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Identification and phenotyping of circulating autoreactive proteinase 3-specific B cells in patients with PR3-ANCA associated vasculitis and healthy controls

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ABSTRACT

Objectives: To develop a method to detect and phenotype circulating proteinase 3 (PR3)-specific B-cells in patients with PR3-ANCA associated vasculitis (AAV).
Methods: Recombinant human PR3 (rPR3) was tagged with FITC or biotin, and its binding characteristics were studied by flow cytometry using three hybridoma cell lines secreting antibodies (Ab) against human PR3, mouse PR3 (no cross-reactivity with human PR3), and human neutrophil elastase. We measured the proportion of PR3-specific B-cells and studied their surface phenotype in patients with PR3-AAV and healthy controls (HC).
Results: Labeled rPR3 efficiently and specifically bound to hybridoma cells producing anti-human-PR3-Ab but not anti-mouse-PR3-Ab or anti-human-elastase-Ab. The proportion of rPR3-stained B cells was higher in patients with PR3-AAV compared to HCs: median (IQR) 1.11% (0.81–2.43) vs 0.45% (0.26–0.62) respectively, $p < 0.001$. There was a trend towards a higher proportion of PR3-specific B cells among patients with active disease compared to patients in remission: 2.91% (1.18–6.52) vs 0.99% (0.72–1.58), $p = 0.09$. In HCs, the proportion of PR3-specific B cells was highest among the transitional B-cell subset, and decreased with the maturation of B cells. Conversely, in patients, the proportion of PR3-specific B cells progressively increased with the maturation of B cells (median 1.90% of naïve B cells, 2.30% of unswitched memory B cells, 2.37% of switched memory B cells, and 3.68% of plasmablasts).
Conclusions: Circulating PR3-specific B cells can be detected in HC and patients with PR3-AAV. Their progressive enrichment during B-cell maturation suggests that they are actively selected and escape peripheral tolerance checkpoints in patients.

1. Introduction

Anti-neutrophil cytoplasm antibody (ANCA)-associated vasculitides (AAV) are a group of severe systemic autoimmune diseases, characterized histologically by necrotizing inflammation of small and medium blood vessels and sometimes granulomatous inflammation. Clinically patients present with a wide variety of manifestations (affecting mainly the respiratory tract and the kidneys, but potentially any other organ), and detectable autoantibodies, ANCA [1]. The two main autoantigens targeted by ANCAs are proteinase 3 (PR3) and myeloperoxidase (MPO) and these two ANCA specificities define distinct groups of patients [2].

While the pathophysiology of AAV is complex, ANCA are likely pathogenic autoantibodies [3]. B lymphocytes play a central role in many autoimmune diseases including AAV [4]. Conclusive evidence supporting the importance of B cells in the disease process was provided by the demonstration of the efficacy of rituximab to treat AAV [5–8]. However, rituximab depletes all CD20-expressing B cells and does not specifically target autoreactive B cells which are the precursor of ANCA-producing plasmablasts and plasma cells.

While PR3-ANCAs have been characterized extensively, little is known about the cells that produce them, namely autoantigen-

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specific plasma cells and their B cell precursors. PR3-ANCA may be produced by B cells actively selected in the granulomatous lesions in patients with granulomatosis with polyangiitis (GPA) [9,10]. A few studies suggested that peripheral blood contains PR3-specific and somatically mutated B cells in patients with active GPA [11], mainly in patients with high-titer serum PR3-ANCA [12]. PBMC from PR3-ANCA positive patients are able to produce IgG-ANCA *in vitro* upon specific stimulation [13–15]. However, these studies only used PR3-ANCA secretion as a surrogate for the presence of PR3-specific B cells, and nothing is known about the characteristics of these circulating autoreactive B cells.

Therefore, the objectives of this study were to develop a new flow-cytometry based method to identify circulating PR3-specific B cells based on the specificity of their B-cell receptor (BCR) targeted with tagged recombinant PR3, in patients with PR3-AAV and healthy controls (HCs), and to further describe their phenotype.

2. Methods

2.1. Patients and controls

Patients with PR3-AAV were prospectively included at Mayo Clinic at a time of a routine clinical visit. Inclusion criteria were diagnosis of GPA or Microscopic Polyangiitis (MPA) according to the Chapel-Hill definitions [1], positive PR3-ANCA, and B-cell reconstitution at the previous visit if the patients previously received rituximab. Data on demographics, diagnosis, disease activity evaluated by the Birmingham Vasculitis Activity Score for Wegener's Granulomatosis (BVAS/WG)[16], current therapies and clinical laboratory tests were collected. Serum PR3-ANCAs were quantified using a commercial bioplex assay, used as a routine test at the Mayo Clinic Immunology facility (BioPlex® 2200 Vasculitis, Bio-Rad, CA). Using this assay, serum PR3-ANCA levels are analyzed as negative (<0.4 U), equivocal (between 0.4 and 1.0 U) and positive (≥1 U). Maximal level of quantification is 8.0 U. Sera were also tested for PR3-ANCA by a previously described in-house capture ELISA using the same recombinant PR3 described below [17]. Apheresis cones (leucocyte concentrates) from HCs were obtained from the Mayo Clinic apheresis facility. All patients signed an informed consent and the study was authorized by the institutional review board (IRB 1566-04).

2.2. Recombinant proteinase 3 production and labelling

A recombinant PR3 (rPR3) was expressed in an epithelial cell line as previously described [18]. This variant consisted of the mature form of the protein (deletion of the N-terminal prodipeptide, allowing a mature conformational state), enzymatically inactive (S195A point mutated to avoid the protease activity which could digest different proteins including immunoglobulins), and produced by stable transfection of HEK-293 cells [19]. This recombinant PR3 is well recognized by PR3-ANCA from patients with GPA [17]. Culture supernatant was harvested after a 48 h starvation, and rPR3 was purified using a column loaded with the anti-human PR3 monoclonal antibody (Ab) MCPR3-2 [20] following recommendations from the supplier (CNBr-Activated Sepharose 4 Fast Flow, GE HealthCare), concentrated, and quantified by Coomassie Plus (Pierce, Rockford, IL). We also produced and purified a mouse rPR3 [21] for the inhibition experiments, following the same methods.

For a first set of experiments, we tagged the rPR3 by fluorescein isothiocyanate (FITC) using a commercial kit, following the manufacturer's instruction (EZ-labeled FITC protein labeling kit, PIERCE). In a second set of experiments, we biotinylated rPR3 using a commercial biotinylation kit (Lightning-Link Rapid Biotin Conjugation Kit, Innova Biosciences, Cambridge, UK). The advantage of biotinylation over direct FITC conjugation is the amplification of the signal on positive cells during the secondary staining step by a fluorescent streptavidine, resulting in higher fluorescence of positive cells and an easier development of a multi-color panel for flow cytometry (FACS) analysis.

2.3. Hybridoma cell culture and FACS analysis

To develop and validate an rPR3-based staining protocol by FACS on living cells, we used hybridoma cells which, in addition to secrete large amounts of monoclonal Ab, also express a variable quantity of their functional Ab on the membrane surface [22,23]. We used two anti-PR3 Ab-producing hybridoma cell lines, previously developed at our center: MCPR3-2 is an Ab recognizing human PR3 [20], whereas MCPR3-13 targets mouse PR3 [24]. There is no cross reactivity between the two species [21,25]. We also used a third hybridoma line specific for anti-human neutrophil elastase (HNE) [21], to assess the specificity of our PR3-based staining against a human protein belonging to the same family as human PR3. We confirmed that >70% of the cells of all hybridoma lines expressed a membrane IgG by FACS analysis using an anti-mouse IgG antibody (data not shown).

Hybridoma cells were cultured at 37 °C in RPMI 1640, 10% FBS, 1% Penicillin/Streptomycin solution, and 10% of the cells were plated in fresh media every 48 h. For FACS analysis, 10^6 hybridoma cells were washed, stained for 20 min on ice in 100 μL of HBSS containing 1% BSA and 0.2 g/L sodium azide using dose ranges of rPR3-FITC, then washed and suspended in 1% PFA-PBS before analysing on a FACS Canto X (BD Biosciences). For experiments using the rPR3-biotin, a supplemental step was included in the protocol by incubating the cells for 15 min on ice with streptavidin-FITC (BD Pharmingen) before the final wash. For competition experiments, MCPR3-2 hybridoma cells were first incubated with a range of unlabeled human or mouse PR3, washed, fixed during 10 min on ice in 1% PFA-PBS, washed, and incubated with rPR3-FITC before analysis.

2.4. PBMCs FACS analysis

Peripheral blood mononuclear cells (PBMCs) from patients and controls were isolated on Ficoll-Paque. The first set of experiments using PR3-FITC was performed on freshly isolated cells (cohort 1). The second set of experiments using PR3-biotin was performed on 10%-DMSO cryopreserved PBMCs (cohort 2). It is well accepted that cryopreservation respects the phenotype of leucocytes, including B cells [26]. We confirmed that the freezing-thawing process did not change the proportion of B cells recognizing PR3 on several samples (see Supplementary Material).

Cells were counted, and 2×10^6 cells were incubated on ice for 20 min with rPR3-FITC and an anti-CD19 antibody for the first set of experiments using samples from cohort 1, then washed, fixed and analyzed by FACS. For the second set of experiments (samples from cohort 2), cells were incubated with rPR3-biotin and a cocktail of different antibodies (anti-CD19-APC-Alexa Fluor 700, Anti-Human IgD-APC, anti-CD27-PC7, CD38-PC5.5 and anti-CD24-APC-Alexa Fluor 750, all from Beckman Coulter, Inc.), washed 3 times, incubated for 15 min with streptavidin-FITC, washed, and fixed. For each experiment, unstained cells as well as single color controls were included. FACS data were analyzed and graphed using KALUZA (Beckman Coulter, Inc., Indianapolis IN) and FlowJo (Ashland, Oregon) softwares.

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