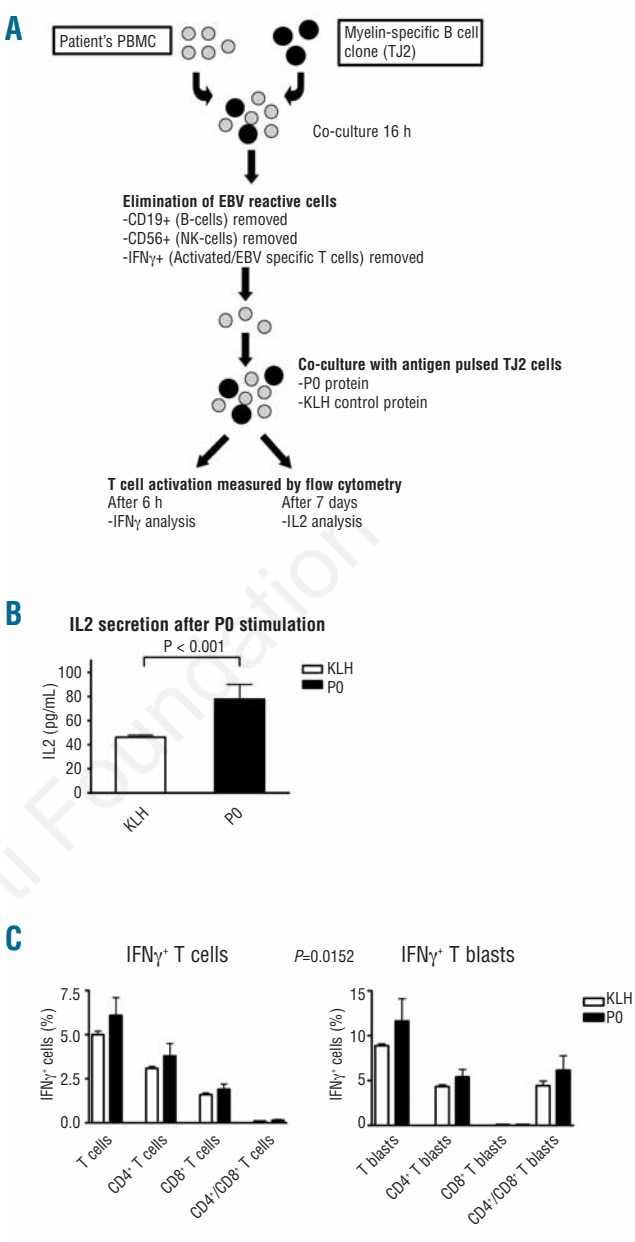

Figure 4. The PN-MGUS derived B-cell line TJ2 is capable of P0-specific T-cell activation. (A) Experimental design of the T-cell activation assay. $CD19^+$ B cells, $CD56^+$ NK cells and IFNγ-secreting, presumably EBV reactive cells, were eliminated from the PN-MGUS patient's PBMC by magnetic separation. PBMC were subsequently co-cultured with antigen-pulsed TJ2 cells for 16 h. Intracellular IFNγ and secreted IL2 were measured after 6 h and 7 days, respectively. (B) Significantly increased levels of secreted IL2 in the 7-day cell cultures were seen after stimulation with P0 pulsed TJ2 cells ($P<0.001$), compared to control antigen, KLH-pulsed TJ2 cells. The results are mean values (±SD) of six separate experimental cultures. More than 30,000 cells from each culture were analyzed in FACS. (C, left panel) An increased level of intracellular IFNγ was seen in T lymphocytes after incubation with P0-primed TJ2 cells as compared to KLH (control antigen)-primed TJ2 cells. This increase was mainly seen in the $CD4^+$ T lymphocyte population although a slight increase was also noted in the $CD8^+$ T lymphocyte population. (C, right panel) T lymphoblasts also exhibited an increased accumulation of intracellular IFNγ after P0-primed TJ2 cell stimulation as compared to KLH (control antigen)-primed TJ2 cells. The IFNγ increase was seen in the $CD4^+$ T blasts and in $CD4^+/CD8^+$ T blasts, but could not be detected in $CD8^+$ T blasts. The P0 *versus.* KLH populations in C (both panels) were statistically analyzed using Fisher's exact test and showed a significantly different response ($P=0.0152$).

Protein zero-specific B cells in monoclonal gammopathy of undetermined significance are efficient antigen-presenting cells

We investigated whether the P0-specific B-cell clone pulsed with P0 or synthetic P0 peptides 194-208 could act as APC for the activation of autologous T cells. For this purpose TJ2 cells were pulsed with P0 or control antigen KLH for 16 h, or with P0 peptides 194-208 for 1 h. The antigen-pulsed TJ2 cells were then co-cultured for 6 h or 7 days with autologous PBMC from a PN-MGUS patient; the PBMC were depleted of $CD56^+$ NK cells, $CD19^+$ B cells and potential EBV-reactive IFNγ-secreting T cells (Figure 4A). T-cell-secreted IL2 was measured in 7 day co-cultures of the TJ2 B-cell clone plus autologous T cells. Significantly increased levels of IL2 were seen after stimulation with P0-pulsed TJ2 cells as compared to control KLH antigen-pulsed TJ2 cells ($P<0.001$) (Figure 4B). B cells pulsed with P0 peptides 194-208 were also potent inducers of T-cell IL2 release ($P<0.001$, *results not shown*).

The induction of IFNγ in a patient's T cells after exposure to peptide-pulsed TJ2 cells was analyzed by flow cytometry after 6 h co-cultures. In the control T-cell population (without any prior antigen pulse), a distinct lymphoblast population was seen in the forward scatter/side scatter dot plot. IFNγ production was therefore analyzed in two separate FACS experiments both in the T lymphocyte population and in the T blast population. Taken together, P0-pulsed TJ2 cells induced a significantly increased IFNγ production above control values of non-stimulated PBMC in five of the six populations ($CD4^+$, $CD8^+$ and $CD4^+/CD8^+$ in the lymphocyte and blast populations). In comparison, KLH-pulsed TJ2 cells did not show an increase in any of six populations ($P=0.0152$, Fisher's exact test). Increase above control was defined as more than 9% above control, which was the total coefficient of variation in this assay (Figure 4C). Interestingly, a $CD4^+/CD8^+$ T lymphoblast population was also present in the PN-MGUS patient. This population also exhibited increased IFNγ levels after stimulation with P0-pulsed TJ2 cells (Figure 4C). TJ2 cells pulsed with P0 peptides 194-208, however, did not stimulate the patient's $CD3^+$ T lymphocytes or T lymphoblasts to increased IFNγ release (*results not shown*). Five percent of T lymphocytes and 9% of T lymphoblasts were IFNγ-positive after control antigen-pulsed TJ2 cell stimulation. This might reflect EBV-reactive T-cell populations not eliminated by the magnetic separation prior to the activation assay.

Bioinformatics: prediction of major histocompatibility complex-binding protein zero peptides

The location of the highest ranking MHCII-binding peptide, amino acids 107-115, in the extracellular domain of P0 can be seen marked in black in the protein crystal structure (Figure 5). Further MHCII and MHCI candidates are detailed in the *Online Supplementary Results* and *Online Supplementary Figure S1*.

Discussion

In this study, we explored the interaction between autoreactive, P0-specific monoclonal $CD5^+$ B cells and T cells in an IgM MGUS patient with chronic progressive sensory-motor polyneuropathy. The main finding is that the patient's authentic MGUS B-cell line, TJ2, had a potent antigen-processing capacity, able to trigger IL2 and IFNγ cytokine production in myelin-specific autologous T cells. The TJ2 cell line expresses a surface IgM BCR with a mutated IGHV3-15*07 gene. The TJ2 IgM binds to an epitope that includes the HNK-1 oligosaccharide linked to Asn93 in the P0 protein sequence.[48] The PN-MGUS patient's B-cell clone expanded *in vitro* was authentic to the patient's monoclonal anti-P0 autoreactive cells, and we found that it retained surface BCR with affinity for P0, efficiently mediated BCR-ligand uptake, processed the antigen and presented myelin peptides in MHCII molecules. We show here for the first time that these naturally processed P0 peptides presented by the B-cell clone induce production of the T-cell-specific cytokine IL2 from autologous T cells and production of the Th1 cytokine IFNγ from both $CD4^+$ and $CD8^+$ autologous T cells.

The involvement of T cells in the pathogenesis of PN-MGUS has been previously suggested[14,16,28-30] and our results presented in this study add further evidence on how these MGUS-derived T helper cells can be triggered by the patient's monoclonal B-cell population that aberrantly presents myelin peptide antigens. The results presented in Figure 3 show that the FPLC peptide fractions induced IFNγ in autologous PBMC, probably due to the presence of anti-P0 antigen memory T cells, which may have arisen after broken tolerance to self-antigen after an infection, as we have interpreted the data. A transforming event in a self-reactive cell may then lead to expansion of an autonomous MGUS-clone. These memory T-cell clones that we observe might be remnants of a disease process that occurred some time previously.

A recent study identified P0 as the key $CD4^+$ T-cell antigen in the NOD-B7-2KO autoimmune peripheral neuropathy mouse model.[49] NOD-B7-2KO mice deficient of IFNγ did not develop peripheral neuropathy, suggesting an inflammatory Th1 response to P0 in these mice. The authors also generated a P0-specific TCR transgenic

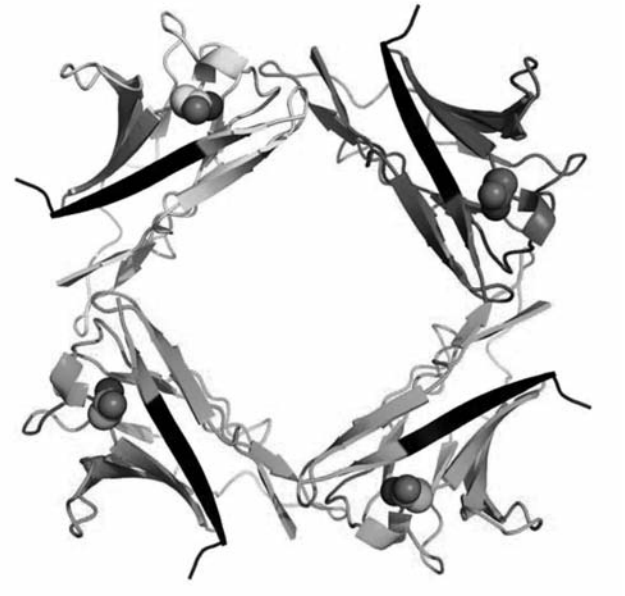

Figure 5. Crystal structure of the P0 extracellular domain from rat according to Shapiro *et al.*[61] and Kursula P.[48] Glycosylation site, Asn93, is shown as spheres and the HLA-DRB1*0701 predicted binding peptide amino acids 107-115 in black.

mouse (NOD-POT) and CD4+ T cells from this mouse proliferated *in vitro* when exposed to peripheral nerve lysate and P0. T cells from the NOD-POT mouse exposed to peripheral nerve lysate or P0 *in vitro* also produced IFNγ and IL17.

A contrasting hypothesis was suggested by Horna *et al.*,[33] who showed that malignant B cells efficiently present tumor antigens to antigen-specific CD4+ T cells, resulting in a strong antitumor effect. This intrinsic antigen-presenting ability of malignant B cells was, however, overridden by tolerogenic bone marrow-derived APC, leading instead to T-cell unresponsiveness and lack of antitumor effect. In the case of MGUS, however, it is difficult to overlook that in PN-MGUS nerve lesion biopsies there are infiltrating T cells, with both CD4+ and CD8+ cells.[14,28] In addition, we previously compared IL4 and IFNγ responses in eight PN-MGUS patients with those in eight normal donors, and in four patients with polyneuropathy other etiology and found significantly more IFNγ than IL4 release in PN-MGUS patients.[16]

The malignant CLL B-cell clone can act as potent APC and aberrantly activate Rh autoreactive T helper cells, thus driving the autoimmune hemolytic anemia seen in many CLL patients.[32] Furthermore, CLL cells have been found to be efficient APC, presenting, in CLL cells, the highly upregulated protein fibromodulin in HLA-A2.[50,51] Pre-activation *in vitro* also enabled the expanded, fibromodulin-specific T cells to secrete IFNγ upon recognition of the antigen.[51] This CD5+ B-cell-specific antigen-presentation thus enabled the expansion of autologous tumor-specific T cells. Another example illustrating the important role of B cells is experimental autoimmune encephalitis, the experimental animal model for multiple sclerosis. Experimental autoimmune encephalitis can be induced by exposing wild-type mice to myelin oligodendrocyte protein, but in mice deficient of B cells, there is no induction, suggesting that B cells have a clear antigen-presenting role in this condition.[52]

In this study (Figure 2 D-F), we show that myelin peptides are processed in the endosome/lysosome compartment (LAMP-2+) and physically associated with MHC class II (Figure 2 G-I). Non-endocytosed myelin could remain on the surface membrane, which would dim the interpretation. However, based on the data illustrated in Figure 2A, we found that all native surface-bound myelin was associated with surface IgM (none was found in other sites). Secondly, the FPLC fractions analyzed (Figure 3) contained peptides only, no native myelin. Specific anti-peptide reagents/monoclonal antibodies would, however, be helpful in distinguishing between processed and unprocessed myelin.

Previous studies have shown Th1 activation and IFNγ production by PBMC from a PN-MGUS patient after synthetic P0 peptide 194-208 stimulation.[16] We confirmed in this study that P0 peptide (194-208)-pulsed TJ2 cells could stimulate a patient's T cells to increase IL2 secretion significantly compared to control antigen. P0 peptide (194-208) did not appear as one of the candidates in the bioinformatic search for HLA-DRB1*0701, HLA-A or HLA-B binding P0 peptides. One possible explanation is that P0 peptide (194-208) is presented in a HLA-DR, DP, DQ receptor of a different genotype. The MHCII peptide binding prediction tools used did not allow prediction of peptides binding to the PN-MGUS patient's HLA-DQ or HLA-DRB1*0403 allele. Interestingly, the bioinformatics data did show that one of the top ranking HLA-DRB1*0701 binding peptides VGKTSQVTL (P0 amino acids 107-115) is localized in the extracellular domain of P0 (Figure 5), thus making it an appealing target for further T-cell activation studies.

During detailed analysis of the IFNγ-producing cell compartment, we noted the presence of an activated CD4+/CD8+ double-positive T blast population (6.2% of T cells) producing IFNγ in the investigated PN-MGUS patient after stimulation with P0. This double-positive population was also found after control KLH antigen stimulation, but at a lower percentage (4.2%). The appearance of double-positive CD4+/CD8+ T lymphocytes had previously been observed after *in vitro* stimulation of human PBMC with mitogenic PHA.[53] It is noteworthy that human herpes viruses are able to induce CD4-expression on human CD8+ T cells.[54] The PBMC from the MGUS patient investigated in this study contained 3.5% CD4+/CD8+ lymphocytes prior to antigen exposure, which is a level similar to that found in normal individuals (2-3%).[55,56] An elevated amount of CD4+/CD8+ lymphocytes had previously been found in MGUS patients,[55] as well as in other autoimmune conditions, such as myasthenia gravis[57] and multiple sclerosis.[58] The appearance of double-positive CD4+/CD8+ T blasts after antigen stimulation reflects an immune abnormality in these autoimmune conditions, as suggested also in our study.

One potential limitation of the present study is that it is based primarily on detailed findings from one patient. However, several different experiments were carried out on this patient and consistently pointed in the same direction. We also expanded the P0 peptide analysis to three additional PN-MGUS patients, which all showed increased P0-peptide-induced IFNγ release as compared to control peptides. This release was abrogated by elimination of CD19+ cells. Hence, there is no reason to believe that the findings presented in this study would not be of general relevance.

The induction of autoantibody production by bacterial molecular mimicry has previously been suggested in PN-MGUS, based on cross-reactivity of the patients' IgM anti-MAG antibodies with bacterial polypeptides.[59] Novel findings also show that the CD5 surface receptor binds certain microbial glycan structures with high affinity.[60] Furthermore, recent data show that the risk of developing multiple myeloma and MGUS is significantly increased among men who have had prior infectious disorders, in particular poliomyelitis for MM (RR = 3.69) and pneumonia for MGUS (RR =2.48).[6] In addition, an association between MGUS and mycobacterial infections was recently revealed in a population of 17,398 patients.[7] Based on these observations and our own results it is tempting to speculate about an infectious background to PN-MGUS. A microbial-triggered B-cell expansion may generate (by molecular mimicry) antigen spreading with B-cell clones reacting with neo-epitopes of myelin molecular motifs in P0 thus aberrantly presenting antigens that induce autoreactive T helper cells. P0 molecular structure studies showing homology to mycobacterial proteins provide support to this idea.[27] Activation of autoreactive T helper cells and production of nerve-specific autoantibodies would, in the end, result in the neuropathy seen in PN-MGUS patients.

In conclusion, the data presented here strongly suggest

that the expanded P0-specific IgM MGUS clone TJ2 is an efficient professional APC able to generate deleterious T-cell activation. These effects are paralleled *in vivo* where $CD4^+$ and $CD8^+$ cells, together with myelin-bound autoantibodies, have been observed in nerve lesions *in situ*, and are most likely involved in the pathological destruction of peripheral nerves seen in PN-MGUS patients.

Authorship and Disclosures

EH and MK planned and performed research, analyzed data, and wrote the paper; AS performed research, analyzed data, and wrote the paper; MV collected patients' samples, planned research, analyzed data and wrote the paper; JE and AR designed and supervised the research, analyzed data and wrote the paper.

The authors declare no conflicts of interest.

References

1. Kyle RA, Therneau TM, Rajkumar SV, Larson DR, Plevak MF, Offord JR, et al. Prevalence of monoclonal gammopathy of undetermined significance. N Engl J Med. 2006;354(13):1362-9.
2. Martín-Jiménez P, García-Sanz R, Balanzategui A, Alcoceba M, Ocio E, Sanchez ML, et al. Molecular characterization of heavy chain immunoglobulin gene rearrangements in Waldenström's macroglobulinemia and IgM monoclonal gammopathy of undetermined significance. Haematologica. 2007;92(5):635-42.
3. Sahota SS, Forconi F, Ottensmeier CH, Provan D, Oscier DG, Hamblin TJ, Stevenson FK. Typical Waldenström macroglobulinemia is derived from a B-cell arrested after cessation of somatic mutation but prior to isotype switch events. Blood. 2002;100(4):1505-7.
4. Kyle RA, Therneau TM, Rajkumar SV, Offord JR, Larson DR, Plevak MF, Melton LJ 3rd. A long-term study of prognosis in monoclonal gammopathy of undetermined significance. N Engl J Med. 2002; 346(8):564-9.
5. Baldini L, Goldaniga M, Guffanti A, Broglia C, Cortelazzo S, Rossi A, et al. Immunoglobulin M monoclonal gammopathies of undetermined significance and indolent Waldenström's macroglobulinemia recognize the same determinants of evolution into symptomatic lymphoid disorders: proposal for a common prognostic scoring system. J Clin Oncol. 2005;23(21):4662-8.
6. Brown LM, Gridley G, Check D, Landgren O. Risk of multiple myeloma and monoclonal gammopathy of undetermined significance among white and black male United States veterans with prior autoimmune, infectious, inflammatory, and allergic disorders. Blood. 2008;111(7):3388-94.
7. Bida JP, Kyle RA, Therneau TM, Melton LJ 3rd, Plevak MF, Larson DR, et al. Disease associations with monoclonal gammopathy of undetermined significance: a population-based study of 17,398 patients. Mayo Clin Proc. 2009;84(8):685-93.
8. Nobile-Orazio E, Barbieri S, Baldini L, Marmiroli P, Carpo M, Premoselli S, et al. Peripheral neuropathy in monoclonal gammopathy of undetermined significance: prevalence and immunopathogenetic studies. Acta Neurol Scand. 1992;85(6):383-90.
9. Vrethem M, Cruz M, Wen-Xin H, Malm C, Holmgren H, Ernerudh J. Clinical, neurophysiological and immunological evidence of polyneuropathy in patients with monoclonal gammopathies. J Neurol Sci. 1993;114(2):193-9.
10. Gosselin S, Kyle RA, Dyck PJ. Neuropathy associated with monoclonal gammopathies of undetermined significance. Ann Neurol. 1991;30(1):54-61.
11. Yeung KB, Thomas PK, King RH, Waddy H, Will RG, Hughes RA, et al. The clinical spectrum of peripheral neuropathies associated with benign monoclonal IgM, IgG and IgA paraproteinaemia. Comparative clinical, immunological and nerve biopsy findings. J Neurol. 1991;238(7):383-91.
12. Kyle RA, Rajkumar SV. Monoclonal gammopathy of undetermined significance. Br J Haematol. 2006;134(6):573-89.
13. Latov N. Pathogenesis and therapy of neuropathies associated with monoclonal gammopathies. Ann Neurol. 1995;37 (Suppl 1):S32-42.
14. Solders G, Nennesmo I, Ernerudh J, Cruz M, Vrethem M. Lymphocytes in sural nerve biopsies from patients with plasma cell dyscrasia and polyneuropathy. J Peripher Nerv Syst. 1999;4(2):91-8.
15. Favereaux A, Lagueny A, Vital A, Schmitter JM, Chaignepain S, Ferrer X, et al. Serum IgG antibodies to P0 dimer and 35 kDa P0 related protein in neuropathy associated with monoclonal gammopathy. J Neurol Neurosurg Psychiatry. 2003;74(9): 1262-6.
16. Ekerfelt C, Ernerudh J, Vrethem M. Th1-like responses to peptides from peripheral nerve myelin proteins in patients with polyneuropathy associated with monoclonal gammopathy. Clin Exp Immunol. 1997;108(3):516-22.
17. Hammer JA, O'Shannessy DJ, De Leon M, Gould R, Zand D, Daune G, Quarles RH, et al. Immunoreactivity of PMP-22, P0, and other 19 to 28 kDa glycoproteins in peripheral nerve myelin of mammals and fish with HNK1 and related antibodies. J Neurosci Res. 1993;35(5):546-58.
18. Murray N, Page N, Steck AJ. The human anti-myelin-associated glycoprotein IgM system. Ann Neurol. 1986;19(5):473-8.
19. Latov N, Hays AP, Sherman WH. Peripheral neuropathy and anti-MAG antibodies. Crit Rev Neurobiol. 1988;3(4):301-32.
20. McGarry RC, Helfand SL, Quarles RH, Roder JC. Recognition of myelin-associated glycoprotein by the monoclonal antibody HNK-1. Nature. 1983;306(5941):376-8.
21. Quarles RH. Myelin-associated glycoprotein (MAG): past, present and beyond. J Neurochem. 2007;100(6):1431-48.
22. Silberman J, Lonial S. Review of peripheral neuropathy in plasma cell disorders. Hematol Oncol. 2008;26(2):55-65.
23. Ilyas AA, Quarles RH, Brady RO. The monoclonal antibody HNK-1 reacts with a human peripheral nerve ganglioside. Biochem Biophys Res Commun. 1984; 122(3):1206-11.
24. Chou DK, Ilyas AA, Evans JE, Costello C, Quarles RH, Jungalwala FB. Structure of sulfated glucuronyl glycolipids in the nervous system reacting with HNK-1 antibody and some IgM paraproteins in neuropathy. J Biol Chem. 1986;261(25):11717-25.
25. Quarles RH, Weiss MD. Autoantibodies associated with peripheral neuropathy. Muscle Nerve. 1999;22(7):800-22.
26. Suneetha LM, Singh SS, Vani M, Vardhini D, Scollard D, Archelos JJ, et al. Mycobacterium leprae binds to a major human peripheral nerve glycoprotein myelin P zero (P0). Neurochem Res. 2003;28(9):1393-9.
27. Vardhini D, Suneetha S, Ahmed N, Joshi DS, Karuna S, Magee X, et al. Comparative proteomics of the Mycobacterium leprae binding protein myelin P0: its implication in leprosy and other neurodegenerative diseases. Infect Genet Evol. 2004;4(1):21-8.
28. Vrethem M, Dahle C, Ekerfeldt C, Nilsson J, Ekstedt B, Ernerudh J. Abnormalities in T-lymphocyte populations in blood from patients with demyelinating polyneuropathy associated with monoclonal gammopathy. J Neurol Sci. 1994;122(2):171-8.
29. Vrethem M, Skogh T, Ernerudh J, Ekstedt B, Andersen O, Lycke J. Soluble interleukin-2 receptor levels in serum of patients with demyelinating polyneuropathy associated with monoclonal gammopathy. J Neurol Neurosurg Psychiatry. 1993;56(6):721-2.
30. Vrethem M, Ernerudh J, Cruz M, Olerup O, Solders G, Ekstedt B, et al. Susceptibility to demyelinating polyneuropathy in plasma cell dyscrasia may be influenced by amino acid position 9 of the HLA-DR beta chain. J Neuroimmunol. 1993;43(1-2):139-44.
31. Latov N, Godfrey M, Thomas Y, Nobile-Orazio E, Spatz L, Abraham J, et al. Neuropathy and anti-myelin-associated glycoprotein IgM M proteins: T cell regulation of M protein secretion in vitro. Ann Neurol. 1985;18(2):182-8.
32. Hall AM, Vickers MA, McLeod E, Barker RN. Rh autoantigen presentation to helper T cells in chronic lymphocytic leukemia by malignant B cells. Blood. 2005;105(5):2007-15.
33. Horna P, Cuenca A, Cheng F, Brayer J, Wang HW, Borrello I, et al. In vivo disruption of tolerogenic cross-presentation mechanisms uncovers an effective T-cell activation by B-cell lymphomas leading to antitumor immunity. Blood. 2006;107(7): 2871-8.

34. Norton WT, Poduslo SE. Myelination in rat brain: method of myelin isolation. J Neurochem. 1973;21(4):749-57.
35. Kadlubowski M, Hughes RA, Gregson NA. Experimental allergic neuritis in the Lewis rat: characterization of the activity of peripheral myelin and its major basic protein, P2. Brain Res. 1980;184(2):439-54.
36. Kvarnström M, Sidorova E, Nilsson J, Ekerfelt C, Vrethem M, Söderberg O, et al. Myelin protein P0-specific IgM producing monoclonal B cell lines were established from polyneuropathy patients with monoclonal gammopathy of undetermined significance (MGUS). Clin Exp Immunol. 2002;127(2):255-62.
37. Nichols TC, Guthridge JM, Karp DR, Molina H, Fletcher DR, Holers VM. Gamma-glutamyl transpeptidase, an ectoenzyme regulator of intracellular redox potential, is a component of TM4 signal transduction complexes. Eur J Immunol. 1998;28(2):4123-9.
38. Sander B, Andersson J, Andersson U. Assessment of cytokines by immunofluorescence and the paraformaldehyde-saponin procedure. Immunol Rev. 1991;119:65-93.
39. Jansson A, Ernerudh J, Kvarnström M, Ekerfelt C, Vrethem M. Elispot assay detection of cytokine secretion in multiple sclerosis patients treated with interferon-beta1a or glatiramer acetate compared with untreated patients. Mult Scler. 2003;9(5):440-5.
40. Dayton MA, Knobloch TJ, Benjamin D. Human B cell lines express the interferon gamma gene. Cytokine. 1992;4(6):454-60.
41. Bhaduri-McIntosh S, Rotenberg MJ, Gardner B, Robert M, Miller G. Repertoire and frequency of immune cells reactive to Epstein-Barr virus-derived autologous lymphoblastoid cell lines. Blood. 2008;111(3): 1334-43.
42. Singh H, Raghava GP. ProPred: prediction of HLA-DR binding sites. Bioinformatics. 2001;17(12):1236-7.
43. Donnes P, Elofsson A. Prediction of MHC class I binding peptides, using SVMHC. BMC Bioinformatics. 2002;3:25.
44. Wang P, Sidney J, Dow C, Mothe B, Sette A, Peters B. A systematic assessment of MHC class II peptide binding predictions and evaluation of a consensus approach. PLoS Comput Biol. 2008;4(4):e1000048.
45. Bui HH, Sidney J, Peters B, Sathiamurthy M, Sinichi A, Purton KA, et al. Automated generation and evaluation of specific MHC binding predictive tools: ARB matrix applications. Immunogenetics. 2005;57(5):304-14.
46. Nielsen M, Lundegaard C, Lund O. Prediction of MHC class II binding affinity using SMM-align, a novel stabilization matrix alignment method. BMC Bioinformatics. 2007;8:238.
47. Sturniolo T, Bono E, Ding J, Raddrizzani L, Tuereci O, Sahin U, et al. Generation of tissue-specific and promiscuous HLA ligand databases using DNA microarrays and virtual HLA class II matrices. Nat Biotechnol. 1999;17(6):555-61.
48. Kursula P. Structural properties of proteins specific to the myelin sheath. Amino Acids. 2008;34(2):175-85.
49. Louvet C, Kabre BG, Davini DW, Martinier N, Su MA, DeVoss JJ, et al. A novel myelin P0-specific T cell receptor transgenic mouse develops a fulminant autoimmune peripheral neuropathy. J Exp Med. 2009;206(3): 507-14.
50. Mikaelsson E, Danesh-Manesh AH, Luppert A, et al. Fibromodulin, an extracellular matrix protein: characterization of its unique gene and protein expression in B-cell chronic lymphocytic leukemia and mantle cell lymphoma. Blood. 2005; 105(12):4828-35.
51. Mayr C, Bund D, Schlee M, Moosmann A, Kofler DM, Hallek M, Wendtner CM. Fibromodulin as a novel tumor-associated antigen (TAA) in chronic lymphocytic leukemia (CLL), which allows expansion of specific CD8+ autologous T lymphocytes. Blood. 2005;105(12):1566-73.
52. Lyons JA, San M, Happ MP, Cross AH. B cells are critical to induction of experimental allergic encephalomyelitis by protein but not by a short encephalitogenic peptide. Eur J Immunol. 1999;29(11):3432-9.
53. Rabinowitz R, Hadar R, Schlesinger M. The appearance of the CD4+CD8+ phenotype on activated T cells: possible role of antigen transfer. Hum Immunol. 1997;55(1):1-10.
54. Lusso P, De Maria A, Malnati M, Lori F, DeRocco SE, Baseler M, Gallo RC. Induction of CD4 and susceptibility to HIV-1 infection in human CD8+ T lymphocytes by human herpesvirus 6. Nature. 1991; 349(6309):533-5.
55. Ortolani C, Forti E, Radin E, Cibin R, Cossarizza A. Cytofluorimetric identification of two populations of double positive (CD4+,CD8+) T lymphocytes in human peripheral blood. Biochem Biophys Res Commun. 1993;191(2):601-9.
56. Nascimbeni M, Shin EC, Chiriboga L, Kleiner DE, Rehermann B. Peripheral CD4(+)CD8(+) T cells are differentiated effector memory cells with antiviral functions. Blood. 2004;104(2):478-86.
57. Schlesinger I, Rabinowitz R, Brenner T, Abramsky O, Schlesinger M. Changes in lymphocyte subsets in myasthenia gravis: correlation with level of antibodies to acetylcholine receptor and age of patient. Neurology. 1992;42(11):2153-7.
58. Munschauer FE, Stewart C, Jacobs L, Kaba S, Ghorishi Z, Greenberg SJ, Cookfair D, et al. Circulating CD3+ CD4+ CD8+ T lymphocytes in multiple sclerosis. J Clin Immunol. 1993;13(2):113-8.
59. Brouet JC, Mariette X, Gendron MC, Dubreuil ML. Monoclonal IgM from patients with peripheral demyelinating neuropathies cross-react with bacterial polypeptides. Clin Exp Immunol. 1994; 96(3):466-9.
60. Vera J, Fenutria R, Canadas O, et al. The CD5 ectodomain interacts with conserved fungal cell wall components and protects from zymosan-induced septic shock-like syndrome. Proc Natl Acad Sci USA. 2009;106(5):1506-11.
61. Shapiro L, Doyle JP, Hensley P, Colman DR, Hendrickson WA. Crystal structure of the extracellular domain from P0, the major structural protein of peripheral nerve myelin. Neuron. 1996;17(3):435-49.