Characterization of Monoclonal Antibodies to the Plasma Cell Alloantigen ENPP1

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The ectonucleotide pyrophosphatase phosphodiesterase 1 (ENPP1) has documented roles in mineralization, nucleotide recycling, and insulin resistance. While ENPP1 was first identified as an alloantigen on mouse plasma cells (PCs), later studies revealed expression in many tissues. Previously described monoclonal antibodies against ENPP1 expressed at the cell surface recognized cells only from mice bearing the *a* allotype, ENPP1^a, precluding studies of mice bearing the alternative allele, ENPP1^b. Here, we characterize a novel anti-ENPP1 monoclonal antibody that recognizes both alleles and can be used for flow cytometry.

Introduction

THE ECTONUCLEOTIDE PYROPHOSPHATASE PHOSPHODIESTERASE 1 (ENPP1) was discovered in 1970 by Takahashi, Old, and Boyse as a membrane alloantigen expressed by neoplastic plasma cells (PCs), termed plasmacytomas, and normal PCs, termed PC-1. PC-1 was shown to be 115 kDa under reducing conditions and 230 kDa under non-reducing conditions.⁽¹⁾ They identified two alleles of PC-1. PC-1^a, recognized by alloantisera, was expressed by strains including BALB/c, NZB, and AKR. Strains that did not express the alloantigen (C57BL/6, C58, DBA/2, and others) were later designated as expressing the PC-1^b allele following molecular identification of the alternative allele. PC-1^a and PC-1^b differ by two amino acids (aa) (H650 and R679) in the extracellular domain.⁽²⁾ Later studies by Goding and colleagues showed that PC-1, encoded by the gene *Enpp1*, is also expressed in many non-lymphoid tissues including chondrocytes, liver, distal convoluted tubules of the kidney, salivary gland, and brain capillary endothelium.⁽³⁾ They succeeded in generating a monoclonal antibody (MAb) (IR518) that recognized the *a* allele⁽⁴⁾ at the H650 position.⁽²⁾

The function of ENPP1 is multifaceted. First, ENPP1 catalyzes 5'-phosphodiesterase bonds in nucleotide triphosphates to generate pyrophosphate (PPi),^(5,6) an important inhibitor of calcification and bone formation. Consistent with this, mice with inactivating mutant alleles of *Enpp1*^(7,8) or a genetically engineered null allele⁽⁹⁾ exhibited "stiff joints" and "tiptoe walking" due to pathologic calcification of the joints and paraspinal ligaments. In addition, mutations of *Enpp1* also cause blood vessel calcification in both mice^(7,10) and hu-

mans.^(11–14) Second, ENPP1 mediates nucleotide recycling by breaking down ATP to AMP, which is then converted to adenosine by 5'-nucleotidase.⁽⁵⁾ Adenosine is then transported freely into cells for metabolism. Third, ENPP1 is involved in regulation of cell adhesion⁽¹⁵⁾ and adipocyte differentiation.⁽¹⁶⁾ Finally, ENPP1 has been shown to modulate insulin receptor signal transduction⁽¹⁷⁾ and purinoceptor signaling⁽¹⁸⁾ such that overexpression of ENPP1 is associated with obesity and insulin resistance (reviewed by Bacci et al.⁽¹⁹⁾).

Although expression of ENPP1 on PCs has been recognized for four decades, little is known about the function of this molecule in B lineage cells. The lack of MAb with specificity for ENPP1^b has impeded study of this molecule in mice bearing the *b* allele. Remarkably, Takei generated a rat MAb, YE1/19.1, against the C57BL/6 EL4 T cell lymphoma that recognized a homodimer of 115 kDa under reducing conditions and 230 kDa under non-reducing conditions.⁽²⁰⁾ The antigen was expressed on a subset of normal T cells, and at high levels on the aberrantly expanded T cell population of *Fas* and *FasL* mutant mice and a non-secretory BALB/c PCT.⁽²¹⁾ In this report, we further characterize YE1/19.1, showing that the MAb recognizes both alleles of ENPP1 and can be used for flow cytometry.

Materials and Methods

The anti-ENPP1 monoclonal antibodies

The rat [IgG2b, κ] MAb YE1/19.1 was described previously.⁽²⁰⁾ The mouse [IgG2a, κ] anti-ENPP1^a MAb (clone IR518) was generated by Goding and colleagues.⁽²²⁾ Both

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antibodies were purified from culture supernatants and labeled with allophycocyanin (APC) using standard procedures from the Custom Antibody Facility, Research Technological Branch (NIAID). A mouse IgG2a isotype control antibody labeled with APC was purchased from Southern Biotech (Birmingham, AL). Purified normal rat IgG (Southern Biotech) was also labeled with APC.

Mice and cells

C57BL/6J (B6) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). *Enpp1*^{-/-} mice, described previously,⁽⁹⁾ were generously provided by Dr. Robert Terkeltaub (University of California–San Diego). Mouse plasmacytoma (PCT) cell lines MPC11 (originated from BALB/c)⁽²³⁾ and BPC4 (originated from B6; generated by Dr. Michael Potter in the National Cancer Institute) were used in this study. All animal studies were performed under protocols of LIP-4 approved by the NIAID IACUC.

Immunoprecipitation and protein identification

The MPC11 and BPC4 PCT cells were cultured in RPMI 1640 supplemented with 10% FBS, 50 μ M 2-mercaptoethanol, 2 mM L-glutamine, and 100 U/mL penicillin. Cells were lysed with lysis buffer containing 50 mM Tris (pH 7.5), 150 mM NaCl, proteinase inhibitors (Roche Molecular Systems, Branchburg, NJ), and 1% Triton X-100. The cell lysate was pre-cleared with protein G beads (Invitrogen, Carlsbad, CA) for 2 h at 4°C and incubated with 20 μ g of YE1/19.1 MAb. Immune complexes were precipitated by incubation with protein G beads (Thermo Scientific, Rockford, IL) and washed seven times before being resolved on a NuPAGE Novex 4–12% Bis-Tris Gel (Invitrogen). After staining with Coomassie Blue, the protein bands between 110 and 260 kDa were dissected and processed for in-gel digestion with trypsin. The peptides extracted from the gel digestion were analyzed by LC-MS (Q-Star, Applied Biosystems, Carlsbad, CA). The LC-MS data were analyzed using NCBI database.

FIG. 1. Identification of YE1/19.1 target protein as ENPP1. **(A)** Murine MPC11 plasmacytoma cells were lysed and precipitated with YE1/19.1. The precipitates were resolved by SDS-PAGE followed by staining with Coomassie Blue. The bands between 260 and 110 kDa were dissected and analyzed by mass spectrometry. The molecular weight standard is shown on left. **(B)** Peptide sequences of mass spectrometry matched the amino acid sequence of ENPP1.

FIG. 2. Binding of anti-ENPP1 MAbs to PCT cell lines MPC11 and BPC4. Cells were stained with indicated antibodies directly labeled with APC and analyzed by FACS. Dead cells were excluded by propidium iodide.

Flow cytometry

Single-cell suspensions prepared from bone marrows (BM) and spleens of B6 mice (8–24 weeks old) were prepared and stained with fluorochrome-labeled MAbs using standard procedures. All antibodies, except as indicated, were purchased from BD Biosciences (San Diego, CA). Cells were an-

alyzed using a FACSCalibur (BD Biosciences, Mountain View, CA), or a LSR II (BD Biosciences).

Transfection and expression of *Enpp1*

HEK 293 cells were transfected with an *Enpp1*^b-pcDNA3.1 vector or the pcDNA3.1 vector only (provided by Dr. Robert

FIG. 3. YE1/19.1 binding to germinal centers (GCs) and ENPP1-overexpressing cells (A). Splenic cells from PC-1^{-/-} (KO) and WT mice were stained with GL7-FITC, anti-FAS-PE, and YE1/19.1-APC. The cells were analyzed by flow cytometry. The top shows gating of GCs and non-GCs. The numbers are percentages of cells falling in each gate. The bottom shows overlays of ENPP1 expression in GCs (left) and non-GCs (right). (B) HEK 293 cells were transfected with an *Enpp1*^b expression vector and analyzed by flow cytometry.

Terkeltaub, University of California–San Diego) using Lipofectamine LTX reagents (Invitrogen, Carlsbad, CA). Stable transfectants were selected with neomycin and tested by FACS.

Results

Characterization of the anti-ENPP1 MAb

ENPP1 has two allotypic alleles, *a* and *b*, distinguished by two aa.⁽²⁾ Mouse strains including BALB/c, C3H, A/J, and NZB express allele *a*, while strains including C57BL/6, DBA/2, and 129 express allele *b*. The YE1/19.1 MAb was raised by immunization of rats with the EL4 T cell lymphoma-derived cell line (B6 origin) and precipitated a molecule of 230 kDa under native and 115 kDa under reducing conditions.⁽²⁰⁾ This MAb also precipitated a protein of 115 kDa from the MPC11 PCT cell line expressing allele *a* (Fig. 1A). We then used mass spectroscopy to determine the aa composition of peptides generated by digestion of the excised band and confirmed the identity of the protein as ENPP1 (Fig. 1B).

To compare the binding of YE1/19.1 with the well-characterized anti-ENPP1^a MAb IR518, we stained PCT cell lines of BALB/c origin (MPC11) and of B6 origin (BPC4) with the two anti-ENPP1 MAbs. As shown in Figure 2, YE1/19.1 bound to both cell lines, whereas IR518 bound only to MPC11, indicating that YE1/19.1 recognizes both *a* and *b* alleles. Preincubation of MPC11 cells with YE1/19.1 partially blocked subsequent binding to APC-labeled IR518 or YE1/19.1 (data not shown). Whether both antibodies could share an overlapping epitope (not the H650) requires further examination. In subsequent analyses using YE1/19.1, we found positive binding of the antibody to peritoneal B1a cells of all strains tested (H. Wang and H.C. Morse III, unpublished data), confirming that YE1/19.1 and IR518 react with at least partially different epitopes of the ENPP1 protein.

Analyses of splenic B cells from normal B6 mice showed that germinal center (GC) B cells, defined as GL7⁺FAS⁺, expressed ENPP1 at considerably higher levels than non-GC B cells (Fig. 3A). To further confirm the binding specificity of YE1/19.1, we proceeded to analyze the reactivity of YE1/19.1

FIG. 4. ENPP1 expression in immune cells. Splenic cells of B6 mice were stained with indicated antibodies and analyzed by flow cytometry. Subsets of immune cells were identified by lineage-specific markers as indicated.

with spleen cells of mice homozygous for a null allele of ENPP1. These mice were generated by insertion of a Neo gene into exon 9, resulting in disruption of transcription of the *Enpp1* gene.⁽⁹⁾ The results showed that reactivity of the YE1/19.1 MAb with both GC and non-GC B cells of the knockout mice was at background levels (Fig. 3A).

Finally, studies of human HEK 293 cells transfected with an *ENPP1^b* expression vector revealed high-level cell surface expression of ENPP1 detected by YE1/19.1 (Fig. 3B). Together, these analyses led to the conclusion that YE1/19.1 recognizes ENPP1^a and ENPP1^b and can be used for flow cytometric studies of ENPP1 expression in all strains of mice carrying either allele.

ENPP1 is differentially expressed in immune cells

To our knowledge, the expression patterns of ENPP1 in different subsets of hematopoietic cells have not been reported. This prompted us to determine the levels of ENPP1 expression on spleen cell subpopulations defined by use of a series of lineage-specific markers and sophisticated flow cytometric analyses. As shown in Figure 4, ENPP1 was expressed at similarly low levels on natural killer (NK) cells, dendritic cells (DC), monocytes/macrophages, and naïve T cells but at higher levels on neutrophils.

As ENPP1 was first described as a marker for plasma cells, we next focused our analysis on B lineage cells. Studies of BM pro-B, pre-B, and immature B cells, as defined by the Hardy nomenclature,⁽²⁴⁾ showed that ENPP1 was expressed at levels slightly higher than background (Fig. 5A). Levels of ENPP1 expression were slightly higher on more mature splenic B cell subsets including transitional, marginal zone (MZ), and follicular (FO) B cells. As noted above, GC B cells (GL7⁺CD95⁺)

expressed significantly higher levels of ENPP1 than non-GC cells (Fig. 5B). As expected from previous studies,⁽²⁰⁾ PCs (B220^{lo/-}CD138^{bri}) expressed high levels of ENPP1 (Fig. 5B).

Discussion

This study shows that the MAb YE1/19.1 recognizes both the *a* and *b* alleles of ENPP1 and can be used for flow cytometric analyses of ENPP1 expression in all strains of mice. While ENPP1 has been well studied in mineralization during bone formation and insulin resistance and diabetes, the correlates of its expression are poorly understood in the immune system, especially in the B cell lineage. This antibody could help to reveal functions of ENPP1 in different types of immune cells under physiological and pathological conditions.

In comparisons with a well-characterized anti-ENPP1^a MAb, IR518, we found that YE1/19.1 recognizes a different epitope of the ENPP1 protein. IR518 binds to a region that includes the aa H650, one of the two aa responsible for the difference between the *a* and *b* alleles. The capability of YE1/19.1 to react with both *a* and *b* allotypes indicates that YE1/19.1 binds to an epitope other than that defined by H650 and R679. Clearly, further work is required to map the binding site of YE1/19.1.

ENPP1 has several documented functions. While expression of ENPP1 in chondrocytes has been attributed to its function in generating PPi, a factor suppressing calcium precipitation,^(5,6) expression of ENPP1 in liver is thought to modulate insulin-mediated sugar metabolism.⁽²⁵⁾ Although ENPP1 is expressed at relatively low levels on a range of hematopoietic cells including NK cells, DCs, monocytes/macrophages, and naïve T and B cells, the function of ENPP1 on these cells remains to be determined. Previous work suggested

FIG. 5. ENPP1 expression in B lineage cells. (A) Bone marrow and splenic cells from B6 mice were stained with antibodies indicated and analyzed by flow cytometry. Each subpopulation of B cells is indicated. The closed line is isotype control background. (B) Splenic cells from immunized (KLH/alum) mice were stained with indicated antibodies and analyzed by flow cytometry.

that the expression of ENPP1 was upregulated in activated T cells and activated B cells⁽²⁶⁾ (H. Wang et al., unpublished data). The fact that ENPP1 is highly expressed by GC B cells and PCs suggests that it may play important roles in the development and function of late-stage B cells. Characterization of YE1/19.1 in this study allows us to further investigate the function of ENPP1 in B cells.

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Author Disclosure Statement

The authors have no financial conflicts to declare.

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