

## Characterization of autoreactive T-cell clones to myeloperoxidase in patients with microscopic polyangiitis and healthy individuals

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### ABSTRACT

**Objective.** To characterize autoreactive T cells against myeloperoxidase (MPO) by generating antigen-specific T-cell clones from patients with microscopic polyangiitis (MPA) and healthy individuals.

**Methods.** Peripheral blood T cells from five patients with MPA and MPO-anti-neutrophil cytoplasmic antibodies (ANCA) and from three healthy donors were used to establish MPO-specific T-cell clones by repeated stimulation with recombinant MPO fragments, followed by limiting dilution. The MPO-specific T-cell clones were subjected to analyses for CD4/CD8 phenotype, human leukocyte antigen (HLA) class II restriction, T-cell receptor (TCR)  $\beta$ -chain gene usage, complementarity-determining region 3 (CDR3) amino acid sequences, and cytokine expression profiles.

**Results.** We successfully generated seven MPO-specific T-cell clones, five from the patients and two from healthy donors. Two clones recognized the light chain of MPO and five recognized the heavy chain. All the clones were HLA-DR-restricted CD4<sup>+</sup>CD8<sup>-</sup> helper T cells. The T-cell clones shared TCR  $\beta$  CDR3 amino acid motifs, depending on their MPO epitope: AGxxN was used by clones recognizing the light chain and TGxS or QGxE by those recognizing the heavy chain, whether the cells were derived from MPA patients or healthy subjects. However, the cytokine expression profiles of the patients' clones were skewed towards the Th1 phenotype, whereas the healthy individuals' clones remained Th0.

**Conclusions.** We have characterized MPO-reactive T cells in detail. This information may be useful for elucidating the mechanism of ANCA production and for developing selective therapeutic strategies for MPO-ANCA-associated vasculitis.

### Introduction

Anti-neutrophil cytoplasmic antibodies (ANCA) with a specificity for myeloperoxidase (MPO) are detected in patients with microscopic polyangiitis (MPA) or with Churg-Strauss syndrome (1). MPO-ANCA are thought to contribute directly to the pathogenic

process of vasculitis by binding to target antigens expressed on the surface of primed neutrophils and monocytes, leading to the release of oxygen metabolites and proteinases (2-4). These responses induce injury and activation of the endothelium, which also plays an important role in development of vasculitis (5).

MPO-ANCA are predominantly of the high-affinity IgG isotype (6), suggesting that their production results from a T-cell-dependent isotype switch and affinity maturation. In addition, treatment with T-cell-directed agents, such as cyclosporine A, reduces the titer of ANCA and induces remission in some patients with ANCA-associated vasculitis (7, 8). Finally, Xiao *et al.* showed that lymphocytes derived from MPO-deficient mice that were pre-immunized with mouse MPO directly induced pauci-immune crescentic glomerulonephritis and pulmonary vasculitis in recombinase-activating gene-2-deficient mice, as well as in wild-type mice (9). These findings strongly suggest that ANCA production requires an antigen-specific collaboration between T and B cells. Thus, although autoreactive T cells that recognize MPO are a potential target for selective immunotherapy for MPO-ANCA-associated vasculitis, their characteristics are largely unknown. Recently, we identified CD4<sup>+</sup> T cells autoreactive to multiple epitopes on MPO in patients with MPO-ANCA-associated vasculitis and in healthy individuals (10). In this study, we successfully established MPO-specific T-cell clones from MPA patients and healthy individuals and characterized their phenotypes, including their T-cell receptor (TCR) and cytokine expression profiles.

### Materials and methods

#### Human subjects

Five patients with MPA (P4, P5, P7, P8, and P13) and three healthy donors (HD1-3) were selected from the subjects examined in our previous study (10), based on the capacity of their peripheral blood T cells to respond to at least one recombinant MPO fragment and the availability of peripheral blood samples. All the MPA patients fulfilled the Chapel Hill criteria (11), and were positive for MPO-ANCA, as determined

Competing interests: none declared.

**Table I.** V $\beta$ -J $\beta$ -C $\beta$  gene rearrangement, CDR3 amino acid sequence, and CDR3 length of the TCR- $\beta$  chain used by MPO-specific T-cell clones.

Antigen	Donor	CD4/CD8 phenotype	HLA class II restriction	Gene segment*			CDR3 amino acid sequence <sup>†</sup>			CDR3 length (amino acids)
				V $\beta$	J $\beta$	C $\beta$	V $\beta$	N-D $\beta$ -N-J $\beta$	J $\beta$	
MPO-L	P4	CD4 <sup>+</sup> /CD8 <sup>-</sup>	DR	2.1	1.5	1	CS	ARVPAGISNQPQHF	GDGTRLSIL	14
	P7	CD4 <sup>+</sup> /CD8 <sup>-</sup>	DR	7	2.4	2	CASS	QDLAGGENIQYF	GAGTRLSVL	12
MPO-HII	P5	CD4 <sup>+</sup> /CD8 <sup>-</sup>	DR	4	2.2	2	CS	<u>VD</u> <u>TGA</u> <u>S</u> GELFF	GEGRSLTVL	11
	P8	CD4 <sup>+</sup> /CD8 <sup>-</sup>	DR	1	1.5	1	CASS	<u>ATGAS</u> NQPQH	GDGTRLSIL	10
	P13	CD4 <sup>+</sup> /CD8 <sup>-</sup>	DR	2.3	1.1	1	CS	ASL <u>QG</u> <u>TE</u> AFF	GQGTRLTVV	10
	HD1	CD4 <sup>+</sup> /CD8 <sup>-</sup>	DR	22.1	1.6	1	CAS	RTGQSTSP <sup>LH</sup>	GNGTRLTVT	10
	HD2	CD4 <sup>+</sup> /CD8 <sup>-</sup>	DR	12.2	1.4	1	CA	AQKR <u>QG</u> <u>DE</u> KLFF	GSGTQLSVL	12

\*Determined based on the nucleotide sequence of the TCR $\beta$  PCR products.

<sup>†</sup>The AGxxN motif in the TCR used by MPO-L-specific T cell clones and the TGxS and QGxE motifs in the TCR used by MPO-HII-reactive T-cell clones are shown as bold, underlined, and doubled-underlined text, respectively.

by a commercially available enzyme-linked immunosorbent assay kit (MBL, Nagoya, Japan). All blood samples were obtained after the subjects gave their written informed consent, as approved by the Institutional Review Board.

#### MPO-specific T-cell lines

We used three recombinant MPO fragments, expressed as maltose-binding protein (MalBP) fusion proteins, for T-cell stimulation. These included MPO-L, the entire 112 amino acid (AA) light chain; MPO-HI, AA 1-227 of the heavy chain; and MPO-HII, AA 212-467 of the heavy chain (10). Antigen-specific T-cell lines were generated according to a previously described method (12) with some modifications. Briefly, peripheral blood mononuclear cells (PBMCs) were cultured in complete medium supplemented with 8% autologous heat-inactivated plasma and one of the recombinant MPO fragments (10  $\mu$ g/ml) that had been known to be capable of stimulating T cells in our previous study (10), in the presence of a monoclonal antibody (mAb) to CD29 (2.5  $\mu$ g/ml) (clone MAR4; BD Pharmingen, San Diego, CA, USA). This procedure improves the viability of T-cell blasts by suppressing antigen-induced cell death *in vitro* (13). After the second stimulation, T-cell blasts were cloned by limiting dilution at 0.3 cells/well, and the cells in growth-positive wells were expanded and maintained in cultures that included interleukin (IL)-2 and irradiated autologous Epstein-Barr virus-transformed lymphoblastoid B cells, and were reape-

tedly stimulated with the antigen. The specificity of each T-cell line was evaluated by a proliferation assay, in which MalBP and a series of MPO fragments were used as the antigen (10). T-cell lines responsive to one of the MPO fragments, but not to MalBP, were regarded as MPO-specific T-cell lines.

#### CD4/CD8 phenotype

The surface expression of CD4 and CD8 was assessed by flow cytometry using a fluorescein isothiocyanate-conjugated anti-CD4 mAb and a phycoerythrin-conjugated anti-CD8 mAb (12).

#### Human leukocyte antigen (HLA) class II restriction

The HLA class II restriction of individual T-cell lines was determined by examining the inhibitory effect of anti-HLA-DR, anti-HLA-DQ, and anti-HLA-DP mAbs on MPO-induced T-cell proliferation (10).

#### Preparation of complementary DNA (cDNA)

MPO-specific T-cell lines or PBMCs were stimulated with phorbol 12-myristate 13-acetate (PMA; 25 ng/ml) and ionomycin (1  $\mu$ g/ml) for 3 days. The CD4<sup>+</sup> T cells were isolated by incubation with anti-CD4 mAb-coupled magnetic beads (Dynal Biotech ASA, Oslo, Norway) (14). The total RNA was extracted using a phenol/guanidine isothiocyanate extraction procedure (Isoagen; Nippon Gene, Tokyo, Japan) and subjected to reverse transcription with oligo-dT priming, to generate cDNAs.

#### Analysis of the TCR $\beta$ chain

The TCR  $\beta$  gene usage of individual MPO-specific T-cell lines was analyzed by polymerase-chain reaction (PCR) using a panel of V $\beta$  region primers corresponding to V $\beta$ 1-24, in combination with a C $\beta$  region primer, as described previously (15, 16). The PCR products were directly sequenced on an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA). The complementarity-determining region 3 (CDR3) was defined as the region starting from the amino acid residue after the CASS sequence of each V $\beta$  segment and ending with the last amino acid before the GxG box in the J $\beta$  region.

#### Cytokine expression profiles

The gene expression of interferon (IFN)- $\gamma$ , IL-2, IL-4, IL-6, IL-10, transforming growth factor (TGF)- $\beta$ , and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was evaluated by PCR using a panel of specific primers, as described elsewhere (14). PBMCs that were stimulated with PMA and ionomycin were used as a control for individual cytokine expression. The PCR products were resolved by electrophoresis on 1.5% agarose gels and visualized by staining with ethidium bromide.

## Results

### Establishment of MPO-specific T-cell clones

We successfully generated a total of seven T-cell lines specific to MPO: five

from patients with MPA and two from healthy donors (Table I). We failed to obtain a T-cell line from one healthy donor. The clonality of each line was confirmed by verifying that each one had only one functionally rearranged TCR  $\beta$  chain. The patterns of reactivity with MalBP and MPO fragments of representative T-cell clones are shown in Figure 1. Two clones derived from MPA patients recognized MPO-L, and the remaining five (three from MPA patients and two from healthy donors) recognized MPO-HII. All the MPO-specific T-cell clones had a CD4<sup>+</sup>CD8<sup>-</sup> helper phenotype and were restricted by HLA-DR.

#### TCR $\beta$ chain

The V $\beta$ -J $\beta$ -C $\beta$  gene rearrangement, CDR3 amino acid sequence, and CDR3 length in the TCR  $\beta$  chain of each MPO-specific T-cell clone are summarized in Table I. There was no common V $\beta$  or J $\beta$  gene segment shared by the MPO-specific T-cell clones. Regarding the CDR3 amino acid sequence, both of the T-cell clones that recognized MPO-L had the amino acid motif AGxxN. In contrast, the TGxS or QGxE motif was found in the TCR V $\beta$  chain of the T-cell clones that recognized MPO-HII. Since MPO-HII encompassed a region spanning 256 amino acid residues, it is likely that two different epitopes are present in this region, in association with different TCR  $\beta$  CDR3 motifs.

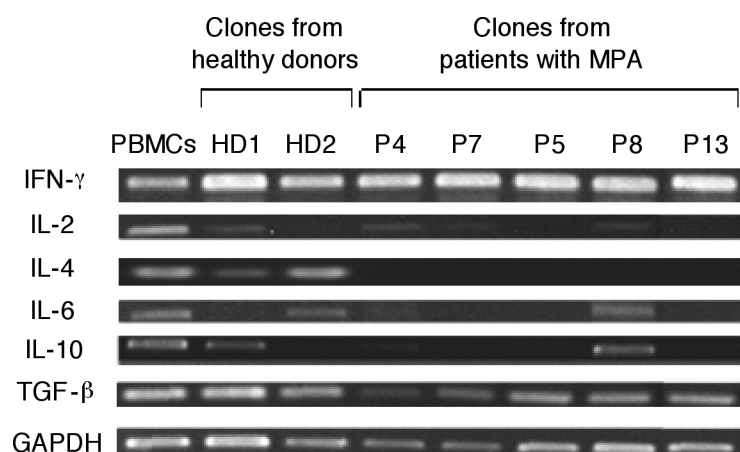
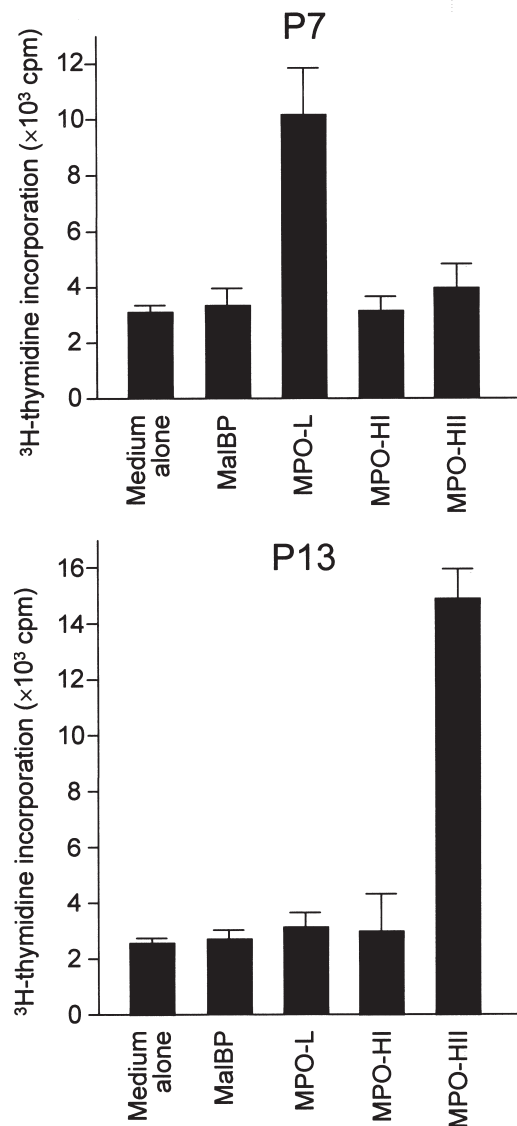
#### Cytokine expression profiles

Individual MPO-specific T-cell clones expressed different sets of cytokines, while all the clones expressed IFN- $\gamma$  and TGF- $\beta$  (Figure 2). Notably, the MPO-specific T-cell clones derived from healthy donors expressed IL-4 in combination with IFN- $\gamma$ , a pattern consistent with a Th0 phenotype. In contrast, none of the clones derived from patients with MPA expressed IL-4, and their cytokine expression pattern was consistent with a Th1 phenotype.

#### Discussion

We successfully established MPO-specific T-cell clones from the peripheral blood of MPA patients and healthy individuals. All the MPO-specific T-cell

**Fig. 1.** Reactivities to MalBP and MPO fragments of MPO-specific T-cell clones established from two patients with MPA (P7 and P13). T-cell clones were cultured with or without MPO-L, MPO-HI, MPO-HII, or MalBP, and irradiated autologous Epstein-Barr virus-transformed lymphoblastoid B cells for 3 days, then the T-cell proliferation was measured by <sup>3</sup>H-thymidine incorporation. All cultures were carried out in triplicate, and the results shown are the mean and standard deviation. Representative results obtained from three independent experiments are shown.



**Fig. 2.** Cytokine expression patterns of MPO-specific T-cell clones, determined by PCR. MPO-specific T-cell clones derived from MPA patients and healthy donors were subjected to PCR to detect the expression of IFN- $\gamma$ , IL-2, IL-4, IL-6, IL-10, TGF- $\beta$ , and GAPDH. PMA/ionomycin-treated PBMCs were used as a control for individual cytokine expression. The PCR products were resolved by electrophoresis on 1.5% agarose gels and visualized by staining with ethidium bromide. Concordant results were obtained in two additional experiments.

clones were HLA-DR-restricted CD4<sup>+</sup> helper T cells; these features were consistent with previously reported MPO-reactive T-cell lines obtained from a patient with MPO-ANCA-associated vasculitis, although the clonality of those lines was not evaluated (17). Analysis of the TCR  $\beta$  chain of the MPO-specific T-cell clones revealed the presence of several distinct CDR3 amino acid motifs in association with the recognition of epitopes on both the light and heavy chains of MPO.

The MPO-specific T-cell clones derived from MPA patients and healthy individuals had common characteristics, including a CD4<sup>+</sup>CD8<sup>-</sup> helper phenotype and restriction by HLA-DR, and some of them shared CDR3 amino acid motifs in the TCR  $\beta$  chain. Thus, there was no difference in properties determined during T-cell development between the MPO-specific T cells derived from the patients and those from healthy individuals. An analogous phenomenon has been reported for several other autoreactive T-cell clones, including those to myelin basic protein in patients with multiple sclerosis, to topoisomerase I in patients with scleroderma, and to  $\beta_2$ -glycoprotein I in patients with antiphospholipid syndrome (15, 16, 18). Taken together, it is likely that T cells autoreactive to MPO are a component of the T-cell repertoire of some healthy individuals.

However, the cytokine expression profiles of the MPO-specific T-cell clones were apparently different: the clones derived from MPA patients had a Th1 phenotype, while those from healthy individuals were Th0. In this regard, the MPO-reactive T-cell lines established from an MPO-ANCA-positive patient reported by Yoshida *et al.* also represented a Th1 cytokine expression profile (17). A difference in cytokine expression profiles between patients and healthy individuals was also shown for T-cell clones autoreactive to topoisomerase I (14). The mechanism responsible for this difference is unclear, but

it is possible that autoreactive T cells in patients are activated due to ongoing or recent antigenic stimulation and differentiate into Th1 cells, whereas those in healthy individuals remain immature Th0 cells, due to a lack of appropriate antigenic stimulation.

The CDR3 amino acid motifs of the TCR  $\beta$  chain unique to MPO-specific T cells might be a potential selective therapeutic target for ANCA-associated vasculitis. TCR-based immunotherapy, such as TCR vaccination, is an effective treatment for various autoimmune diseases in animal models, and active investigations into its appropriateness for clinical applications are on-going (19).

In summary, we have identified characteristics unique to autoreactive T cells against MPO by generating MPO-specific T-cell clones. This information may be useful in elucidating the pathogenesis of ANCA-associated vasculitis and in developing selective immunotherapy for this intractable disease.

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