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TRANSFUSION

Characterization of CD177-reactive iso- and auto-antibodies

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Abstract

Background: CD177 is a surface protein on neutrophils and a main mediator for the surface expression of proteinase 3 (PR3). Its functions are largely unknown. At least three types of antibodies have been described to target CD177: isoantibodies, which are formed in CD177-null individuals as a result of an immune reaction following transfusion or pregnancy; autoantibodies present in sera from patients with autoimmune neutropenia; and antineutrophil cytoplasmic antibodies in sera from patients with glomerulonephritis with polyangiitis. In this study, we aimed to compare the binding characteristics of auto- and iso-antibodies to optimize their detectability in the neutrophil serology laboratory.

Study Design and Methods: The reactivity of iso- and auto-antibodies against CD177 was studied using granulocytes, "native" CD177/PR3 complex, and recombinant CD177 or PR3.

Results: All iso- and auto-antibodies were reactive with CD177/PR3 when immobilized with monoclonal antibody (moab) 7D8. Seventy-five percent of autoantibodies, but none of the isoantibodies, did not react with CD177/PR3 immobilized with moab MEM166. The majority of autoantibodies did not react with recombinant CD177, whereas most isoantibodies tested positive.

Discussion: Our results suggest that iso- and auto-antibodies against CD177 target different epitopes. Isoantibodies mainly target CD177 alone, while the majority of autoantibodies target a native epitope present on the neutrophil surface, but absent from recombinant CD177 which lacks PR3. Moab MEM166 binds to the native epitope and hinders the binding of CD177 autoantibodies. The results may help to design diagnostic strategies, especially for the identification of autoantibodies.

KEYWORDS

autoimmune neutropenia, CD177, HNA-2, neonatal alloimmune neutropenia (NAIN), proteinase 3

1 | INTRODUCTION

CD177 is a glycosyl-phosphatidly-inositol–anchored glycoprotein that is exclusively expressed on neutrophils. It

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belongs to the Ly6 family of proteins and is also known as NB1 or human neutrophil antigen-2 (HNA-2).[1,2] CD177 is absent from the neutrophil surface in 3%–5% of healthy individuals, known as CD177-null. In CD177-positive individuals, the expression of CD177 follows a bimodal pattern. It is absent from the surface of some neutrophil subsets.[1]

The function of CD177 remains largely unknown. Multiple interactions between CD177 and other proteins such as proteinase 3 (PR3) and CD11b/CD18 on the neutrophil surface have been described.[3,4] CD177 is known as a ligand for PECAM-1 heterophilic interactions on endothelial cells.[5] Previous studies addressed the CD177 interaction with PECAM-1 as a mediator for neutrophil transmigration through endothelial cells; CD177 binding to PECAM-1 initiates signaling pathways that lead to VE-cadherin degradation from endothelial junctions and endothelial barrier destabilization, which assist the neutrophil's migration through the endothelial layer.[6]

PR3 is a key neutrophil serine protease and found in azurophil and specific granules from where it is released upon neutrophil activation.[7–9] Interestingly, the membrane expression of PR3 on the neutrophils' surface is mainly mediated by CD177.[10] A hydrophobic patch on PR3 was shown to mediate the binding of PR3 to CD177.[11] There is evidence that CD177/PR3 forms a larger protein complex with CD11b/CD18 in membrane lipid rafts.[12, 4]

Three different disorders can result from immunization against PR3 and CD177. First, glomerulonephritis with polyangiitis (GPA; previously known as Wegener's disease). The major antigen in GPA is PR3, the target for antineutrophil cytoplasmic antibodies. Second, autoimmune neutropenia (AIN). Although most autoantibodies that cause AIN recognize CD16b,[13] HNA-2 autoantibodies were reported in some patients.[1] Third, neonatal alloimmune neutropenia (NAIN). In CD177-null individuals, contact with CD177-positive cells during pregnancy or transfusion induces immunization against CD177 and, consequently, the production of HNA-2 isoantibodies. These antibodies are transferred to the fetus during pregnancy and lead to immune neutropenia of the newborn.

If and to which extent these different antibodies bind to CD177/PR3 and which serological approach is appropriate for their detection have not been investigated so far. Here, we characterize the reaction of iso- and autoantibodies against CD177 by different approaches.

2 | MATERIALS AND METHODS

2.1 | Sera cohort

HNA-2 isoantibodies (n = 10) were obtained from mothers of children with alloimmune neutropenia. HNA-2 autoantibodies (n = 12) were obtained from patients with secondary AIN diagnosed with autoimmune lymphoproliferative syndrome or B-cell chronic lymphatic leukemia. Sera from healthy blood donors (n = 12) were used as controls.

2.2 | Monoclonal antibodies

Monoclonal antibody (moab) 7D8 was produced in our laboratory. Hybridoma clone 7D8 producing a moab against CD177 was a generous gift from Dr. D. Stroncek (National Institutes of Health, Bethesda, MD, United States). Hybridoma cells were cultured in RPMI (Capricorn Scientific, Ebsdorfergrund, Germany) containing 10% fetal calf serum (FCS; PAN-Biotech, Germany) and 0.5% penicillin/streptomycin (Invitrogen, Karlsruhe, Germany). Moab was purified from the collected cell supernatant by the use of a protein G column. Moab MEM166 against a different epitope residing on CD177 was purchased from BioLegend (San Diego, CA, United States).

2.3 | Human neutrophils

Neutrophils were isolated from ethylenediaminetetraacetate (EDTA)-anticoagulated whole blood from healthy donors by dextran sedimentation and Ficoll gradient centrifugation. Expression of CD177 was studied by flow cytometry. Aliquots of 3×10^5 neutrophils were incubated with 7D8 or mIgG (as isotype control; Dako, Hamburg, Germany). Cells were washed, and bound antibodies were detected with Fluorescein isothiocyanate (FITC)-labeled anti-mouse (Dako) using a FACS Canto (BD Bioscience, Heidelberg, Germany).

2.4 | Granulocyte immunofluorescence test (GIFT)

GIFT was performed as described before.[14] Briefly, in 96-well plates (Greiner Bio One, Kremsmunster, Austria), 2×10^5 Paraformaldehyde (PFA)-fixed granulocytes were incubated with 40 µl of serum (1:2 diluted) for 30 min at 37°C. Cells were washed (3x) with phosphate-buffered saline (PBS; without Ca^{2+}) and thereafter incubated with FITC-anti-human (40 µl, 1:40 diluted; Dako, Los Altos, Canada) for 30 min at room temperature in the dark. Next, the cells were washed (3x) with PBS (without Ca^{2+}), and one drop of PBS/glycerin was added to each well, mixed, and dropped on the slide. The fluorescence intensity was evaluated by fluorescence microscopy and scored from 1 (negative) to 4 (strongly positive).

2.5 | Monoclonal antibody immobilization of granulocyte antigens assay (MAIGA)

The MAIGA was performed as previously described.[13] Briefly, in 96-well plates, 10^4 fixed granulocytes from an individual expressing CD177 were incubated with 50 µl of undiluted human serum for 30 min at 37°C. After washing steps, granulocytes were incubated with 10 µl supernatant of moab 7D8 (anti-CD177; ~ 20 µg/ml) for 30 min at 37°C. Following this, cells were washed and solubilized with 100 µl of lysis buffer (1% Triton X100, 5 mmol/L EDTA, 2 mmol/L phenylmethylsulfonyl fluoride, 150 mmol/L NaCl in 20 mmol/L Tris buffer, pH 7.4) for 30 min at 4°C. After lysis, samples were centrifuged at 15,000 g for 30 min. Then, the supernatant was collected (70 µl) and diluted with washing buffer (180 µl). The diluted supernatant of 100 µl was transferred to a microtiter well coated with goat anti-mouse (Dianova, Hamburg, Germany) in duplicate and incubated overnight. Next day, wells were washed and subsequently goat anti-human conjugated with peroxidase (Jackson Research, West Grove, PA, United States) was added. The reactions were then visualized by addition of a substrate containing ortho-phenylenediamine (o-phenylenediamine; Dako, Hamburg, Germany). After 15 min, the reaction was stopped with 2.5 M H_2SO_4, and optical density was measured at 492 nm in an enzyme-linked immunosorbent assay (ELISA) plate reader (Tecan, Crailsheim, Germany).

2.6 | CD177-ELISA for detection of human anti-CD177

Soluble rCD177 was synthesized as described before.[15] Full-length CD177 cDNA was cloned in the expression vector pIB/V5-HisTOPO containing blasticidin resistance gene by T/A cloning strategy (Invitrogen) and afterward transformed into TOP10 competent *Escherichia coli* (Invitrogen). The clones were screened, and positive clones were purified using QIAprep (Qiagen) and were validated by sequencing analysis.

High Five insect cells were then transfected with CD177 plasmid. After 3 days, cells were cultured in a culture medium containing blasticidin. Using 7D8 affinity column, the CD177 were purified from the medium supernatant. Purified protein was dialyzed against PBS, and the concentration was estimated in bicinchoninic acid assay (Thermo Scientific, Bonn, Germany). For CD177 ELISA, a 96-well plate (Greiner Bio One, Kremsmunster, Austria) was coated overnight with 100 µl/well of moab 7D8 (4 µg/ml). Without washing, the plate was blocked for 1 h with 100 µl/well of 3% bovine serum albumin (BSA; Serva Electrophoresis GmbH, Heidelberg, Germany) in PBS (BSA/PBS). After washing with 150 µl/well of 0.2% BSA/PBS with 0.05% Tween 20, wells were incubated with 100 µl recombinant human CD177 protein (2.5 µg/ml) in washing buffer. After incubation for 1 h at room temperature, 100 µl/well of 1:50 diluted human sera in washing buffer was added to the plate. Wells were washed four times and subsequently incubated with 100 µl horseradish peroxidase (HRP)-labeled goat anti-human (diluted 1:4000 in washing buffer; Jackson Research, West Grove, PA, United States) for 30 min at room temperature. After four washing steps, 200 µl of o-phenylenediamine in substrate buffer was added and incubated for 30 min in the dark. The reaction was stopped with 50 µl of 2.5 M H_2SO_4, and optical density of each well was measured at 492 nm in an ELISA plate reader (Tecan, Crailsheim, Germany).

2.7 | PR3-ELISA for detection of human anti-PR3 in serum

Anti-PR3 was identified using a commercial solid-phase ELISA (anti-PR3 hn-hr ELISA; Euroimmun AG, Lübeck, Germany). In brief, microtiter stripes coated with native and recombinant PR3 protein were incubated with 1:100 diluted sera samples (or calibrator samples) for 30 min at room temperature. After incubation, the wells were washed and 100 µl of peroxidase-labeled anti-human was added and incubated for 30 min at room temperature. The wells were washed again and filled with 100 µl of chromogen/substrate solution and incubated in the dark for 15 min at room temperature. Reactions were stopped with 100 µl/well stop solution, and optical density was measured in an ELISA plate reader (Tecan, Crailsheim, Germany) at 450/620 nm.

3 | RESULTS

3.1 | Analysis of HNA-2 iso- and auto-antibodies in GIFT and MAIGA

All sera were initially tested in the GIFT with CD177-positive and CD177-negative neutrophils, where they gave the characteristic pattern of anti-CD177 (data not shown). No reactivity was detected with CD177-negative cells but all sera reacted positively with CD177-positive cells. They resulted in a "mixed pattern" when the CD177-positive cell suspension contained 40%–60% positive and 60%–40% negative neutrophil subpopulations. Next, all sera were analyzed in MAIGA using two moabs, 7D8 and MEM166, against CD177. The cut-off for each moab was established using sera from 12 healthy donors in three independent experiments. In the MAIGA with 7D8 as capture

antibody, all samples from both cohorts were positive (Figure 1(A)). Isoantibodies, in general, gave stronger signals than autoantibodies. However, samples 1, 2, 10, and 11 showed borderline reactivity. When MEM166 was used to capture CD177 (Figure 1(B)), HNA-2 isoantibodies showed stronger reactions (70% positive). However, only three out of 12 autoantibodies (25%) were positive. We conclude from these data that moab MEM166, but not moab 7D8, can block epitopes on CD177. These data show that auto- and iso-antibodies against HNA-2 differ in the spectrum of recognized epitopes.

3.2 | Analysis of HNA-2 iso- and auto-antibodies in CD177-ELISA

The cut-off was established using sera from 12 healthy donors tested in three independent experiments. Out of 10 HNA-2 isoantibodies, eight gave positive results with rCD177 (80%). Two sera (samples 5 and 6) did not react with rCD177 (Figure 2(A)). In the cohort of HNA-2 autoantibodies, two sera (samples 3 and 7) gave positive reactions with recombinant CD177; of which, sample 3 gave a strong reaction. However, the majority (83.3%) did not react with rCD177.

These results indicate that for the majority of HNA-2 autoantibodies, rCD177 ELISA, failed to provide a suitable target antigen. These data confirm that iso- and auto-antibodies recognize different epitopes on CD177 (Figure 3).

3.3 | Analysis of HNA-2 iso- and auto-antibodies in PR3-ELISA

All sera were tested for the presence of anti-PR3. No reactivity with PR3 was detected in both cohorts.

4 | DISCUSSION

In this paper, we present evidence that HNA-2 isoantibodies and HNA-2 autoantibodies bind to different epitopes on CD177.

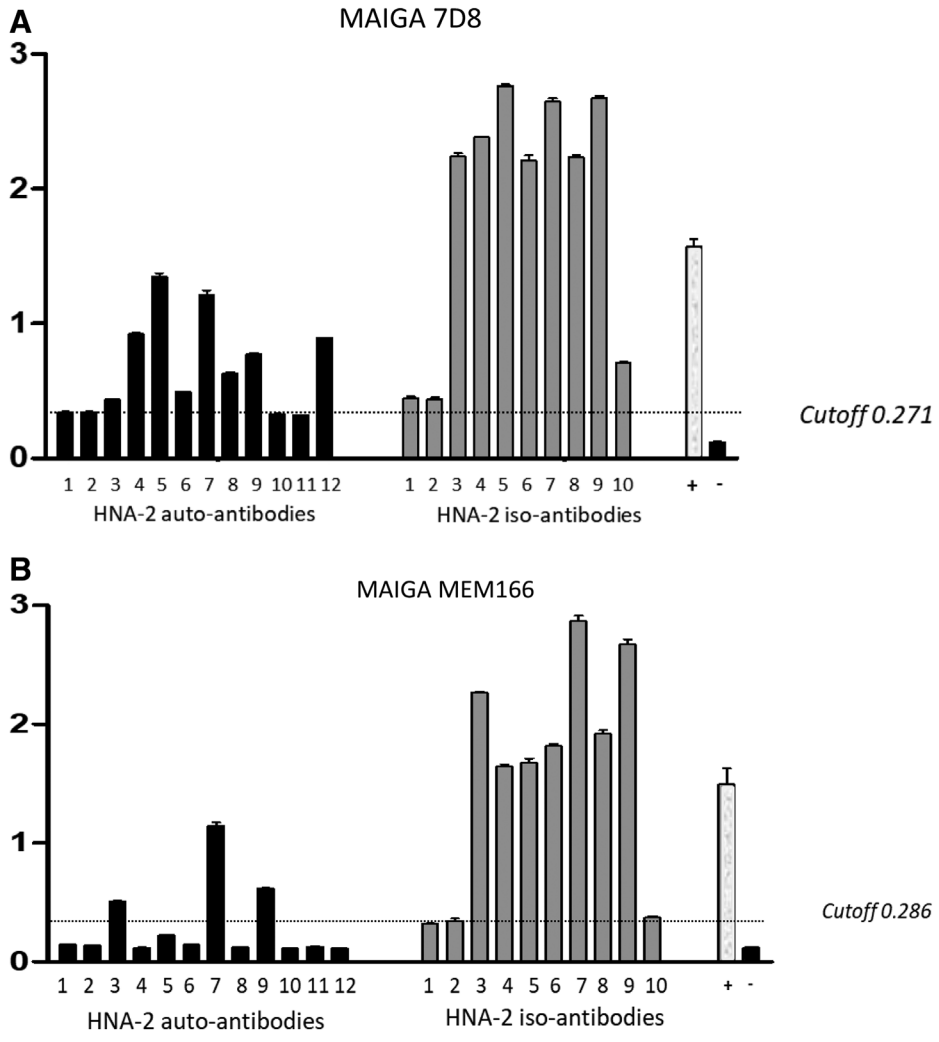

FIGURE 1 Detection of CD177 antibodies in MAIGA. The samples of two cohorts of CD177 auto- (left) and iso-antibodies (right) were tested in MAIGA using 7D8 (A) or MEM166 (B). Serum from a healthy male donor and serum from an NAIN case containing CD177 isoantibodies were used as controls. Cut-off for each moab was determined using sera from healthy donors (n = 12), calculated as mean + 3SD. Shown are representative diagrams from three independent experiments

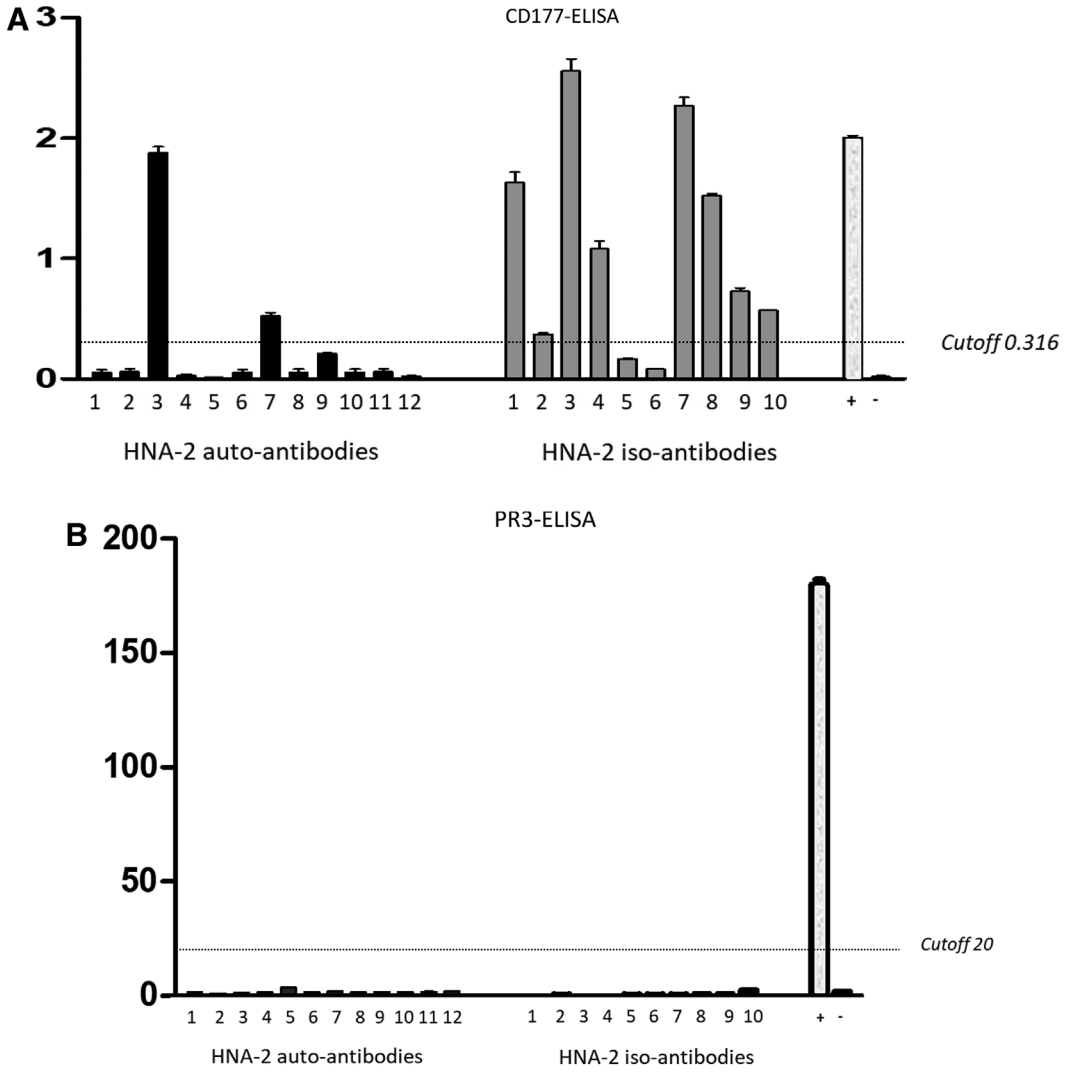

FIGURE 2 Analysis of CD177 antibodies in ELISA. The samples of two cohorts were further characterized using CD177 (A) and PR3 (B) ELISA. For CD177-ELISA, rCD177 was immobilized on 7D8 precoated microtiter plates. Serum was added, and bound CD177 auto- or iso-antibodies in human serum were detected with HRP-labeled goat anti-human. Serum from a healthy male donor and serum from an NAIN case containing CD177 isoantibodies were used as controls. In PR3-ELISA antibodies in serum were bound to immobilize PR3 and detected with HRP-labeled goat anti-human. Shown are representative diagrams from three independent experiments

Most antibodies present in sera from patients with AIN had the following reaction characteristics: they did not react with recombinant PR3 and with recombinant CD177, but they reacted with the native CD177/PR3 complex from neutrophils. Apparently, most autoantibodies bind to epitopes present on the CD177/PR3 complex only. In contrast, most antibodies present in sera from mothers who gave birth to newborns with NAIN had the following reaction characteristics: they did not react with recombinant PR3, but they reacted with both recombinant CD177 and the native CD177/PR3 complex. Apparently, most isoantibodies bind to epitopes present on CD177 as such, that is, they are not dependent on the presence of PR33.

One could argue that the lack of epitopes for autoantibodies on recombinant CD177 is related to structural or post-translational differences between the native and the recombinant protein rather than the absence or presence of PR3. PR3 is selectively expressed on CD177-positive neutrophils,[3,4,16] and the CD177/PR3 complex mediates PECAM-1-dependent transendothelial migration.[5,16,17] The interaction site between PR3 and CD177 has been partially unraveled.[11, 12] In functional studies, neutrophil pretreatment with moab MEM166 (but not moab 7D8) interfered with neutrophil migration,[5,17] indicating that moab MEM166 binds to an epitope relevant to CD177/PR3 complex interaction with PECAM-1. CD177 as such, in the absence of PR3, does not interact with PECAM-1.[12] Interestingly, when moab MEM166 was used to investigate the binding of auto- and iso-antibodies to the native CD177/PR3 complex, it exclusively blocked the binding of autoantibodies, but not isoantibodies. This finding supports our assumption

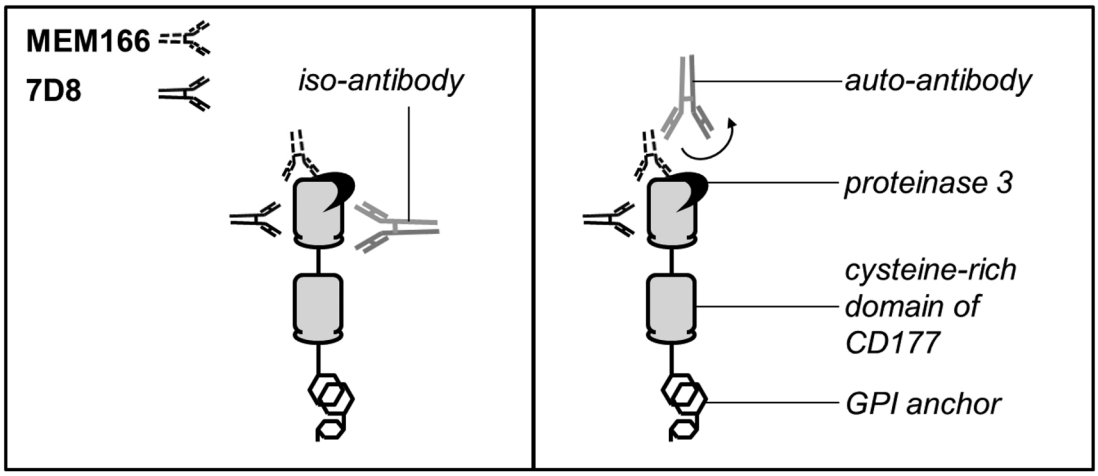

FIGURE 3 The CD177 protein is a glycosyl-phosphatidly-inositol (GPI) anchor protein consists of two cysteine-rich domains. Proteinase 3 (PR3) binds to the membrane distal domain of CD177. Two monoclonal antibodies were studied, MEM166 (dashed lines) and 7D8 (solid line). The majority of HNA-2 isoantibodies recognize epitopes on CD177, which is independent from PR3 and/or binding sites for MEM166 and/or 7D8 (left panel). In contrast, the majority of HNA-2 autoantibodies recognize PR3-dependent epitopes on CD177 and/or involve binding sites for MEM166. In the presence of MEM166, most HNA-2 autoantibodies do not bind to CD177. In contrast, autoantibody binding is not hindered in the presence of 7D8 (right panel)

that epitopes of most autoantibodies (>80%) are specific to the CD177/PR3 complex. Autoepitopes are usually absent from recombinant CD177. Autoepitopes are blocked on native CD177/PR3 in the presence of moab MEM166 (but not in the presence of moab 7D8) because moab MEM166 binds to functionally relevant domains formed by CD177 and PR3. Therefore, we recommend to use moab 7D8 for the characterization of autoantibodies by MAIGA, rather than MEM166.

This finding also has implications for routine testing in the neutrophil immunology laboratory. MAIGA represents the gold standard for the characterization of neutrophil antibodies. In a first step, following the incubation of neutrophils with a specific moab and the serum, a trimolecular complex is formed (consisting of the human anti-HNA-2, the native target protein, and the CD177 moab), which is then immobilized to a microtiter plate. Two moabs against CD177 are widely used, MEM166 and 7D8. Our data show that CD177 immobilized with moab 7D8 provides a suitable target antigen for both HNA-2 isoantibodies and HNA-2 autoantibodies. As far as HNA-2 isoantibodies are considered, moab MEM166 can be well used instead of moab 7D8 because a comparison of optical densities obtained with the two different moabs for 10 HNA-2 isoantibodies did not show any difference ($p = .315$, Mann–Whitney U test). In contrast, moab MEM166 and HNA-2 autoantibodies compete for the same epitope(s), leading to false-negative test results in the majority of auto-antisera.

Our data also demonstrate that recombinant CD177 lacking PR3 is unsuitable for the detection of autoantibodies for test formats other than MAIGA, such as ELISA or comparable applications.

We do not have functional data to elucidate potential differences between HNA-2 autoantibody and HNA-2 isoantibody binding to CD177 on neutrophils in vivo. Moab MEM166 inhibits neutrophil migration,[5, 18] and it is intriguing to speculate that autoantibodies might have similar effects. Functional impairment of circulating neutrophils might be an attractive way to explain why sometimes in patients with AIN, infection severity and severity of neutropenia are not associated.

In conclusion, we have demonstrated that HNA-2 isoantibodies and HNA-2 autoantibodies bind to distinguishable epitopes. We recommend to use moab 7D8 for the characterization of autoantibodies by MAIGA, rather than moab MEM 166. Other moabs against CD177 should be studied carefully before they are used for diagnostic purposes. Further studies are also required to determine whether HNA-2 isoantibody-mediated and HNA-2 autoantibody-mediated neutropenias also differ functionally.

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AUTHORS CONTRIBUTIONS

B.B. and U.J.S. designed the study, analyzed the data, and wrote the manuscript. C.H., S.S., S.H., and A.T. performed the test. G.B. contributed to the manuscript.

CONFLICT OF INTEREST

Authors have no conflict of interest to declare.

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