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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Supplementary Design and Methods

Antibody reagents

The following antibodies were used: mouse anti-human HLA-DP, -DQ, -DR monoclonal antibodies (Dako A/S, Glostrup, Denmark), mouse anti-human IgM monoclonal antibody (Dako A/S), mouse anti-human LAMP-2 (Southern Biotech, Birmingham, AL, USA), AlexaFluor594-conjugated goat anti-mouse IgG antibody (Molecular Probes Inc., Eugene, OR, USA), AlexaFluor488-conjugated streptavidin (Molecular Probes), phycoerythrin-conjugated mouse anti-human IFN-γ monoclonal antibody (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), mouse anti-human IFN-γ monoclonal antibody (Mabtech AB, Stockholm, Sweden), biotinylated mouse anti-human IFN-γ monoclonal antibody (Mabtech AB). The following antibodies were purchased from BD Biosciences, San Diego, CA: mouse anti-human CD46 monoclonal antibody, FITC-conjugated mouse anti-human CD3, APC-conjugated mouse anti-human CD3 monoclonal antibody, PerCP-conjugated mouse anti-human CD3 monoclonal antibody, PE-conjugated mouse anti-human CD4 monoclonal antibody, FITC-conjugated mouse anti-human CD4 monoclonal antibody, APC-Cy-7-conjugated mouse anti-human CD8 monoclonal antibody, PE-Cy-7-conjugated mouse anti-human CD19 monoclonal antibody, APC-conjugated mouse anti-human CD56, FITC-conjugated mouse anti-human CD56 monoclonal antibody, PE-conjugated mouse anti-human CD69 monoclonal antibody, and PerCP-conjugated mouse anti-human HLA-DR monoclonal antibody.

Antigens

Native myelin and P0 full length protein were purified from bovine peripheral nerves as described previously[1,2] with some modifications.[3] In short, the nerves were homogenized on ice and centrifuged on a 0.85 M sucrose solution for 60 min at 75,000 g in a Beckman L8-70M ultracentrifuge (Beckman Coulter, Fullerton, USA). The interphase was collected; the myelin was washed three times with cold water and subsequently freeze-dried. P0 was extracted as previously described.[3] The purified native myelin and P0 was biotinylated using EZ-Link NHS-Biotin (Pierce Biotechnology, Inc., Rockford, IL, USA).

Synthetic P0 peptides representing amino acids 194-208 (P0 194-208) were synthesized based on the human P0 sequence by Dr. Åke Engström (Uppsala, Sweden) as described previously.[4] This peptide was previously shown to be immunogenic in PN-MGUS patients including the patient in the current study.[4] Keyhole limpet hemocyanin (KLH) from Calbiochem, (Lab Kemi, Stockholm, Sweden) was used as a control antigen and phytohemagglutinin (PHA) from Sigma-Aldrich, (Stockholm, Sweden) was used as a positive control.

Major histocompatibility complex class I and class II typing

Genomic DNA from TJ2 cells was isolated using Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, WI, USA), and high resolution HLA typing of HLA-DRB1, HLA- DQA1 and HLA-DQB1 was performed using the following high-resolution sequence specific primer (SSP) kits: DQA1-SSP, DQB1*02-SSP, DQB*03-SSP, DRB1*04-SSP, DRB1*07-SSP (Olerup SSP AB, Saltsjöbaden, Sweden). The following SSP kits were used for the HLA-A and HLA-B typing: HLA-A low resolution, HLA-B low resolution, HLA-A*02, HLA-A*31, HLA-B*13, HLA-B*40. The PCR was run according to the manufacturer's instructions on a GeneAmp PCR system 9700 (Applied Biosystems Inc, Foster City, CA, USA). The PCR product was separated on a 2% agarose gel containing ethidium bromide and visualized under ultraviolet light.

Immunoglobulin heavy chain gene sequencing

The IGHV gene sequence of the TJ2 MGUS B-cell line was previously published by us,[3] but the sequence was incomplete in the CDR3 region, lacking a W-anchor. Repeated sequencing experiments performed in this study have clarified that a mutated codon 118 gave rise to a W to V replacement. RNA was extracted from TJ2 cells using a RNeasy Mini Kit (Qiagen) and cDNA was synthesized using the First Strand cDNA Synthesis Kit for RT-PCR (AMW) (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's description. PCR amplification of IGHV gene rearrangement was performed using consensus V_H/J_H primers as described previously[5,6] and sequenced on a MegaBACE 500 instrument (GE Healthcare, Uppsala, Sweden). A DYEnamic™ET dye terminator kit (GEHealthcare) was used for the gene sequencing reactions. The sequences were aligned against Ig sequences in the IMGT database (http://www.imgt.org),[7] and a 2% deviation from the germline sequence was used as the cut-off for mutated IGHV genes.

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 previously been described.[8] TJ2 cells were washed in serum-free medium, resuspended to 10×10^6 cells/mL and incubated for 30 min at 37°C in the presence of 40 μg/mL biotinylated myelin or biotinylated P0. The cells were washed twice in serum-free medium and further incubated with non-biotinylated myelin or P0. Incubation times investigated were 2.5 h, 5 h, 8 h, 16 h and 24 h. The cells were washed in PBS with 2% BSA and receptor clustering was induced by incubating the cells with a mouse anti-human IgM monoclonal antibody (3.65 μg/mL) for 30 min at 37°C followed by a 1 h incubation with the secondary Alexa594-conjugated goat anti-mouse IgG antibody (4 μg/mL). Cells were washed twice in cold PBS with 0.02% NaN_3 and stained with Alexa488-conjugated streptavidin (2 μg/mL) for 20 min on ice. Cells were then washed twice in PBS, fixed with 4% paraformaldehyde (PFA) and examined on a Carl Zeiss Axiovert 200M fluorescence microscope. Two hun-

dred cells were examined for antigen-receptor co-capping. In parallel, a similar procedure was used to detect co-capping of myelin or P0 with MHCII. Mouse anti-human HLA-DP, -DQ, -DR monoclonal antibody (5 μg/mL) was used for the MHCII clustering.

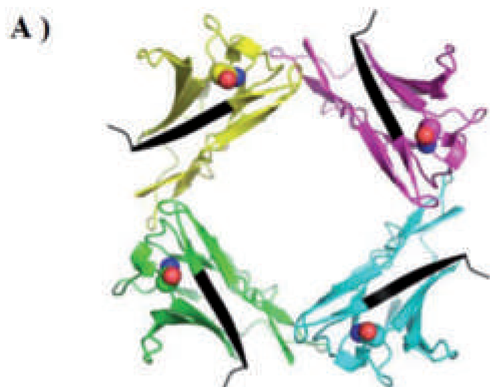
Interferon-γ enzyme-linked immunsorbent spot assay

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.[9] In brief, plates were coated with 100 μL mouse anti-human IFNγ monoclonal antibody diluted to 15 mg/mL in PBS (pH 7.4). The freeze-dried myelin peptide fractions were dissolved in 200 μL PBS, and 10 μL were added to each well. One hundred thousand lymphocytes/well were incubated with or without peptide fractions for 48 h. Biotinylated mouse anti-human IFNγ detection antibody was diluted in PBS, 0.05% Tween-20. Triplicate samples were analyzed and the spots were counted under a dissection microscope. The mean values of the triplicates were used and the mean value of the non-stimulated cells, the spontaneous secretion, was subtracted from that of the stimulated cells.

Bioinformatic prediction of major histocompatibility complex class II-binding protein zero peptides

Four MHCII binding prediction tools were used to generate P0 peptide candidates binding to HLA-DR unique to the PN-MGUS patient used in this study (HLA-DRB1*0403, *0701). None of the bioinformatics tools could predict peptides for HLA-DQ or for the DRB1*0403 allele; therefore, only the DRB1*0701 allele was included in the analysis. Human myelin P0 protein amino acid sequence without the signal

A)



B) HLA-DRB1*0701

Myelin P0 protein - Homo Sapiens:
IVVYTDREVH GAVGSRVTLH CSFWSSEWVS DDISFTWRYQ PEGGRDAISI
FHYAKGQPYI DEVGTFKERI QWVGDPRWKD GSIVIHNLDY SDNGTFTCDV
KNPPDIVGKT SQVTLYVFEK VPTRYGVVLG AVIGGVLGVV LLLLLLFYVV
RYCWLRRQAA LQRRLSAMEK GKLHKPGKDA SKRGRQTPVL YAMLDHSRST
KAVSEKKAKG LGESRKDKK

C) HLA-A*0201

Myelin P0 protein - Homo Sapiens:
IVVYTDREVH GAVGSRVTLH CSFWSSEWVS DDISFTWRYQ PEGGRDAISI
FHYAKGQPYI DEVGTFKERI QWVGDPRWKD GSIVIHNLDY SDNGTFTCDV
KNPPDIVGKT SQVTLYVFEK VPTRYGVVLG AVIGGVLGVV LLLLLLFYVV
RYCWLRRQAA LQRRLSAMEK GKLHKPGKDA SKRGRQTPVL YAMLDHSRST
KAVSEKKAKG LGESRKDKK

D) HLA-A*3101

Myelin P0 protein - Homo Sapiens:
IVVYTDREVH GAVGSRVTLH CSFWSSEWVS DDISFTWRYQ PEGGRDAISI
FHYAKGQPYI DEVGTFKERI QWVGDPRWKD GSIVIHNLDY SDNGTFTCDV
KNPPDIVGKT SQVTLYVFEK VPTRYGVVLG AVIGGVLGVV LLLLLLFYVV
RYCWLRRQAA LQRRLSAMEK GKLHKPGKDA SKRGRQTPVL YAMLDHSRST
KAVSEKKAKG LGESRKDKK

Online Supplementary Figure S1. The PN-MGUS patient's specific predicted MHCI and MHCII binding P0 peptides. (A) Crystal structure of P0 extracellular domain from rat. Glycosylation site, Asn93, is shown as spheres and the HLA-DRB1*0701 binding peptide amino acids 107-115 in black. (B) The top ranking HLA-DRB1*0701 binding P0 peptides as predicted by PROPRED, SVMHC, IEDB-AR and HLA-pred are marked in the human P0 amino acid sequence. The nine amino acid core sequence of the peptides is shown in blue with the starting residue in red. The extended peptides, predicted by IEDB-AR, are shown in magenta. Asn93 is also shown (green). (C, D) HLA-B*40, HLA-A*0201 and HLA-A*3101 associated peptides as predicted by PROPRED, and IEDB-AR. The predicted P0 peptides are marked in blue in the human P0 amino acid sequence. The starting residue is shown in red.

peptide, accession number P25189, from Swiss-Prot/TrEMBL (*http://www.expasy.org/*) was used. The prediction tools used were PROPRED (*http://www.imtech.res.in/raghava/propred/*),[10] SVMHC (*http://www-bs.informatik.uni-tuebingen.de/SVMHC/*),[11] IEDB-AR (*http://tools.immuneepitope.org/main/index.html*)[12-15] and HLA-pred (*http://www.imtech.res.in/raghava/hlapred/index.html*). PROPRED, SVMHC, and HLA-pred are based on the TEPITOPE method and predict the nine core amino acids binding to the MHCII molecule. HLA-pred also enables a search in the MHCBN database for experimentally proven MHCII binding peptides in the query antigen sequence. IEDB-AR uses four different analysis methods (Consensus, SMM-align, Sturniolo and ARB). The consensus method was chosen in this analysis since it combines SMM-align, Sturniolo and ARB. IEDB-AR also extends the nine core amino acids to a 15 amino acid long peptide.

Bioinformatic prediction of major histocompatibility complex class I-binding protein zero peptides

Bioinformatic tools Propred1(*http://www.imtech.res.in/raghava/propred1/*)[16] and IEDB-AR (*http://tools.immuneepitope.org/analyze/html/mhc_binding.html*)[17] were used to generate P0 peptide candidates both cleaved by immunoproteasomes and binding to MHCI alleles (HLA-A*0201, HLA-A*3101 and HLA-B*40) specific for the PN-MGUS patient in this study. The P0 amino acid sequence used was the same as for the MHCII binding peptide prediction.

Results

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

MHCII peptide binding prediction tools PROPRED, SVMHC, IEDB-AR and HLA-pred were used to generate possible antigenic HLA-DRB1*0701 binding peptides in the human myelin protein P0 amino acid sequence. The seven highest ranking MHCII (HLA-DRB1*0701) binding P0 peptide candidates from PROPRED, SVMHC and HLA-pred, and the ten highest ranking peptides for IEDB-AR are presented (*Online Supplementary Figure S1B*). 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 (*Online Supplementary Figure S1A*). For MHCI, the prediction tools Propred1 and IEDB-AR were used to generate P0 peptide candidates (8, 9 and 10 amino acids) both cleaved by immunoproteasomes and binding to MHCI alleles (HLA-A*0201, HLA-A*3101 and HLA-B*40) specific for the PN-MGUS patient in this study. The highest scoring peptides for HLA-A*0201 and HLA-B*40 could be found in the transmembrane domain of P0 (*Online Supplementary Figure S1B, D*). For HLA-A*3101 peptide candidates could be seen in either the transmembrane domain or intracellular domain of P0 (*Online Supplementary Figure S1C*).

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