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ORIGINAL ARTICLE

Autoreactive T-cell responses to myeloperoxidase in patients with antineutrophil cytoplasmic antibody-associated vasculitis and in healthy individuals

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Abstract The aim of this study was to evaluate the characteristics of autoreactive T cells to myeloperoxidase (MPO) in patients with MPO-antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis. Peripheral blood T cells from 15 patients with MPO-ANCA-associated vasculitis and 14 healthy individuals were cultured with three recombinant proteins that together comprised the entire MPO sequence (L, all 112 amino acids (AA) of the light chain; HI, AA 1-227 of the heavy chain; HII, AA 212-467 of the heavy chain), and the antigen-specific T-cell proliferative response was measured by ^{3}H-thymidine incorporation. T-cell responses to MPO-L and HI were both detected in four patients and three healthy donors, and responses to MPO-HII were detected in four patients and seven healthy donors. These findings indicate that at least three independent T-cell epitopes exist on the MPO molecule. Interestingly, the patients whose T cells showed these MPO-induced responses were mainly in remission. Peripheral blood T cells reactive with MPO were primarily of the HLA-DR-restricted CD4$^+$ phenotype. In summary, we successfully used recombinant MPO fragments to detect autoreactive CD4$^+$ T cells to multiple MPO epitopes in blood samples from patients with MPO-ANCA-associated vasculitis and healthy individuals.

Keywords ANCA-associated vasculitis · MPO-reactive T cell · Myeloperoxidase

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Introduction

Antineutrophil cytoplasmic antibodies (ANCAs) are frequently detected in patients with small-vessel vasculitis, including Wegener's granulomatosis (WG), microscopic polyangiitis (MPA), and Churg-Strauss syndrome (CSS) [1]. ANCAs with specificity for proteinase 3 (PR3) are predominantly associated with WG, whereas ANCAs with specificity for myeloperoxidase (MPO) are more common in patients with MPA or CSS [2]. Direct pathogenic roles of MPO-ANCAs have been demonstrated by their binding to target antigens expressed on the surface of primed neutrophils and monocytes, leading to the induction and release of oxygen metabolites and proteinases, which trigger vascular injury [3–5].

MPO-ANCAs are predominantly of the high-affinity IgG isotype [6], suggesting that their production results from T-cell-dependent isotype-switching and affinity maturation. The human leukocyte antigen (HLA)-DRB1*0901 is associated with MPO-ANCA-associated vasculitis among Japanese patients [7]. In addition, treatment with T-cell-directed agents, such as cyclosporin A, reduces the titer of ANCAs and induces remission in some patients with ANCA-associated vasculitis [8, 9]. Finally, Xiao et al. [10] showed that lymphocytes derived from MPO-deficient mice that were pre-immunized with mouse MPO directly induce pauci-immune crescentic glomerulonephritis and pulmonary vasculitis in recombinase-activating gene-2-deficient

mice and in wild-type mice. These findings strongly suggest that ANCA production requires an antigen-specific collaboration between T and B cells. Thus, autoreactive T cells to MPO are a potential target of selective immunotherapy for MPO-ANCA-associated vasculitis, but the characteristics of these T cells are largely unknown. In this study, responses to MPO by autoreactive T cells from patients with MPO-ANCA-associated vasculitis and healthy individuals were evaluated using recombinant MPO fragments as the antigen source.

Materials and methods

Patients and healthy subjects

Fifteen patients (5 men, 10 women, 20–84 years old) with a diagnosis of MPO-ANCA-associated vasculitis, including 12 patients with MPA and 3 with CSS, were studied. All the patients fulfilled the Chapel Hill criteria [11] for MPA or CSS, and were positive for MPO-ANCAs, as determined by a commercially available enzyme-linked immunosorbent assay kit (MBL, Nagoya, Japan). Disease activity was assessed for all the patients at time of blood sampling, using the Birmingham Vasculitis Activity Score [12]. Remission was defined as the state in which no more than one item on the score showed persistent disease activity [13]. Controls included 14 healthy volunteers (6 men, 8 women, 26–51 years old). All blood samples were obtained after the subjects gave their written informed consent, as approved by the Institutional Review Board.

HLA class II genotyping

Genomic DNA was isolated from peripheral blood leukocytes using a standard phenol extraction procedure. The HLA-DRB1 and DQB1 alleles were determined using polymerase-chain reaction (PCR) followed by analysis of restriction fragment length polymorphisms [14].

Preparation of recombinant MPO fragments

Fusion proteins of maltose-binding protein (MalBP) and human MPO fragments were prepared using a bacterial expression system, as described previously [15]. The MPO fragments consisted of all 112 amino acid residues (AA) of the light chain (MPO-L), AA 1-227 of the heavy chain (MPO-HI), and AA 212-467 of the heavy chain (MPO-HII). Together these fragments comprise the entire MPO protein sequence. Briefly, this was done by amplifying a series of MPO complementary DNAs (cDNAs) from human neutrophil cDNAs by PCR using the following specific primer pairs: MPO-L, 5′-AGGAATTCTGCCCGGAGCAGGACAA-3′ (sense) and 5′-GCGAATTCTCAGCCAGTGACGAAG-3′ (antisense); MPO-HI, 5′-GGGAATTCGTCAACTGCGAG-3′ (sense) and 5′-CCGAATTCTCAGATGAGGGTGTG-3′ (antisense); and MPO-HII, 5′-GCAGAATTCATCGCCAACGTCTTC-3′ (sense) and 5′-TCGGATCCCTAGGAGGCTTCCCT-3′ (antisense). The cDNA constructs were subcloned in-frame at the 3′ end of the MalBP gene in the pMAL-c2 expression vector (New England Biolabs, Beverly, MA) and were introduced into competent *Escherichia coli* strain DH5α (Toyobo, Osaka, Japan). The sequences of both DNA strands were verified on an ABI Prism 3100 genetic analyser (Applied Biosystems, Foster City, CA) using the BigDye Terminator Cycle Sequence Ready Reaction kit (Applied Biosystems). Recombinant MalBP fusion proteins and control MalBP were expressed by induction with isopropyl-β-D-thiogalactopyranoside and purified using amylase-resin affinity chromatography [16]. The recombinant proteins were then dialyzed against phosphate-buffered saline and filter-sterilized for cell culture. All preparations were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by Coomassie brilliant blue staining or immunoblotting with rabbit anti-MPO (Chemicon, Temecula, CA) or anti-MalBP (New England Biolabs) polyclonal antibodies. Semi-quantitative densitometric analysis was then performed using ChemiDox XRS and Quantity One software (Bio-Rad Laboratories, Hercules, CA).

Cell preparations

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized venous blood by Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) density-gradient centrifugation. PBMCs were cultured in RPMI1640 containing 10% fetal bovine serum, 2 mM L-glutamine, 50 units/ml penicillin, and 50 μg/ml streptomycin at 37°C in 5% CO_2. In some experiments, $CD4^+$ or $CD8^+$ cell-depleted PBMCs were prepared using anti-CD4 or anti-CD8 monoclonal antibody (mAb)-coupled magnetic beads (Dynabeads®; Invitrogen, Carlsbad, CA), respectively, according to the manufacturer's instructions.

T-cell proliferation assays

Antigen-induced T-cell proliferation was assayed as described previously [17] with some modifications. PBMCs (10^5/well) were cultured in triplicate with or without antigen in 96-well flat-bottomed culture plates for 6 days and then incubated with 0.5 μCi/well of ^{3}H-thymidine for an additional 16 h. The cells were harvested, and the ^{3}H-thymidine incorporation was determined on a Top-Count microplate scintillation counter (Packard, Meriden, CT). MalBP-MPO fusion proteins (MPO-L, HI, and HII) as well as MalBP (10 μg/ml) were added to the cultures as antigens. Antigen-specific T-cell proliferation was

expressed as the stimulation index (SI), which was calculated as the cpm incorporated by the antigen-stimulated cultures divided by the cpm incorporated by the control cultures. A significant antigen-induced T-cell response was defined as SI $\geq$2. Phytohemagglutin (PHA; 1 μg/ml) was used to stimulate PBMCs to exclude non-specific T-cell unresponsiveness.

The secondary stimulation of peripheral blood T cells was also performed as described [18]. Briefly, PBMCs were primed with MPO-L, HI, or HII for 7 days. The viable cells were then cultured for an additional 3 days with individual MPO fragments or MalBP (10 μg/ml) in the presence of 50 U/ml recombinant interleukin-2 (Biogen Idec, San Diego, CA) and irradiated (3,000 rad) autologous PBMCs. Antigen-induced T-cell proliferation was measured by the incorporation of ^{3}H-thymidine.

Blocking of the antigen-specific T-cell response by anti-HLA class II antibody

To examine the effects of individual anti-HLA class II mAbs on antigen-specific T-cell proliferation, anti-HLA-DR (clone L248), anti-HLA-DQ (clone 1a3), anti-HLA-DP (clone B7/21), or isotype-matched IgG2a control mouse mAbs (Leinco Technologies, St. Louis, MO) were added at the initiation of the culture at a final concentration of 1 μg/ml [19]. A significant inhibition was defined as the cpm incorporated by the cultures with anti-HLA class II mAb divided by the cpm incorporated by the cultures incubated with an isotype-matched control mAb of <0.5.

Statistical analysis

All continuous variables are expressed as the mean ± standard deviation (SD). Comparisons between two groups were tested for statistical significance using the Mann-Whitney *U*-test or Kruskal-Wallis test, when applicable. Associations between T-cell responses induced by individual recombinant MPO fragments and HLA class II alleles were tested using the chi-square test or Fisher's test, as appropriate.

Results

Preparation of recombinant MPO fusion proteins

Three recombinant fusion proteins (MPO-L, HI, and HII) that together comprised the whole MPO sequence were successfully expressed and purified. Dominant protein bands with molecular weights of 56, 69, 75, and 44 kDa were detected in the MPO-L, HI, HII, and MalBP preparations, respectively, but all the preparations except for the MalBP also contained several degradation products. Their identity was confirmed on immunoblots probed with the anti-MalBP antibody. In addition, anti-MPO polyclonal antibodies were reactive with the dominant proteins in the MPO-L, HI, and HII preparations. The recombinant MPO fragments, including the intact and degraded products, represented >95% of the total protein in each preparation.

Clinical features and HLA class II alleles in patients with MPO-ANCA-associated vasculitis

Table 1 summarizes the clinical features as well as the HLA-DRB1 and DQB1 alleles present in the 15 patients with MPO-ANCA-associated vasculitis examined in this study. All the MPA patients had renal involvement, whereas three patients with CSS had mononeuritis multiplex alone. At the time of blood sampling, 12 patients were receiving prednisolone alone or in combination with cyclophosphamide, while three patients (P9, P14, and P15) were newly diagnosed and had never received immunosuppressive treatment. Five patients maintained clinical remission on low-dose prednisolone, and the remaining ten had active disease status. There was no common DRB1 or DQB1 allele detected for patients with MPO-ANCA-associated vasculitis. Contrary to a previous report [8], only three (20%) had DRB1*0901.

T-cell proliferative responses to MPO fragments

Representative results of the T-cell proliferation induced by MalBP, MPO-L, HI, and HII, and PHA obtained from two patients with MPO-ANCA-associated vasculitis are shown in Fig. 1. In patient 8 (P8), MPO-HII, but not MPO-L or HI, induced a proliferative response, whereas T cells from P13 responded to all three MPO fragments. The T-cell proliferative responses to individual MPO fragments in the 15 patients with MPO-ANCA-associated vasculitis and the 14 healthy controls are summarized in Fig. 2. T-cell responses to MPO-L, HI, and HII were detected in four (27%) patients each, but these responses were also detected in three (21%), three (21%), and seven (50%) healthy controls. There was no difference in the degree of response to MPO-L, HI, or HII as expressed by the SI between the patients and healthy controls. Interestingly, all the responders to MPO-L, including four patients and three healthy controls, also responded to MPO-HI, although MPO-L and MPO-HI did not share any homologous amino acid sequences. Therefore, a T-cell response to any MPO fragment was detected in samples from six patients (40%) and eight healthy controls (57%). This experiment was repeated for three patients and one healthy control, and the results were consistently reproducible. Moreover, the specificity of the T-cell response to the MPO fragments in the three patients and one healthy control was confirmed by experiments testing the secondary response: T cells primed by MPO-L, HI, or HII were again specifically reactive with the fragments used for priming in the secondary cultures (data not shown).

Table 1 Clinical features and HLA class II alleles in patients with MPO-ANCA-associated vasculitis

Patients	Age/sex	Diagnosis	Organ involvement	MPO-ANCA (EU)[a]	Therapy at blood examination	Disease status	HLA class II alleles	
							DRB1	DQB1
P1	68/M	CSS	PN	46	PSL 10 mg/day	Remission	*0406/*0803	*0302/*0601
P2	65/M	MPA	K	126	PSL 40 mg/day	Active	*0404/*1501	*0302/*0402
P3	58/M	MPA	K, L	573	PSL 60 mg/day	Active	*0405/*1502	*0401/*0601
P4	63/F	MPA	K, PN	25	PSL 10 mg/day, CY 80 mg/day	Active	*0802/*0803	*0302/*0601
P5	20/F	MPA	K, L	24	PSL 18 mg/day, IVCY 750 mg/Mo	Active	*0403/*1501	*0302/*0602
P6	55/F	MPA	K, PN	34	PSL 8 mg/day	Remission	*0901	*0303
P7	68/M	MPA	K, L	22	PSL 5 mg/day	Remission	*1402/*1501	*0301/*0602
P8	69/F	MPA	K	31	PSL 10 mg/day	Remission	*1402/*1501	*0501/*0604
P9	79/M	MPA	K, L	26	None	Active	*0901/*1201	*0301/*0303
P10	28/F	MPA	K, L	56	PSL 10 mg/day	Active	*0410/*0901	*0303/*0402
P11	68/F	CSS	PN	81	PSL 40 mg/day	Active	*0701/*1302	*0201/*0604
P12	84/F	MPA	K, PN	365	PSL 30 mg/day	Active	*0406/*1501	*0302/*0602
P13	60/F	MPA	K, L	24	PSL 9 mg/day	Remission	*0803/*1401	*0502/*0601
P14	42/F	CSS	PN	99	None	Active	*0101/*1502	ND
P15	75/F	MPA	K, L	640	None	Active	*0101/*0405	ND

HLA human leukocyte antigen; *MPO* myeloperoxidase; *ANCA* antineutrophil cytoplasmic antibody; *EU* ELISA units; *M* male; *F* female; *CSS* Churg-Strauss syndrome; *MPA* microscopic polyangiitis; *PN* peripheral nerve; *K* kidney; *L* lung; *PSL* prednisolone; *CY* oral cyclophosphamide; *IVCY* intravenous cyclophosphamide; *ND* not determined

[a] Normal range <20 EU

Associations of MPO-induced T-cell responses with clinical and immunogenetic parameters

The potential associations of T-cell responses to individual MPO fragments with age, sex, diagnosis (MPA vs. CSS), organ involvement, and MPO-ANCA titer were evaluated in the patients with MPO-ANCA-associated vasculitis, but none of those parameters was associated with the responses. On the other hand, a T-cell response to any MPO fragment was detected in all five patients in remission, but in only one (P3) of the ten patients with active disease ($P = 0.002$; see Fig. 2). The therapeutic regimen was associated with a T-cell response to MPO; the response tended to be more frequent among the ten patients who received $\leq$10 mg/day prednisolone than among the five patients on >10 mg/day prednisolone (50 vs. 20%), although there was no statistically significant difference between these two frequencies ($P = 0.08$).

We failed to find any correlations between the T-cell responses induced by MPO fragments and the HLA class II alleles in the patients and healthy controls, but there was a trend toward a higher frequency of DQB1*03 in the responders to MPO-L and HI than in the non-responders (86 vs. 50%, $P = 0.18$), when the data from all 27 subjects in which the DQB1 allele were determined were combined.

Characteristics of MPO-reactive T cells

To examine whether the MPO-reactive T cells had a CD4 or CD8 phenotype, we evaluated the effects of depleting the $CD4^+$ or $CD8^+$ cells on the T-cell proliferation induced by MPO-L, HI, or HII. Figure 3 illustrates representative results from a healthy control who responded to all three MPO fragments. The antigen-induced T-cell responses to the three fragments were almost completely lost after the depletion of $CD4^+$ cells, but not after the depletion of $CD8^+$ cells. Concordant findings were observed in an additional two healthy control subjects.

The effects of anti-HLA-DR, anti-HLA-DQ, or anti-HLA-DP mAbs on the T-cell proliferative responses to MPO fragments were examined in samples from seven healthy control subjects. The T-cell responses induced by MPO-L, HI, and HII were inhibited by the anti-HLA-DR mAb, but not by the anti-HLA-DP or anti-HLA-DQ mAbs, in all the subjects examined (see Fig. 4 for representative results).

Discussion

We successfully detected autoreactive T cells to MPO in peripheral blood samples from patients with MPO-ANCA-associated vasculitis and healthy individuals, using

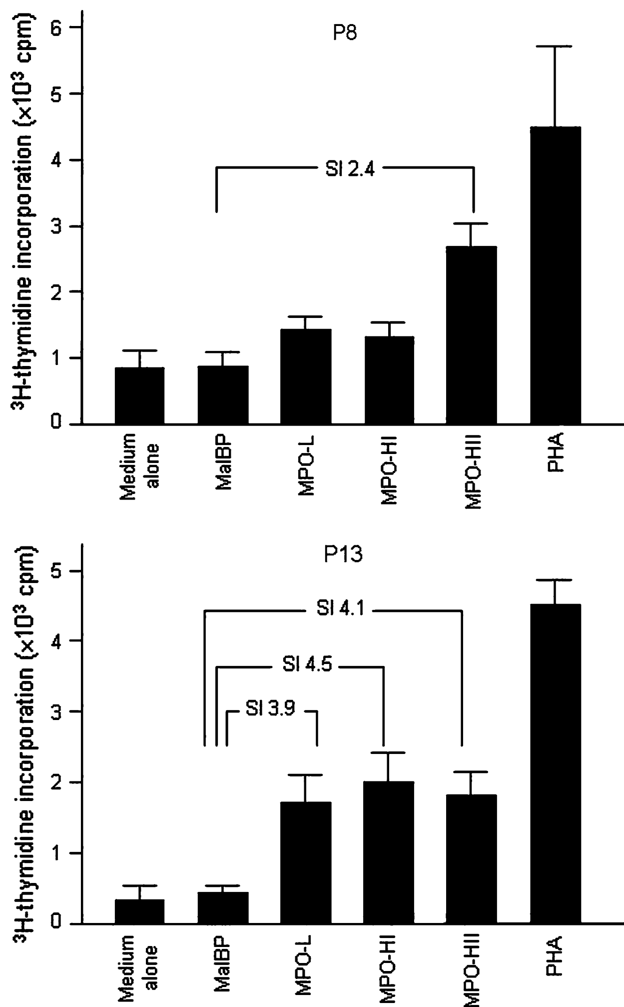


Fig. 1 T-cell proliferative responses to MPO fragments in two patients with MPO-ANCA-associated vasculitis (P8 and P13). PBMCs were cultured with MPO-L, HI, HII, MalBP, or phytohemagglutin (PHA) for 7 days, and then T-cell proliferation was measured by ^{3}H-thymidine incorporation. The stimulation index (SI) was calculated as the cpm incorporated in antigen-stimulated cultures divided by the cpm incorporated in control cultures exposed to MalBP. All cultures were carried out in triplicate, and the results shown are the mean and SD

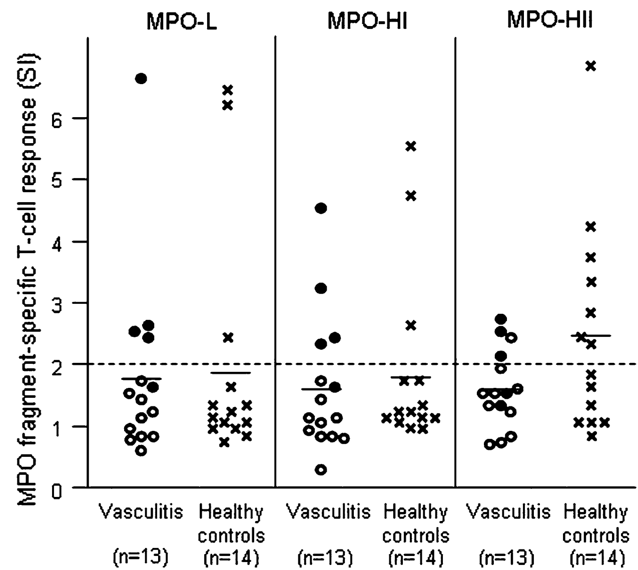


Fig. 2 T-cell proliferative responses specific to the three MPO fragments (MPO-L, HI, and HII) in the 15 patients with MPO-ANCA-associated vasculitis and the 14 healthy donors. A *broken line* denotes the cutoff for a positive response. *Horizontal lines* indicate mean values. *Open circles* and *closed circles* indicate patients with active disease and those in remission, respectively

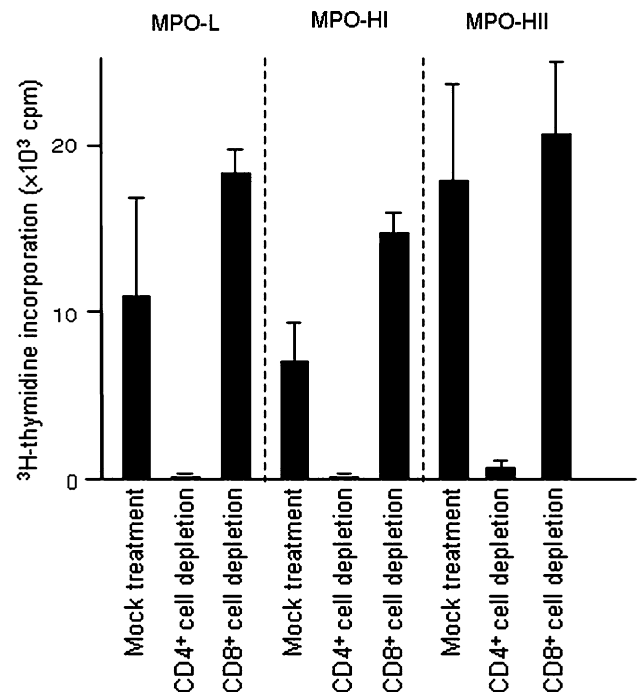
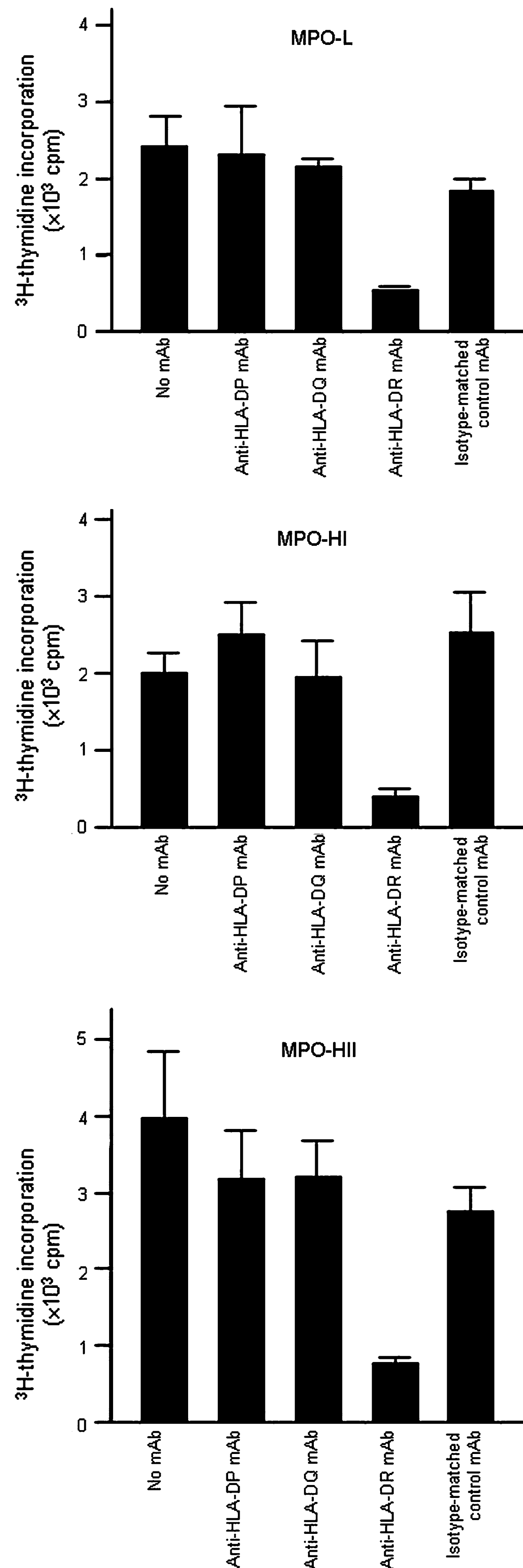


Fig. 3 Effects of depleting CD4$^+$ or CD8$^+$ cells from PBMCs on the T-cell responses induced by MPO fragments. Mock-treated, CD4$^+$ cell-depleted, and CD8$^+$ cell-depleted PBMCs from a representative healthy donor were cultured with MPO-L, HI, or HII for 7 days, and ^{3}H-thymidine incorporation in the cultures was measured. All the experiments were carried out in triplicate cultures, and the results show the mean and SD

recombinant MPO fragments as the antigen source. MPO-reactive T cells were mainly HLA-DR-restricted CD4$^+$ T cells that recognized multiple epitopes on the light and heavy chains of MPO.

Several groups have evaluated in vitro T-cell proliferative responses to MPO in patients with MPO-ANCA-associated vasculitis. Griffith et al. [20] demonstrated a significant T-cell proliferative response to purified native MPO in 7 of 12 patients with MPO-ANCA-associated vasculitis and in 5 of 12 healthy donors. Moreover, Yoshida et al. [21] established MPO-reactive T-cell lines from one patient with MPO-ANCA-associated vasculitis by repeated stimulation with native MPO. These T-cell lines were positive for CD4 and restricted by HLA-DR, consistent with our findings.

Fig. 4 Effects of anti-HLA class II mAbs on the T-cell proliferation induced by MPO fragments. PBMCs from healthy donors were cultured with MPO-L, HI, or HII in the presence of anti-HLA-DR, anti-HLA-DQ, anti-HLA-DP, or isotype-matched control mAbs for 7 days, and ^{3}H-thymidine incorporation by the cultures was measured. All experiments were carried out in triplicate, and the results show the mean and SD



However, there has been some concern that the native MPO might have enzyme activity at the concentration used in these cultures [22] and that the native molecule could be cytotoxic and induce apoptosis of the lymphocytes. These concerns suggested that it might not be feasible to evaluate MPO-specific T-cell responses accurately using native MPO as the antigen. In fact, many previous publications have demonstrated that peripheral blood T cells fail to respond to various native autoantigens, including topoisomerase I, glycoprotein IIb-IIIa, and β_2-glycoprotein I [17, 23, 24]. Two groups have used heat-inactivated MPO instead of native MPO as an antigen to improve the in vitro culture systems: in these studies, a significant T-cell proliferative response was detected in none of nine patients and in only one of five patients with MPO-ANCA-associated vasculitis [25, 26]. T-cell responses to autoantigens can also be affected by chemically modified autoantigens or bacterially expressed recombinant fragments [23, 24]. These results could be explained by a change in the antigen's conformation that affected antigen processing, resulting in an alteration of the peptide repertoire generated at the endosome in antigen-presenting cells [27]. Therefore, the heat inactivation of MPO may be insufficient to reveal its antigenic peptides from being recognized by the MPO-reactive T cells from patients with MPO-ANCA-associated vasculitis.

Here, we used bacterially expressed recombinant MPO fragments, which lack enzymatic activity, to detect MPO-reactive T cells in 6 (40%) of 15 patients with MPO-ANCA-associated vasculitis. This approach was reasonable, because the preparation of recombinant fragments of many autoantigens using a bacterial expression system promotes the display by antigen-presenting cells of the antigenic peptides recognized by autoreactive T cells [17, 18, 28].

Despite our use of MPO fragments that comprised the entire MPO sequence, T-cell responses to MPO were detected in samples from only 40% of the patients with MPO-ANCA-associated vasculitis. In addition, MPO-reactive T cells were detectable predominantly in patients in remission. We believe that this finding does not indicate a lack of MPO-reactive T cells in patients with active vasculitis. Rather, the patients' immunosuppressive treatment regimens may have interfered with our detection of in vitro T-cell responses to MPO: half of the patients with active vasculitis received high-dose prednisolone alone or in combination with cyclophosphamide. Alternatively, this

finding could be explained by in vitro activation-induced T-cell death, as observed in patients with active systemic lupus erythematosus and those with active multiple sclerosis [29, 30]. A T-cell response to MPO was detected in none of samples from three patients with active vasculitis (P9, P14, and P15), although they had never received any immunosuppressive therapy at the time of blood sampling. Therefore, it is likely that autoreactive T cells in patients with active vasculitis have been already activated in vivo and thereby sensitized to activation-induced cell death when they are exposed to strong antigenic stimulation in vitro.

We detected a T-cell response to MPO fragments in the samples from more than half of the healthy control subjects. In this regard, in vitro T-cell proliferative responses to autoantigens such as topoisomerase I, glycoprotein IIb-IIIa, β_2-glycoprotein I, and insulin are known to occur in healthy individuals [17, 18, 19, 31]. In addition, Griffith et al. [20] showed that peripheral blood T cells from some healthy subjects exhibited an in vitro proliferative response to MPO, although that study used native MPO as the antigen source. Taken together with our findings, these results strongly suggest that autoreactive T cells to MPO, like many other autoreactive T cells, represent a subset of the normal T-cell repertoire.

Conclusion

We found that autoreactive CD4$^+$ T cells to several distinct epitopes on MPO were present in patients with MPO-ANCA-associated vasculitis as well as in healthy individuals and that the use of recombinant MPO fragments as antigens was an effective way to assess the T-cell responses. Further studies aimed at characterizing the MPO-reactive T cells may be useful for evaluating the pathogenesis of MPO-ANCA-associated vasculitis and for developing novel therapies targeting these autoreactive T cells.

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Conflicts of interest statement The authors declare that there are no conflicts of interest associated with this article.

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