

Myeloperoxidase/HLA Class II Complexes Recognized by Autoantibodies in Microscopic Polyangiitis

Ryosuke Hiwa,¹ Koichiro Ohmura,² Noriko Arase,³ Hui Jin,⁴ Kouyuki Hirayasu,⁵
Masako Kohyama,⁴ Tadahiro Suenaga,⁴ Fumiji Saito,³ Chikashi Terao,² Tatsuya Atsumi,⁶
Hirotsugu Iwatani,³ Tsuneyo Mimori,² and Hisashi Arase⁴

Objective. Autoantibodies against myeloperoxidase (MPO) that are expressed in neutrophils play an important role in the pathogenesis of microscopic polyangiitis (MPA). We recently observed that misfolded cellular proteins are transported to the cell surface by HLA class II molecules and are targeted by autoantibodies in patients with rheumatoid arthritis or antiphospholipid syndrome, suggesting that HLA class II molecules play an important role in autoantibody recognition. The aim of this study was to address the role of HLA class II molecules in the cell surface expression of MPO in patients with MPA.

Methods. The association of MPO with HLA-DR was analyzed using MPO and HLA-DR transfectants as well as neutrophils from healthy donors and patients with MPA. Autoantibody binding to the MPO/HLA-DR complex was analyzed by flow cytometry. The association of

MPO with HLA-DR was assessed using the immunoprecipitation technique. The function of MPO-antineutrophil cytoplasmic antibody (ANCA) was assessed using a neutrophil-like cell line expressing HLA-DR and MPO.

Results. MPO protein was detected on the cell surface in the presence of HLA-DR, and the MPO/HLA-DR complex was recognized by MPO-ANCA. A competitive inhibition assay suggested that MPO associated with HLA-DR expresses cryptic autoantibody epitopes for MPO-ANCA. Autoantibody binding to the MPO/HLA-DR complex was correlated with disease susceptibility conferred by each HLA-DR allele, suggesting that the MPO/HLA-DR complex is involved in the pathogenicity of MPA. Indeed, MPO-HLA class II complexes were detected in neutrophils from a patient with MPA as well as in cytokine-stimulated neutrophils from healthy donors. Moreover, MPO-ANCA stimulated MPO/HLA-DR complex-expressing HL-60 cells.

Conclusion. Our findings suggest that MPO complexed with HLA class II molecules is involved in the pathogenesis of MPA as a target for MPO-ANCA.

Microscopic polyangiitis (MPA) is a type of systemic small vessel vasculitis that affects multiple organs (1). It is categorized as an antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (1). Approximately 70% of patients with MPA possess myeloperoxidase (MPO)-ANCAs (2) that recognize MPO expressed in neutrophils (3). Although MPO is not expressed on the cell surface of neutrophils in a steady state, it is transported to the cell surface via cytokine stimulation, which enables MPO-ANCAs to bind to the cell surface of neutrophils (4,5). ANCAs bind to and stimulate neutrophils (4,6–8) and cause vasculitis in small vessels (9,10). A mouse model of MPA has demonstrated that the transfer of anti-MPO antibodies causes vasculitis (11), suggesting that MPO-ANCAs are directly involved in the pathogenesis of MPA

Supported in part by the Japan Society for the Promotion of Science (KAKENHI grant 15H02545), the Japan Agency for Medical Research and Development (Practical Research Project for Allergic Diseases and Immunology), the Mochida Memorial Foundation for Medical and Pharmaceutical Research, the Uehara Memorial Foundation, Bristol-Myers K.K. (BRAVE RA Clinical Investigation grant), the Terumo Life Science Foundation, and the Tokyo Biochemical Research Foundation.

¹Ryosuke Hiwa, MD: World Premier International Immunology Frontier Research Center and Osaka University, Suita, Japan, and Kyoto University, Kyoto, Japan; ²Koichiro Ohmura, MD, PhD, Chikashi Terao, MD, PhD, Tsuneyo Mimori, MD, PhD: Kyoto University, Kyoto, Japan; ³Noriko Arase, MD, PhD, Fumiji Saito, PhD, Hirotsugu Iwatani, MD, PhD (current address: Osaka National Hospital, Osaka, Japan): Osaka University, Suita, Japan; ⁴Hui Jin, PhD, Masako Kohyama, PhD, Tadahiro Suenaga, MD, PhD, Hisashi Arase, MD, PhD: World Premier International Immunology Frontier Research Center and Osaka University, Suita, Japan; ⁵Kouyuki Hirayasu, PhD: World Premier International Immunology Frontier Research Center, Suita, Japan; ⁶Tatsuya Atsumi, MD, PhD: Hokkaido University Graduate School of Medicine, Sapporo, Japan.

Address correspondence to Hisashi Arase, MD, PhD, Department of Immunochemistry, Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan. E-mail: arase@biken.osaka-u.ac.jp.

Submitted for publication February 20, 2017; accepted in revised form June 1, 2017.

(12,13). However, it has not been fully elucidated how MPO is transported to the cell surface of neutrophils after cytokine stimulation.

We previously observed that misfolded endoplasmic reticulum (ER) proteins are transported to the cell surface, without processing to peptides, by HLA class II molecules. This occurs when misfolded proteins are associated with the peptide-binding groove of HLA class II molecules instead of the invariant chain (14). Furthermore, misfolded proteins that were transported to the outside of the cell by HLA class II molecules were efficiently recognized by autoantibodies from patients with rheumatoid arthritis (RA) or the antiphospholipid syndrome (15–17). More importantly, autoantibody binding to the IgG heavy chain/HLA class II complex is strongly associated with susceptibility to RA conferred by each HLA-DR allele, suggesting that the IgG heavy chain/HLA class II complex is involved in the pathogenesis of RA (15). Although HLA class II expression is generally limited to certain cell populations, such as dendritic cells and B cells, HLA class II expression is induced on a variety of cells upon stimulation by cytokines, such as interferon- γ (IFN γ). Therefore, certain types of inflammation or infection would induce expression of HLA class II molecules. Because the antigenicity of misfolded proteins seems to be different from that of normally folded proteins, we hypothesized that the misfolded proteins that are transported to the cell surface by these HLA class II molecules might be involved in the pathogenesis of autoimmune diseases (18).

Resting neutrophils do not express HLA class II molecules. However, HLA class II expression is induced in neutrophils by cytokine stimulation (19,20). In addition, a recent genome-wide association study (GWAS) revealed that susceptibility to MPA is associated with polymorphisms in HLA class II genes (21). In Japanese individuals, MPA is significantly associated with HLA-DRB1*09:01 (22,23). However, the role of HLA class II molecules expressed on neutrophils in the pathogenesis of MPA has remained unknown. In this study, we addressed the role of HLA class II molecules in the cell surface expression of MPO in patients with MPA. Our findings provide new insight into the role of HLA class II molecules in the pathogenesis of MPO-ANCAs in patients with MPA.

PATIENTS AND METHODS

Patient samples. Sera, peripheral blood neutrophils, and DNA from patients with MPA were collected from Kyoto University, Hokkaido University, and Osaka University. Diagnoses of MPA were based on the European Medicines Agency algorithm-based criteria (24) and the Chapel Hill Consensus Conference definition (1). Human peripheral blood neutrophils were obtained from healthy volunteers or patients with MPA,

using the Ficoll-dextran method (25). IgG was purified from the plasma of MPA patients or healthy donors by affinity chromatography using protein G–Sepharose, as described previously (26).

Study approval. The protocol for the collection and use of human sera, peripheral blood cells, and DNA was approved by the institutional review boards (IRBs) of Kyoto University (E458, G1006–1), Hokkaido University (010–0326), and Osaka University (25–2, 12246). Written informed consent in accordance with the Declaration of Helsinki was obtained from all participants according to the relevant guidelines of the IRBs.

Plasmids. Complementary DNAs (cDNAs) for different HLA class II alleles and the invariant chain (accession no. NM_004355.2) were prepared as previously described (14–16). Human MPO (accession no. NC_000017.11) was cloned from cDNA prepared from human bone marrow total RNA (Clontech). Some HLA-DR alleles were generated using QuikChange multimutagenesis kits (Agilent) from HLA genes with similar sequences. All cDNA sequences for HLA were based on information in the IMGT/HLA Database (www.ebi.ac.uk/imgt/hla/index.html). The nucleotide sequences of all of the constructs were confirmed by DNA sequencing (ABI 3130xl DNA Sequencer; Applied Biosciences). HLA-DRB1*01:01 containing a covalently attached transferrin receptor peptide (TfRpep-HLA-DRB1*01:01) and HLA-DRB1*04:04 containing a covalently attached HLA-Cw3 peptide (HLA-Cw3-pep-HLA-DRB1*04:04) were generated as previously described (27).

Antibodies. Anti-HLA-DR monoclonal antibody (L243; American Type Culture Collection) was used for the detection of HLA-DR by flow cytometry and immunoprecipitation (IP) of HLA-DR proteins. Anti-HLA-DR/DP monoclonal antibody (HL-38; Sigma-Aldrich) was used for IP detected by flow cytometry. Anti-human MPO monoclonal antibody (16E3; GeneWay Research) was used for flow cytometry. Anti-DYKDDDDK-tagged (clone L5) monoclonal antibody (BioLegend) was used for IP. Rabbit anti-HLA-DR α antibody (FL-254; Santa Cruz Biotechnology), anti-FLAG monoclonal antibody (clone M2; Sigma-Aldrich), and anti-human MPO polyclonal antibody (HPA021147; Sigma-Aldrich) were used for Western blotting.

Cell lines. HEK 293T cells (RCB2202) were purchased from the RIKEN BioResource Center (Tsukuba, Japan). HL-60 cells (JCRB0085) were purchased from the Japanese Collection of Research Bioresources Cell Bank (JCRB) (Osaka, Japan). We used polymerase chain reaction to confirm that the cells were negative for mycoplasma contamination. Expression plasmids containing each cDNA were transfected into cells, using Polyethylenimine Max (Polysciences).

Flow cytometric analysis. Cells were incubated with mouse primary monoclonal antibodies (anti-HLA-DR α antibody [L243] or anti-human MPO antibody [16E3]), followed by allophycocyanin (APC)-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch). For intracellular staining, cells were fixed and permeabilized with Fixation/Permeabilization solution (BD Biosciences). Intracellular MPO was detected with anti-human MPO followed by APC-conjugated anti-mouse IgG antibody. Stained cells were analyzed using a FACSCalibur (Becton Dickinson) or FACSVerse cytometer (Becton Dickinson).

Analysis of autoantibody binding to the MPO/HLA-DR complex. Human MPO was cotransfected together with HLA-DR α and green fluorescent protein (GFP). The transfectants were mixed with sera (dilution 1:300) from patients with MPA, followed by APC-labeled anti-human IgG Fc antibody

(Jackson ImmunoResearch). To determine specific autoantibody titers against MPO/HLA-DR complex, the mean fluorescence intensity (MFI) of autoantibody binding to HLA-DR9 alone transfectants was subtracted from the MFI of autoantibody binding to HLA-DR9 and human MPO transfectants. Anti-MPO/HLA-DR complex antibody titers were calculated based on the MFI of IgG autoantibody binding to MPO/HLA-DR9 cotransfectants, using a standard MPA serum for which the MPO-ANCA titer (720 units/ml) is known, as determined by enzyme-linked immunosorbent assay (ELISA).

Immunoprecipitation and immunoblotting. Cells were lysed in lysis buffer (10 mM Tris, 150 mM NaCl, pH 7.5) containing 0.5% Nonidet P40 (Sigma). HLA-DR and human MPO were precipitated with anti-HLA-DR monoclonal antibody (L243) and protein G-Sepharose (GE Healthcare) or anti-DYKDDDDK monoclonal antibody (L5) and protein G-Sepharose. Mouse IgG2a (BD Biosciences) was used as an isotype-matched control. The immunoprecipitates were eluted by boiling with sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer, separated on 10% (weight/volume) polyacrylamide gels (Atto), and transferred onto PVDF membranes (Millipore). The membranes were incubated with anti-HLA-DR α antibody (FL-254) or anti-human MPO antibody (HPA021147), followed by horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Thermo Fisher Scientific). The membranes were also incubated with anti-FLAG antibody (M2), followed by HRP-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch). Peroxidase activity was detected with SuperSignal reagent (Thermo Fisher Scientific).

HLA-DRB1 genotyping and ELISA. HLA-DRB1 alleles were determined as previously described (28). Briefly, a WAKFlow system (Wakunaga Pharmaceutical) was used for the HLA-DRB1 typing.

MPO protein purified from human neutrophils (Sigma-Aldrich) was coated onto 96-microwell plates (3690; Costar), and IgG bound to the plates was detected by HRP-conjugated donkey anti-human IgG antibody (Jackson ImmunoResearch). Peroxidase activity was detected using an OptEIA kit (BD Biosciences). Anti-MPO antibody titers were calculated using standard serum from a patient with MPA, for which the MPO-ANCA titer is known (720 units/ml). Interleukin-8 (IL-8) expression in the culture supernatant was determined by ELISA (eBioscience).

Competitive inhibition assay. Sera from MPA patients were mixed with various concentrations of MPO protein purified from human neutrophils (Sigma) and incubated overnight at 4°C. Anti-MPO/HLA-DR complex antibodies and anti-MPO antibodies were detected using flow cytometry and ELISA, respectively. Anti-MPO/HLA-DR complex antibody titers and anti-MPO antibody titers were calculated using a standard MPA serum, as described above. The percent inhibition was defined as $100 - (\text{titer of inhibited serum} / \text{titer of noninhibited serum}) \times 100$.

Immunoprecipitation detected by flow cytometry (IP-FCM). For the IP-FCM assay, we used a modified version of the protocol described by Schrum et al (29). Anti-HLA-DR α monoclonal antibody HL-38 was coupled to Aldehyde/Sulfate Latex Beads (Life Technologies). The beads were washed twice with MES buffer. Anti-HLA-DR α monoclonal antibody (HL-38) was mixed with the beads and shaken at room temperature overnight. The antibody-coupled beads were then washed 3 times and suspended in phosphate buffered saline (PBS) containing 0.1% glycine and 0.1% NaN₃. Next, the antibody-coupled beads were mixed with cell lysate at 4°C for 1 hour. The beads were

subsequently stained with biotinylated primary antibody, followed by APC-streptavidin (Jackson ImmunoResearch). Finally, the stained beads were analyzed using a FACSVerse system (Becton Dickinson).

Stimulation of neutrophils. Isolated neutrophils were resuspended with RPMI 1640 medium containing 10% autologous serum. Neutrophils (1×10^7) were cultured for 48 hours in the presence of IFN γ (final concentration 100 units/ml) plus autologous serum in a 6-well plate. Thereafter, neutrophils were stained with anti-HLA-DR or anti-MPO antibody and analyzed by flow cytometry.

Stimulation of HLA-DR-transfected HL-60 cells. HLA-DR9 was stably transfected into HL-60 cells using a Platinum-E (Plat-E) system (30). HLA-DR9 and an amphotropic envelope were transfected into Plat-E cells. Culture supernatant containing a retroviral vector was collected 2 days after replacement and added to medium for the HL-60 cells. HLA-DR-positive cells were then sorted with a Sony SH800 cell sorter. GFP was stably transfected into HL-60 cells in the same manner, and these were used as mock cells. HL-60 cells were cultured in medium containing 10 μ M all-trans-retinoic acid for 5 days in order to differentiate into neutrophil-like cells (31,32). Next, 20 μ g/ml IgG purified from plasma was coated onto a 96-well plate. After incubation at 4°C overnight, the plate was washed twice with PBS. A total of 100 μ l of 1×10^6 cells/ml of differentiated HL-60 cells was cultured with the precoated plate for 3 hours. The IL-8 concentration in culture supernatant was determined by ELISA.

Statistical analysis. Pearson's product-moment correlation coefficient was used to assess the significance of correlations, and the correlation coefficient and *P* value of the linear regression line were calculated. Mann-Whitney U test, Student's *t*-test, or one-way analysis of variance with Tukey's post hoc test was used to determine the significance of differences. The odds ratios (ORs) for the association between different HLA-DRB1 alleles and MPA were log-transformed to normalize the distribution. HLA-DRB1 alleles with a frequency of >2% in MPA patients were analyzed for their association with autoantibody binding to the MPO/HLA-DR complex. *P* values less than 0.05 were considered significant.

RESULTS

MPO expression on the cell surface in association with HLA class II molecules. In order to address the role of HLA class II molecules in the cell surface expression of MPO, we transfected human MPO with MPA-susceptible HLA-DR9 (HLA-DRA*01:01/DRB1*09:01) (22) into HEK 293T cells and used flow cytometry to analyze the cell surface expression of MPO. Cell surface expression of MPO was low when MPO alone was transfected. However, when MPO and HLA-DR9 were cotransfected, the cell surface expression of MPO was markedly increased; the intracellular level of MPO was not affected by HLA-DR9 expression (Figure 1A). We then investigated whether MPO-ANCAs could bind to cell surface-expressed MPO induced by HLA-DR. IgG autoantibodies from an MPA patient bound to MPO induced on the cell surface by HLA-

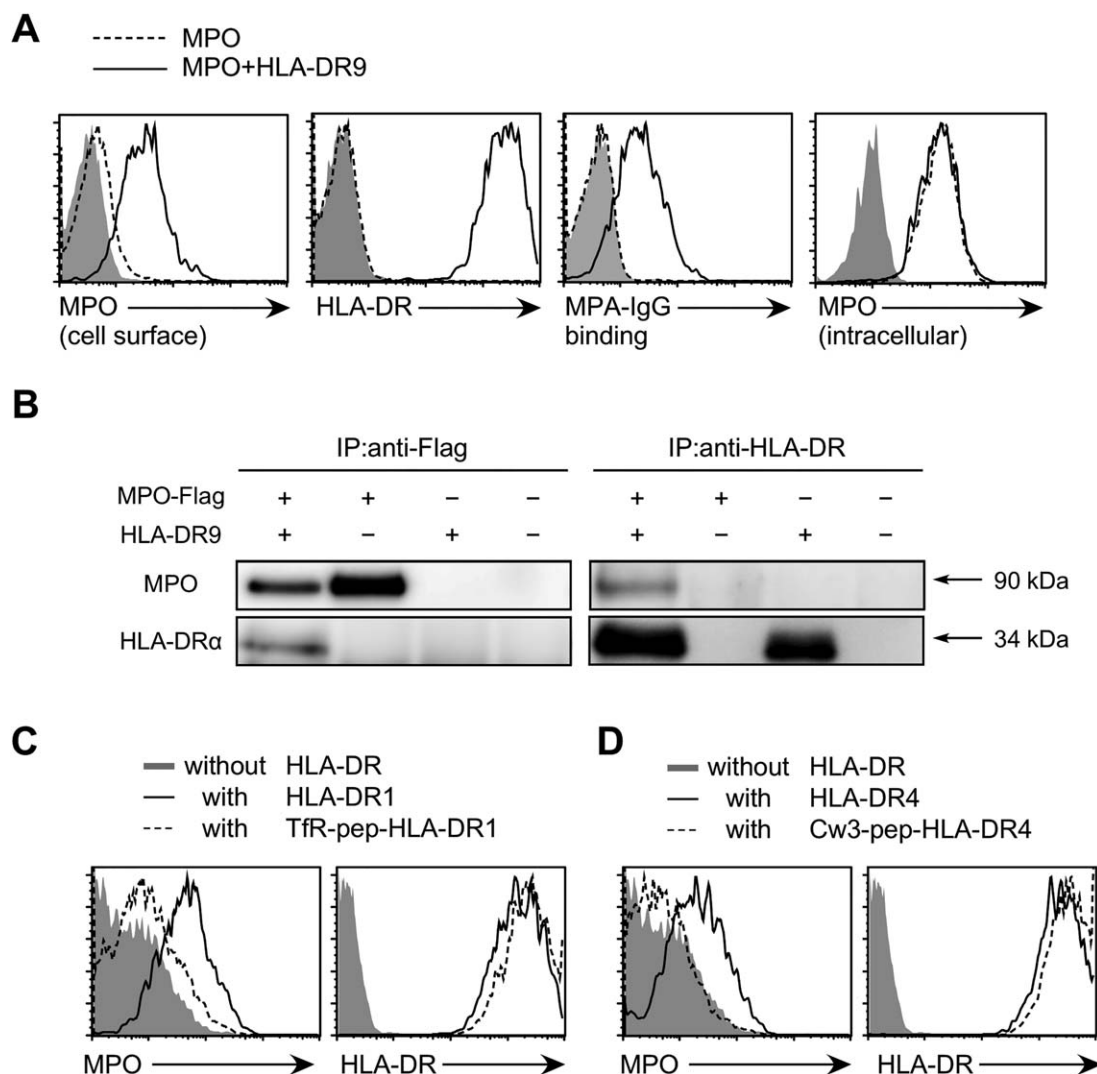


Figure 1. Myeloperoxidase (MPO) is expressed on the cell surface in association with HLA-DR. **A**, MPO and green fluorescent protein (GFP) were transfected into HEK 293T cells with (solid line) or without (broken line) HLA-DRB1*01:01 and HLA-DRB1*09:01 (HLA-DR9). The transfectants were stained with anti-MPO antibodies, anti-HLA-DR antibodies, or serum from a patient with microscopic polyangiitis (MPA-IgG). Intracellular MPO was also stained. Antibody binding to GFP-expressing cells is shown. Cells transfected with GFP alone were stained as a control (shaded histograms). **B**, MPO protein was coimmunoprecipitated with HLA-DR9. HLA-DR9 and/or MPO was transfected into HEK 293T cells. Cell lysates were immunoprecipitated (IP) with anti-FLAG or anti-HLA-DR antibody. Immunoprecipitates were blotted with anti-FLAG, anti-MPO, or anti-HLA-DRα antibodies. **C**, HLA-DR1 (solid lines), HLA-DRB1*01:01 containing a covalently attached transferrin receptor peptide (Tfr-pep-HLA-DR1) (broken lines), or control plasmids (shaded histograms) were cotransfected with MPO and GFP. Cell surface expression of MPO and HLA-DR on GFP-positive cells is shown. **D**, HLA-DR4 (solid lines), HLA-DRB1*04:04 containing a covalently attached HLA-Cw3 peptide (HLA-Cw3-pep-HLA-DR4) (broken lines), or control plasmids (shaded histograms) were cotransfected with MPO and GFP. Cell surface expression of MPO and HLA-DR on GFP-positive cells is shown. Results are representative of at least 3 independent experiments.

DR. In contrast, MPO-ANCAs did not bind to the cells that were transfected with MPO alone.

Next, we used an IP assay to investigate whether full-length MPO is associated with HLA-DR. Full-length 90-kD MPO protein was coimmunoprecipitated with HLA-DR9, whereas MPO was not detected in the

absence of HLA-DR9, indicating that full-length but not fragmented MPO is associated with HLA-DR (Figure 1B). Our group previously showed that various misfolded proteins are associated with the peptide-binding groove of major histocompatibility complex (MHC) class II molecules (14–16). To test the possibility that MPO also is

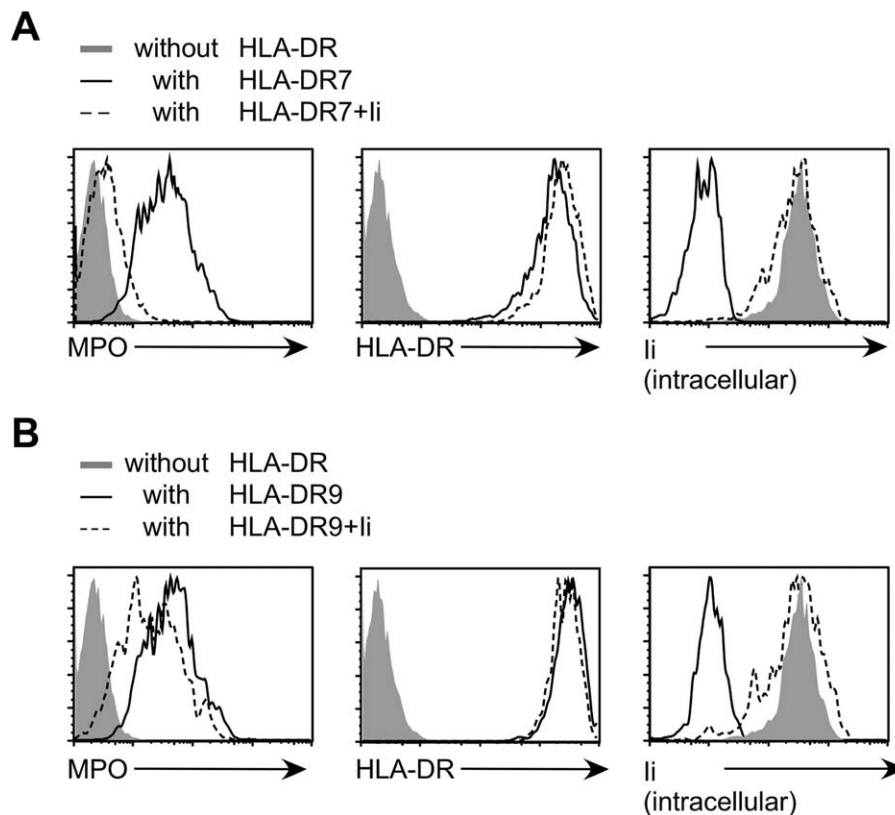


Figure 2. Cell surface expression of MPO induced by HLA-DR with an MPA-susceptible allele is less affected by the invariant chain (Ii) than by an MPA-nonsusceptible allele (HLA-DR7). MPO and GFP were cotransfected with HLA-DR into HEK 293T cells in the presence (broken lines) or absence (solid lines) of the invariant chain. Cell surface expression of HLA-DR and MPO on GFP-positive cells was analyzed. Cells transfected without HLA-DR were stained as a control (shaded histograms). Expression of the invariant chain was analyzed by intracellular staining. **A**, MPO was transfected with HLA-DR7. **B**, MPO was transfected with HLA-DR9. Results are representative of at least 3 independent experiments. See Figure 1 for other definitions.

associated with the peptide-binding groove of HLA-DR, we measured MPO cell surface expression using HLA-DR1 that was covalently attached to a transferrin receptor peptide, which reportedly binds to the peptide-binding groove of HLA-DR1 with relatively high affinity (14,27,33). We also used HLA-DR4 that was covalently attached to a HLA-Cw3 peptide, which also binds to the peptide-binding groove of HLA-DR4 (15,34). When covalently attached to a peptide, both HLA-DR1 and HLA-DR4 transported less MPO to the cell surface than did wild-type HLA-DR (Figures 1C and D). These findings indicate that full-length MPO is associated with the peptide-binding groove of HLA class II molecules.

The invariant chain binds to the peptide-binding groove of newly synthesized MHC class II molecules at the ER and transports them to the endolysosomal pathway. In addition, the invariant chain inhibits the binding of certain proteins to MHC class II molecules at the ER (35). We investigated the effect of the invariant chain on the

association of MPO with HLA-DR. In the absence of the invariant chain, HLA-DR7, an allele not associated with susceptibility to MPA, transported MPO to the cell surface in a manner similar to that of HLA-DR9 (Figures 2A and B). In the presence of the invariant chain, the cell surface expression of MPO induced by HLA-DR7 was clearly inhibited (Figure 2A). On the other hand, inhibition of MPO cell surface expression by the invariant chain was low when HLA-DR9 was transfected (Figure 2B). Therefore, the affinities of HLA class II molecules to the invariant chain and MPO differed depending on the alleles of HLA class II molecules that were expressed.

Binding of autoantibodies from MPA patients to MPO complexed with MHC. We determined the anti-MPO/HLA-DR complex antibody titers in sera obtained from patients with MPA at the time of disease onset as well as those in sera obtained from healthy controls. Anti-MPO/HLA-DR complex antibodies were detected in 87.5% of patients with MPA when the cutoff

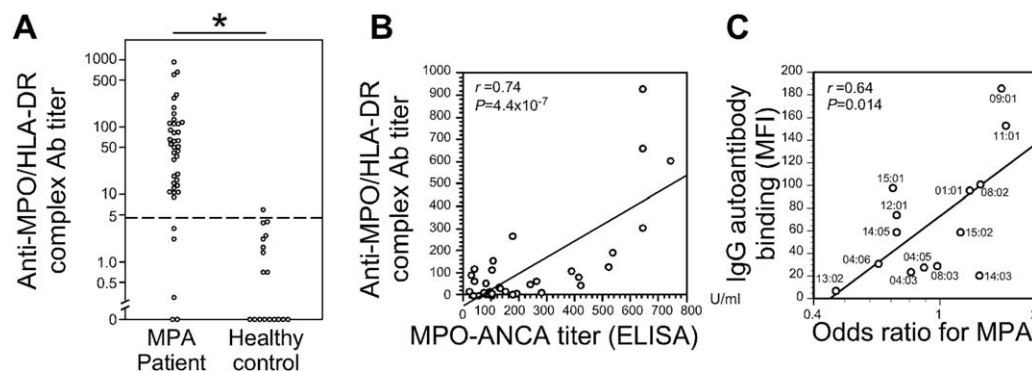


Figure 3. Autoantibodies from MPA patients bind to MPO complexed with HLA-DR. **A**, Anti-MPO/HLA-DR complex antibody (Ab) titers were determined in sera obtained from patients with MPA ($n = 40$) and from healthy controls. The cutoff value was defined as the mean + 2SD of the titers in healthy controls. Differences between anti-MPO/HLA-DR complex antibody titers in patients with MPA and healthy controls were analyzed by Mann-Whitney U test. Each data point represents an individual subject; the horizontal line shows the cutoff value. $* = P < 0.05$. **B**, MPO-antineutrophil cytoplasmic antibody (ANCA) titers in serum samples from 40 MPA patients were plotted in relation to IgG autoantibody titers in each serum to MPO complexed with HLA-DR9 (anti-MPO/HLA-DR antibody titer). **C**, MPO was cotransfected with HLA-DRA*01:01 in combination with each HLA-DRB1 allele indicated, and the mean fluorescence intensity (MFI) of binding of autoantibodies from an MPA patient to MPO complexed with HLA-DR was plotted against the odds ratio for MPA susceptibility for each HLA-DRB1 allele. The odds ratios for the association between different HLA-DRB1 alleles and MPA were log-transformed to normalize the distribution. In **B** and **C**, Pearson's product-moment correlation coefficient was used for the analysis. Results are representative of at least 3 independent experiments. ELISA = enzyme-linked immunosorbent assay (see Figure 1 for other definitions).

value was defined as the mean + 2SD of the titers in healthy controls. The titers of anti-MPO/HLA-DR complex antibody in patients with MPA were significantly higher than those in healthy controls (Figure 3A). This finding suggests that the MPO/HLA-DR complex is recognized by autoantibodies from patients with MPA. We next analyzed whether anti-MPO/HLA-DR complex antibody titers were correlated with MPO-ANCAs detected by conventional ELISA. The anti-MPO/HLA-DR complex antibody titer was significantly correlated with the MPO-ANCA titer determined by ELISA (Figure 3B). These results imply that MPO/HLA-DR complexes are targets for MPO-ANCAs in patients with MPA.

We investigated the effects of the HLA-DR of different alleles on the transport of MPO to the cell surface. MPO was transfected into HEK 293T cells with different HLA-DR alleles, and its cell surface expression was analyzed (Figure 3C). The results indicated that the cell surface expression of MPO differed depending on the HLA-DR alleles. As observed in other autoimmune diseases, susceptibility to MPA is associated with certain HLA-DRB1 alleles (22,23). The ORs for the association between MPA and each HLA-DR allele were plotted against autoantibody binding to the MPO/HLA-DR complex for each allele (Figure 3C). There was a significant positive correlation between autoantibody binding to the MPO/HLA-DR complex and the OR for the correlation conferred by each HLA-DR allele ($r = 0.64$, $P = 0.014$).

These findings suggest that the MPO/HLA-DR complex is involved in the pathogenesis of MPA.

Cryptic autoantibody epitopes expressed by MPO associated with HLA-DR. Because the structure of the proteins associated with HLA-DR differs from that of native proteins (14–16), there is a possibility that cryptic autoantibody epitopes that are not exposed on the surface of native MPO proteins are exposed on MPO by association with HLA-DR. To test this possibility, we neutralized patient sera containing MPO-ANCAs using native MPO protein purified from human neutrophils. Anti-native MPO antibody titers detected by ELISA were decreased in the presence of native MPO proteins, in a dose-dependent manner (Figure 4). In contrast, autoantibodies against the MPO/HLA-DR complex were still detected in sera from MPA patients even after neutralization with native MPO proteins (Figure 4). In particular, there was a significant difference between the anti-MPO/HLA-DR antibody titer and the anti-MPO antibody titer after neutralization with $3.16 \mu\text{g/ml}$ native MPO. These results suggest that MPO associated with HLA-DR might express cryptic autoantibody epitopes that are not exposed on native MPO.

Formation of the MPO/HLA-DR complex in neutrophils stimulated with $\text{IFN}\gamma$. The presence of autoantibodies specific to MPO complexed with HLA-DR suggests that under certain conditions, MPO is associated with HLA-DR in neutrophils. Although neutrophils

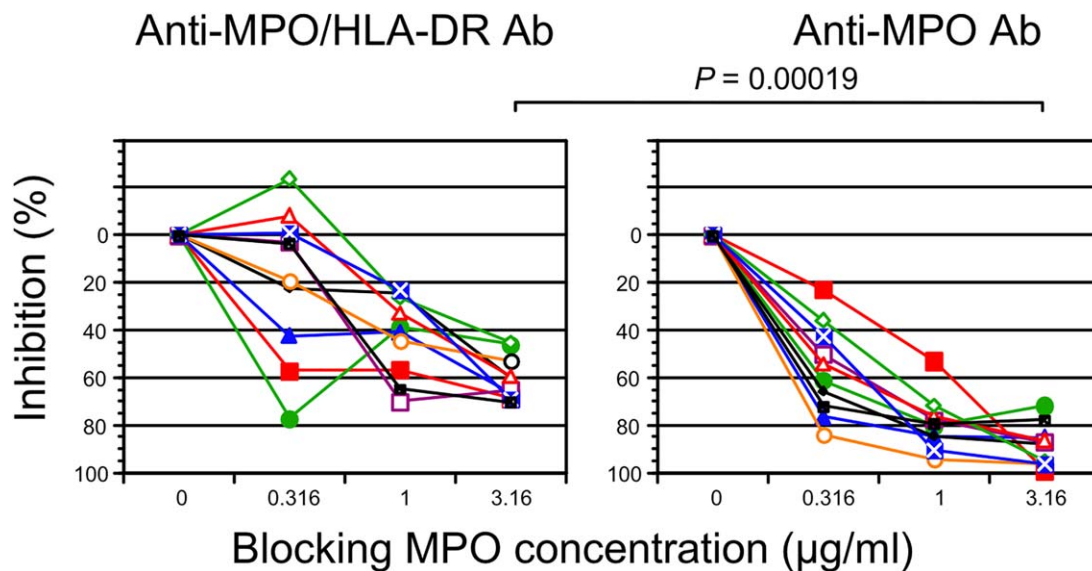


Figure 4. Cryptic autoantibody epitopes on myeloperoxidase (MPO) are exposed by association with HLA-DR. Serum samples from patients with microscopic polyangiitis (MPA) ($n = 10$) were mixed with various concentrations of MPO protein purified from human neutrophils and incubated overnight at 4°C. After incubation, anti-MPO/HLA-DR complex antibodies (Ab) were detected using flow cytometry, and anti-MPO antibodies were determined using enzyme-linked immunosorbent assay. Autoantibody titers were calculated using standard serum. Inhibition (%) was defined as $100 - (\text{titer of inhibited serum/titer of noninhibited serum}) \times 100$. Results are representative of at least 3 independent experiments. Student's *t*-test was used to analyze the difference between the anti-MPO/HLA-DR antibody titer and the anti-MPO antibody titer after incubation with MPO at a concentration of 3.16 µg/ml native MPO. Lines connect the data points for each subject. Each patient was distinguished using different colors and symbols.

contain high levels of intracellular MPO, resting neutrophils express few HLA class II molecules. However, it has been reported that human neutrophils express HLA class II molecules after stimulation with IFN γ (19,20). We thus analyzed HLA-DR expression on the surface of neutrophils isolated from healthy donors, following IFN γ stimulation (Figure 5A). As reported previously, neutrophils expressed HLA-DR following stimulation with IFN γ .

When HLA-DR was immunoprecipitated from the cell lysates of neutrophils from healthy donors, MPO was coprecipitated with HLA-DR from the cell lysates of IFN γ -stimulated neutrophils with MPA-susceptible HLA-DRB1*09:01, but not from those of unstimulated neutrophils. In addition, MPO was not coprecipitated with HLA-DR from the cell lysates of IFN γ -stimulated neutrophils with MPA-nonsusceptible HLA-DRB1*12:01/15:02 (Figure 5B). These findings indicate that MPO can be complexed with HLA class II molecules in neutrophils, under pathophysiologic conditions.

Detection of the MPO/HLA-DR complex in neutrophils isolated from a patient with MPA. We next analyzed neutrophils obtained from a patient with a diagnosis of MPA. The patient, a 75-year-old female, had an MPO-ANCA titer of 220 units/ml. She had a fever and interstitial pneumonia but no glomerulonephritis or neuropathy. We isolated neutrophils from her peripheral

blood at onset and after immunosuppressive therapy and analyzed them using flow cytometry. A small but significant amount of HLA-DR was expressed on the surface of neutrophils at onset, but this decreased after treatment (Figure 5C). We next investigated whether the MPO/HLA-DR complex was present in the patient's neutrophils, using the IP-FCM method, which is much more sensitive for the detection of molecular interactions compared with conventional methods using IP and Western blotting (29,36). The association of MPO with HLA-DR was detected in neutrophil cell lysates obtained from the patient at onset (Figure 5D). The amount of MPO complexed with HLA-DR decreased after treatment. In contrast, the association of MPO with HLA-DR was not detected in cell lysates from healthy donors. These results indicate that MPO forms a complex with HLA-DR in the neutrophils of MPA patients.

Activation of neutrophil-like cell lines by MPO-ANCA autoantibodies. Next, we investigated the pathophysiologic function of the MPO/HLA-DR complex on the surface of neutrophils. We hypothesized that MPO-ANCAs activate neutrophils through MPO/HLA-DR complexes. However, in vitro functional analyses of the MPO/HLA-DR complex on IFN γ -stimulated neutrophils were not successful, because resting peripheral neutrophils are nonspecifically activated by IFN γ . Accordingly, we stably transfected

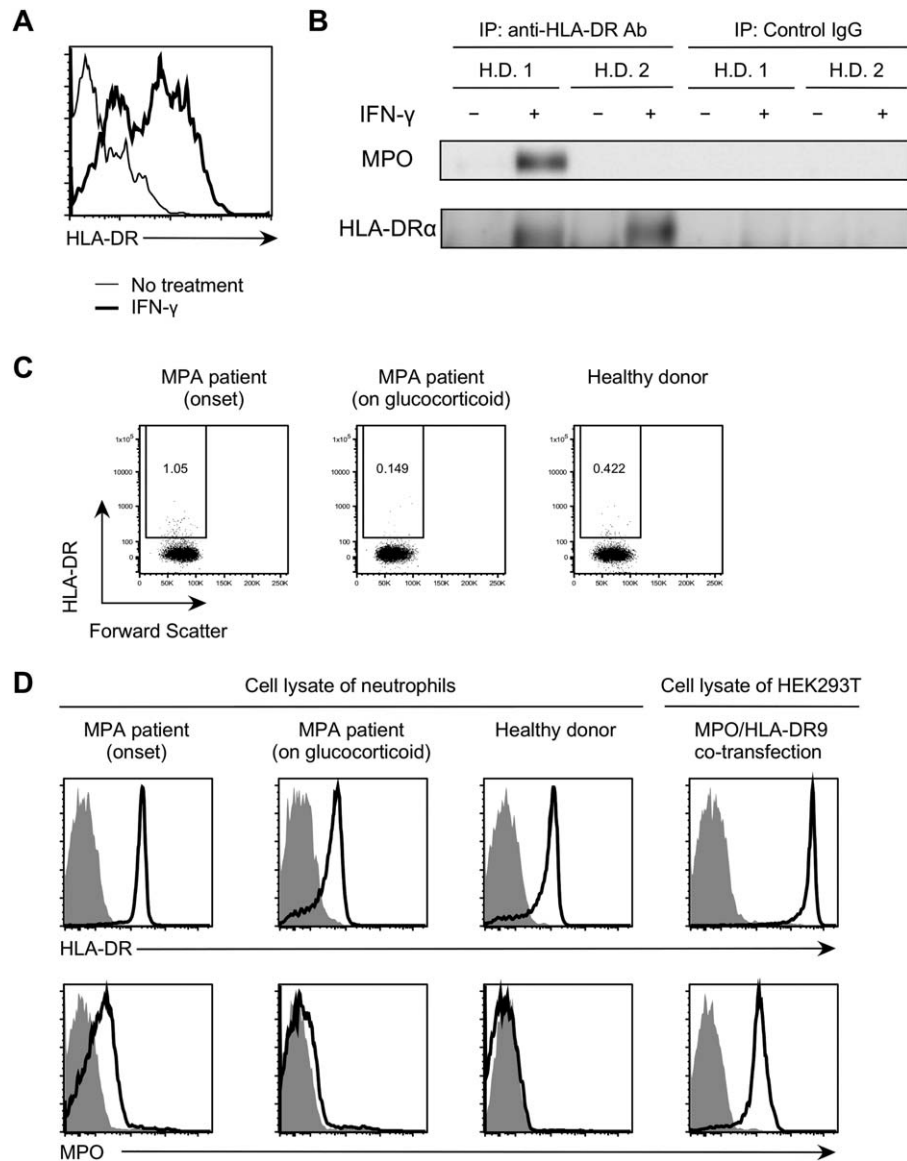


Figure 5. The MPO/HLA-DR complex is detected in neutrophils stimulated with interferon- γ (IFN γ) and neutrophils isolated from patients with MPA. **A**, Neutrophils from healthy donors (HDs) were isolated and incubated for 2 days in the presence or absence of IFN γ . After stimulation, neutrophils were stained with anti-HLA-DR antibody (Ab). The expression of HLA-DR on neutrophils with (thick line) or without (thin line) stimulation by IFN γ is shown. **B**, MPO protein was coimmunoprecipitated with HLA-DR9. After stimulation with IFN γ , cell lysates were immunoprecipitated with anti-HLA-DR or isotype-matched control IgG antibody. Immunoprecipitates were blotted with anti-MPO or anti-HLA-DR antibody. HD 1 = a donor carrying HLA-DRB1*09:01/09:01; HD 2 = a donor carrying HLA-DRB1*12:01/15:02. Results are representative of at least 3 independent experiments. **C**, Neutrophils were isolated from a patient with MPA at disease onset and after immunosuppressive therapy. Freshly isolated neutrophils were stained with anti-HLA-DR antibody. Numbers in the boxes are the proportions of HLA-DR-positive cells. **D**, Anti-HLA-DR antibody-coupled beads were mixed with cell lysates of neutrophils obtained from a patient with MPA and shaken at 4°C for 1 hour. The beads were stained with biotinylated anti-HLA-DR or anti-MPO antibody, followed by allophycocyanin-streptavidin. Staining of isotype-matched control IgG-coupled beads is shown as shaded histograms. Results are representative of at least 3 independent experiments. See Figure 4 for other definitions.

HLA-DR9 into HL-60 cells, which constitutively express MPO and differentiate into mature neutrophil-like cells upon stimulation with all-*trans*-retinoic acid (31,32). Intrinsic MPO expressed in HL-60 cells was expressed on the cell

surface in the presence of HLA-DR9, suggesting that MPO formed a complex with HLA-DR (Figure 6A). HL-60 cells were differentiated into neutrophil-like cells by stimulation with all-*trans*-retinoic acid and then were stimulated with

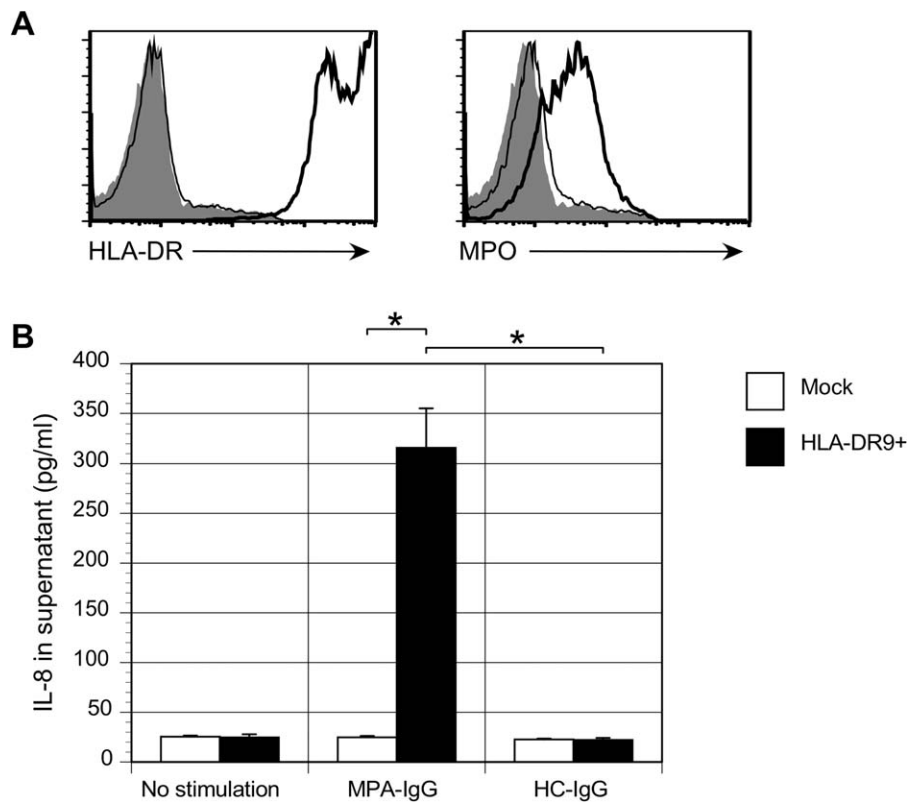


Figure 6. MPO-antineutrophil cytoplasmic antibodies activate the HL-60 neutrophil-like cell line expressing MPA-susceptible HLA-DR. **A**, HLA-DR9 was stably transfected into HL-60 cells via a retroviral vector. HLA-DR-positive cells were sorted. The sorted cells (thick lines) and mock cells (thin lines) were stained with anti-MPO antibody or anti-HLA-DR antibody. Shaded histograms represent control staining. Results are representative of at least 3 independent experiments. **B**, HLA-DR-transfected HL-60 cells were differentiated into neutrophil-like cells with all-*trans*-retinoic acid. Cells were stimulated with plate-bound IgG purified from a patient with MPA or a healthy control (HC). The interleukin-8 (IL-8) concentration of culture supernatants was determined using enzyme-linked immunosorbent assay. Values are the mean $\pm$ SD of triplicate experiments. * = $P < 0.05$ by one-way analysis of variance and Tukey's post hoc test. See Figure 4 for other definitions.

IgG purified from the sera of MPA patients or healthy donors. IL-8 was secreted by HLA-DR9-expressing HL-60 cells upon stimulation with IgG from MPA patients, but not with IgG from healthy donors (Figure 6B). These results suggest that the MPO-ANCAs stimulate neutrophils through the MPO/HLA-DR complex.

DISCUSSION

In the current study, we demonstrated that intracellular MPO is transported to the cell surface by HLA class II molecules with an MPA-susceptible allele. MPO expressed on the cell surface by HLA class II molecules was recognized by MPO-ANCAs, which was involved in neutrophil activation by MPO-ANCAs. These findings suggest that HLA class II molecules are involved in the pathogenesis of MPA by direct association with MPO.

The major function of HLA class II molecules is to present antigenic peptides to T cells. However, it has

remained unclear how HLA class II molecules control susceptibility to autoimmune diseases. However, we have observed that misfolded ER proteins are transported to the cell surface by MHC class II molecules without processing to peptides when they are associated with MHC class II molecules (14,18). In the current study, we demonstrated that MPO proteins are also transported to the cell surface by associating with HLA-DR. Although mature MPO consists of a heavy subunit (60 kd) and a light subunit (12 kd), the MPO protein that coimmunoprecipitated with HLA-DR was 90 kd, which is a molecular weight compatible with apopro-MPO or pro-MPO (37). Because both ends of the peptide-binding groove of HLA class II are open (38-40), it is possible (structurally) that the peptide-like structure exposed on full-length MPO proteins binds to the peptide-binding groove. Although we cannot determine the conformation of MPO proteins that are bound to HLA class II molecules, it is likely that MPO proteins associated with HLA class II molecules are

structurally different from native MPO proteins, because only misfolded proteins are associated with HLA class II molecules and are transported to the cell surface (14,15). Indeed, native MPO protein efficiently blocked anti-native MPO autoantibodies, whereas autoantibodies against the MPO/HLA-DR complex were not completely blocked by native MPO protein. These results suggest that cryptic autoantibody epitopes on MPO might be exposed by an association with HLA-DR. These findings are consistent with our hypothesis that MPO associated with HLA-DR is structurally altered compared with native MPO protein.

The ORs for the association between each HLA-DRB1 allele and MPA were significantly correlated with autoantibody binding to MPO complexed with HLA-DR for each allele. This is the first study to show a molecular mechanism that is associated with MPA susceptibility conferred by each HLA-DR allele. HLA-DR of MPA-susceptible alleles efficiently transported MPO to the cell surface, suggesting a relatively high affinity for MPO. The invariant chain binds to newly synthesized HLA class II molecules at the ER and blocks the binding of ER proteins to HLA class II molecules (35). Interestingly, the effect of the invariant chain on the binding of MPO to HLA-DR was lower for susceptible alleles than for nonsusceptible alleles. The affinities of MPO to HLA-DR seem to be higher than the affinities of the invariant chain to HLA-DR in the case of MPA-susceptible HLA-DR alleles.

Significant associations of HLA-DRB1*09:01 with MPA and MPO-ANCA-positive vasculitis in Japanese patients have been reported (22,23). HLA-DRB1*09:01 is the most common HLA-DRB1 allele in Japanese individuals but is rare in Caucasians (Allele*Frequencies in Worldwide Populations [<http://www.allele-frequencies.net/>]) (41). Our results demonstrating the efficient binding of MPO protein with HLA-DRB1*09:01 might explain why MPA is more common than granulomatosis with polyangiitis (Wegener's) (GPA) in Japan, while GPA is more common in Europe (42). A previous GWAS demonstrated that positivity for MPO-ANCAs, not susceptibility to MPA, is significantly associated with a single-nucleotide polymorphism (rs5000634) in the HLA-DQ region (21). Because the HLA-DR region was not included in the GWAS, and there is strong linkage disequilibrium between HLA-DQ and HLA-DR, HLA-DR is also a possible candidate gene. Because HLA-DRB1*09:01 is a dominant allele in Japan, the high frequency of MPO-ANCAs in Japanese individuals (43), unlike that in Caucasians, might be a result of the high-affinity binding between MPO and HLA-DR9.

The presence of autoantibodies against MPO/HLA-DR complexes suggested that MPO/HLA-DR complexes are generated in vivo. Neutrophils possess high levels of MPO but express very few HLA-DR molecules in a

steady state. Previous studies have shown that HLA-DR molecules are induced on neutrophils after stimulation with cytokines such as IFN γ (20,44). Indeed, it has been reported that neutrophils isolated from patients with GPA express MHC class II molecules (45,46). Similarly, we showed that neutrophils from MPA patients express HLA-DR. In addition, we demonstrated that HLA-DR9-positive neutrophils but not HLA-DR12-positive or HLA-DR15-positive neutrophils from healthy donors produce MPO/HLA-DR complexes following stimulation with IFN γ . Therefore, in certain pathologic conditions such as viral infections in which various cytokines are produced, HLA-DR induced by cytokines forms a complex with MPO. Indeed, MPO/HLA-DR complexes were detected in freshly isolated neutrophils from MPA patients. These results imply that the MPO/HLA-DR complex is involved in the pathogenesis of MPA.

We demonstrated that MPO-ANCAs activated HL-60 cells that expressed the MPO/HLA-DR complex. Pathologic conditions in which cytokines such as IFN γ are overproduced might lead to cell surface expression of the MPO/HLA-DR complex in neutrophils, which could be a target for MPO-ANCAs. Several studies have demonstrated that HLA class II molecules transduce activating signals in MHC class II-expressing cells such as dendritic cells and B cells (47–50). Although the precise mechanism of neutrophil activation by autoantibodies is unclear, it is possible that HLA-DR complexed with MPO transduces activating signals in neutrophils by MPO-ANCAs.

The mechanism by which MPO-ANCAs are produced in patients with MPA remains unclear. The presence of autoantibodies against the MPO/HLA-DR complex suggests that MPO proteins complexed with HLA-DR are the targets of autoantibodies against MPO. It is possible that structurally altered MPO proteins that are associated with HLA-DR could be recognized as “altered-self” or “neo-self” antigens by immune cells and initiate autoantibody production (18). Indeed, misfolded protein–HLA class II complexes stimulate antigen-specific B cells (14). At present, it remains unclear how T cells are involved in the production of autoantibodies against the complex. Considering that IgG autoantibodies are the most common, T cells must be involved in production of autoantibodies against the misfolded protein–HLA class II complexes. Further analyses are necessary to elucidate how autoantibodies against MPO/HLA class II complexes are produced.

ACKNOWLEDGMENTS

We thank K. Shida, S. Matsuoka, K. Furuno, and M. Matsumoto for providing technical assistance and C. Kita for providing secretarial assistance. We also thank N. Tsuchiya for

granting permission to use data about the HLA-DRB1 allele frequency in MPA.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Hiwa had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Hiwa, Ohmura, N. Arase, Hirayasu, Kohyama, Suenaga, Atsumi, Mimori, H. Arase.

Acquisition of data. Hiwa, Ohmura, N. Arase, Jin, Saito, Terao, Atsumi, Iwatani.

Analysis and interpretation of data. Hiwa, Ohmura, N. Arase, Jin, Hirayasu, Kohyama, Suenaga, Saito, Terao, Atsumi, Iwatani, Mimori, H. Arase.

REFERENCES

- Jennette JC, Falk RJ, Bacon PA, Basu N, Cid MC, Ferrario F, et al. 2012 revised International Chapel Hill Consensus Conference nomenclature of vasculitides. *Arthritis Rheum* 2013;65:1–11.
- Guillevin L, Durand-Gasselin B, Cevallos R, Gayraud M, Lhote F, Callard P, et al. Microscopic polyangiitis: clinical and laboratory findings in eighty-five patients. *Arthritis Rheum* 1999;42:421–30.
- Falk RJ, Jennette JC. Anti-neutrophil cytoplasmic autoantibodies with specificity for myeloperoxidase in patients with systemic vasculitis and idiopathic necrotizing and crescentic glomerulonephritis. *N Engl J Med* 1988;318:1651–7.
- Falk RJ, Terrell RS, Charles LA, Jennette JC. Anti-neutrophil cytoplasmic autoantibodies induce neutrophils to degranulate and produce oxygen radicals in vitro. *Proc Natl Acad Sci U S A* 1990;87:4115–9.
- Huugen D, Xiao H, van Esch A, Falk RJ, Peutz-Kootstra CJ, Buurman WA, et al. Aggravation of anti-myeloperoxidase antibody-induced glomerulonephritis by bacterial lipopolysaccharide. *Am J Pathol* 2005;167:47–58.
- Kettritz R, Jennette JC, Falk RJ. Crosslinking of ANCA-antigens stimulates superoxide release by human neutrophils. *J Am Soc Nephrol* 1997;8:386–94.
- Kessenbrock K, Krumbholz M, Schonermarck U, Back W, Gross WL, Werb Z, et al. Netting neutrophils in autoimmune small-vessel vasculitis. *Nat Med* 2009;15:623–5.
- Nakazawa D, Shida H, Tomaru U, Yoshida M, Nishio S, Atsumi T, et al. Enhanced formation and disordered regulation of NETs in myeloperoxidase-ANCA-associated microscopic polyangiitis. *J Am Soc Nephrol* 2014;25:990–7.
- Ewert BH, Jennette JC, Falk RJ. Anti-myeloperoxidase antibodies stimulate neutrophils to damage human endothelial cells. *Kidney Int* 1992;41:375–83.
- Lu X, Garfield A, Rainger GE, Savage CO, Nash GB. Mediation of endothelial cell damage by serine proteases, but not superoxide, released from antineutrophil cytoplasmic antibody-stimulated neutrophils. *Arthritis Rheum* 2006;54:1619–28.
- Xiao H, Heeringa P, Hu P, Liu Z, Zhao M, Aratani Y, et al. Antineutrophil cytoplasmic autoantibodies specific for myeloperoxidase cause glomerulonephritis and vasculitis in mice. *J Clin Invest* 2002;110:955–63.
- Kallenberg CG, Stegeman CA, Abdulahad WH, Heeringa P. Pathogenesis of ANCA-associated vasculitis: new possibilities for intervention. *Am J Kidney Dis* 2013;62:1176–87.
- Jennette JC, Falk RJ. Pathogenesis of antineutrophil cytoplasmic autoantibody-mediated disease. *Nat Rev Rheumatol* 2014;10:463–73.
- Jiang Y, Arase N, Kohyama M, Hirayasu K, Suenaga T, Jin H, et al. Transport of misfolded endoplasmic reticulum proteins to the cell surface by MHC class II molecules. *Int Immunol* 2013;25:235–46.
- Jin H, Arase N, Hirayasu K, Kohyama M, Suenaga T, Saito F, et al. Autoantibodies to IgG/HLA class II complexes are associated with rheumatoid arthritis susceptibility. *Proc Natl Acad Sci U S A* 2014;111:3787–92.
- Tanimura K, Jin H, Suenaga T, Morikami S, Arase N, Kishida K, et al. β 2-Glycoprotein I/HLA class II complexes are novel autoantigens in antiphospholipid syndrome. *Blood* 2015;125:2835–44.
- Arase N, Tanimura K, Jin H, Yamaoka T, Kishibe M, Nishioka M, et al. Novel autoantibody against the β 2-glycoprotein I/HLA-DR complex in patients with refractory cutaneous ulcers. *Br J Dermatol* 2017. E-pub ahead of print.
- Arase H. Rheumatoid rescue of misfolded cellular proteins by MHC class II molecules: a new hypothesis for autoimmune diseases. *Adv Immunol* 2016;129:1–23.
- Gosselin EJ, Wardwell K, Rigby WF, Guyre PM. Induction of MHC class II on human polymorphonuclear neutrophils by granulocyte/macrophage colony-stimulating factor, IFN- γ , and IL-3. *J Immunol* 1993;151:1482–90.
- Fanger NA, Liu C, Guyre PM, Wardwell K, O'Neil J, Guo TL, et al. Activation of human T cells by major histocompatibility complex class II expressing neutrophils: proliferation in the presence of superantigen, but not tetanus toxoid. *Blood* 1997;89:4128–35.
- Lyons PA, Rayner TF, Trivedi S, Holle JU, Watts RA, Jayne DR, et al. Genetically distinct subsets within ANCA-associated vasculitis. *N Engl J Med* 2012;367:214–23.
- Tsuchiya N, Kobayashi S, Kawasaki A, Kyogoku C, Arimura Y, Yoshida M, et al. Genetic background of Japanese patients with antineutrophil cytoplasmic antibody-associated vasculitis: association of HLA-DRB1*0901 with microscopic polyangiitis. *J Rheumatol* 2003;30:1534–40.
- Kawasaki A, Hasebe N, Hidaka M, Hirano F, Sada KE, Kobayashi S, et al. Protective role of HLA-DRB1*13:02 against microscopic polyangiitis and MPO-ANCA-positive vasculitides in a Japanese population: a case-control study. *PLoS One* 2016;11:e0154393.
- Watts R, Lane S, Hanslik T, Hauser T, Hellmich B, Koldingsnes W, et al. Development and validation of a consensus methodology for the classification of the ANCA-associated vasculitides and polyarteritis nodosa for epidemiological studies. *Ann Rheum Dis* 2007;66:222–7.
- Weinrauch Y, Drujan D, Shapiro SD, Weiss J, Zychlinsky A. Neutrophil elastase targets virulence factors of enterobacteria. *Nature* 2002;417:91–4.
- Andrew SM, Titus JA. Purification and fragmentation of antibodies. *Curr Protoc Immunol* 1997;7.1–2.7.12.
- Scott CA, Peterson PA, Teyton L, Wilson IA. Crystal structures of two I-Ad-peptide complexes reveal that high affinity can be achieved without large anchor residues. *Immunity* 1998;8:319–29.
- Terao C, Ohmura K, Kochi Y, Ikari K, Maruya E, Katayama M, et al. A large-scale association study identified multiple HLA-DRB1 alleles associated with ACPA-negative rheumatoid arthritis in Japanese subjects. *Ann Rheum Dis* 2011;70:2134–9.
- Schrum AG, Gil D, Dopfer EP, Wiest DL, Turka LA, Schamel WW, et al. High-sensitivity detection and quantitative analysis of native protein-protein interactions and multiprotein complexes by flow cytometry. *Sci STKE* 2007;2007:pl2.
- Morita S, Kojima T, Kitamura T. Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther* 2000;7:1063–6.
- Millius A, Weiner OD. Chemotaxis in neutrophil-like HL-60 cells. *Methods Mol Biol* 2009;571:167–77.
- Breitman TR, Selonick SE, Collins SJ. Induction of differentiation of the human promyelocytic leukemia cell line (HL-60) by retinoic acid. *Proc Natl Acad Sci U S A* 1980;77:2936–40.

33. Chicz RM, Urban RG, Lane WS, Gorga JC, Stern LJ, Vignali DA, et al. Predominant naturally processed peptides bound to HLA-DR1 are derived from MHC-related molecules and are heterogeneous in size. *Nature* 1992;358:764–8.
34. Hayden JB, McCormack AL, Yates JR III, Davey MP. Analysis of naturally processed peptides eluted from HLA DRB1*0402 and *0404. *J Neurosci Res* 1996;45:795–802.
35. Neefjes J, Jongstra ML, Paul P, Bakke O. Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nat Rev Immunol* 2011;11:823–36.
36. Schrum AG. Visualization of multiprotein complexes by flow cytometry. *Curr Protoc Immunol* 2009;Chapter 5:Unit5 9.
37. Andersson E. The role of the propeptide for processing and sorting of human myeloperoxidase. *J Biol Chem* 1998;273:4747–53.
38. Stern LJ, Brown JH, Jardetzky TS, Gorga JC, Urban RG, Strominger JL, et al. Crystal structure of the human class II MHC protein HLA-DR1 complexed with an influenza virus peptide. *Nature* 1994;368:215–21.
39. Ghosh P, Amaya M, Mellins E, Wiley DC. The structure of an intermediate in class II MHC maturation: CLIP bound to HLA-DR3. *Nature* 1995;378:457–62.
40. Fremont DH, Hendrickson WA, Marrack P, Kappler J. Structures of an MHC class II molecule with covalently bound single peptides. *Science* 1996;272:1001–4.
41. Gonzalez-Galarza FF, Takeshita LY, Santos EJ, Kempson F, Maia MH, da Silva AL, et al. Allele frequency net 2015 update: new features for HLA epitopes, KIR and disease and HLA adverse drug reaction associations. *Nucleic Acids Res* 2015;43:D784–8.
42. Fujimoto S, Watts RA, Kobayashi S, Suzuki K, Jayne DR, Scott DG, et al. Comparison of the epidemiology of anti-neutrophil cytoplasmic antibody-associated vasculitis between Japan and the U.K. *Rheumatology (Oxford)* 2011;50:1916–20.
43. Sada KE, Yamamura M, Harigai M, Fujii T, Dobashi H, Takasaki Y, et al. Classification and characteristics of Japanese patients with antineutrophil cytoplasmic antibody-associated vasculitis in a nationwide, prospective, inception cohort study. *Arthritis Res Ther* 2014;16:R101.
44. Kambayashi T, Laufer TM. Atypical MHC class II-expressing antigen-presenting cells: can anything replace a dendritic cell? *Nat Rev Immunol* 2014;14:719–30.
45. Hansch GM, Radsak M, Wagner C, Reis B, Koch A, Breitbart A, et al. Expression of major histocompatibility class II antigens on polymorphonuclear neutrophils in patients with Wegener's granulomatosis. *Kidney Int* 1999;55:1811–8.
46. Iking-Konert C, Vogt S, Radsak M, Wagner C, Hansch GM, Andrassy K. Polymorphonuclear neutrophils in Wegener's granulomatosis acquire characteristics of antigen presenting cells. *Kidney Int* 2001;60:2247–62.
47. Al-Daccak R, Mooney N, Charron D. MHC class II signaling in antigen-presenting cells. *Curr Opin Immunol* 2004;16:108–13.
48. Haylett RS, Koch N, Rink L. MHC class II molecules activate NFAT and the ERK group of MAPK through distinct signaling pathways in B cells. *Eur J Immunol* 2009;39:1947–55.
49. Nagaraj S, Nelson A, Youn JI, Cheng P, Quiceno D, Gabrilovich DI. Antigen-specific CD4+ T cells regulate function of myeloid-derived suppressor cells in cancer via retrograde MHC class II signaling. *Cancer Res* 2012;72:928–38.
50. Lang P, Stolpa JC, Freiberg BA, Crawford F, Kappler J, Kupfer A, et al. TCR-induced transmembrane signaling by peptide/MHC class II via associated Ig- α/β dimers. *Science* 2001;291:1537–40.