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Molecular and Functional Characterization of Fcγ Receptor IIIb-Ligand Interaction: Implications for Neutrophil-Mediated Immune Mechanisms in Malaria

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ABSTRACT The Fcγ receptor IIIb (FcγRIIIb) is a low-affinity receptor of IgG and is essential in neutrophil-mediated effector functions. Different allelic forms of FcγRIIIb carrying human neutrophil antigen (HNA-1a, -1b, -1c, and -1d) have been identified. Here, we have generated stable transfected HEK293 cell lines expressing HNA-1aa, -1bb, and -1bc. Of these, cells expressing HNA-1bc interacted significantly stronger (binding affinities, 2.277 versus 0.743) with human IgG than cells expressing the HNA-1aa or -1bb alloforms. The higher affinity of IgG toward the HNA-1c alloform was confirmed using neutrophils derived from German blood donors. Neutrophils from HNA-1abc-phenotyped individuals bound IgG significantly stronger (1.825 versus 0.903) than did neutrophils from HNA-1ab-typed individuals. These findings were confirmed by surface plasmon resonance (SPR) analysis demonstrating that recombinant HNA-1bc had a higher affinity (dissociation constant [K_d], 7.24×10^{-6} M) than recombinant HNA-1bb (K_d, 1.15×10^{-5} M) against normal IgG. Finally, we demonstrated that *Plasmodium falciparum* merozoites opsonized with human IgG affinity purified against *P. falciparum* glutamate-rich protein (GLURP) enhanced stronger reactive oxygen species (ROS) emission in neutrophils obtained from HNA-1abc donors than in neutrophils from HNA-1ab donors. Collectively, these results indicate that the amino acid substitution $Ala_{78}Asp$ resulting in the HNA-1c allotype leads to higher affinity toward human IgG, enhancement of neutrophil activation, and possibly effective clearance of malaria by intracellular ROS.

KEYWORDS human neutrophil antigen-1, alloantibodies, IgG binding, malaria

The Fcγ receptor IIIb (FcγRIIIb) is a low-affinity receptor for the Fc region of multimeric IgG. It is exclusively expressed on neutrophils and is essential in many effector functions of the immune system, including phagocytosis, antibody-dependent cellular cytotoxicity, and release of inflammatory mediators (1).

Currently, five different FcγRIIIb alleles of the gene encoding human neutrophil antigen 1a (HNA-1a), HNA-1b, -1c, and -1d are found (2). Molecular analysis demonstrated that HNA-1a differs from HNA-1b in five nucleotides (positions 141, 147, 227, 277, and 349) resulting in four amino acid changes (positions 36, 65, 82, and 106) in the

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membrane-distal domain of FcγRIIIb. The point mutations at positions 65 and 82 lead to two additional N-linked glycosylation sites, resulting in a higher apparent molecular mass of HNA-1b than of HNA-1a (3). A recent study showed that the HNA-1b alloform carries two alloantigenic determinants; HNA-1c and HNA-1d (4). Whereas an amino acid change at position 78 (Ala78Asp) controls the formation of HNA-1c epitopes (5), the HNA-1d antigenic determinants depend on two amino acid residues at positions 78 (Ala) and 82 (Asp) (4). Alloantibodies (aabs) against HNA-1 are responsible for the development of neonatal alloimmune neutropenia (NAIN) and transfusion-related acute lung injury (6).

Several studies have indicated that amino acid changes resulting in differential glycosylation of FcγRIIIb not only are responsible for the formation of HNA-1a and -1b antigenic determinants but also may control the affinity of the receptor and consequently functionality (7, 8). However, the role of HNA-1c, expressed on the same protein as the HNA-1b epitope, is unknown. Population studies showed that 23 to 31% of Africans, 5% of Caucasians, and $<$1% of Asians express the HNA-1c alloform of FcγRIIIb on their neutrophils (6).

In infectious diseases such as malaria, FcγRIIIb-dependent neutrophil-mediated effector mechanisms, including phagocytosis and respiratory burst, are among the first line of defense against the invading parasite (9–11). So far, only few studies have investigated the possible implications of FcγRIIIb allotypes on the risk of malaria in areas of endemicity. Recently, we found that the frequency of the HNA-1c allotype (*FCGR3B-c.233A*) was significantly lower (0.067 versus 0.201) in Ghanaian children with febrile malaria than in healthy controls (12). Furthermore, the absolute risk of clinical malaria decreased with increasing amounts of antibodies against the *Plasmodium falciparum* glutamate-rich protein (GLURP) in HNA-1c-positive but not in HNA-1c-negative Ghanaian children (13). We proposed that HNA-1c-negative FcγRIIIb might be less efficient in engaging IgG antibodies to mediate parasite multiplication control mechanisms; however, the binding capacities of most FcγRIIIb alloforms are yet to be properly characterized.

In this study, we introduce the use of stable transfectants expressing HNA-1aa, -1bb, and -1bc to characterize the binding properties of FcγRIIIb alloforms toward IgG and substantiate their relevance for immune protection against clinical malaria.

RESULTS

Characterization of stable transfected HEK293 cells expressing FcγRIIIb alloforms (HNA-1aa, -1bb, and -1bc). The expression of HNA-1aa, -1bb, and-1bc FcγRIIIb allotypes on the surface of transfected HEK293 cell lines was investigated by flow cytometry-based methods. All cell lines stained equally well with a monoclonal antibody (MAb), LNK16, against FcγRIIIb, demonstrating that they expressed comparable amounts of FcγRIIIb on their cell surfaces (Fig. 1A). To confirm the allospecificity of the transfected cell lines, a monoclonal antibody-immobilized granulocyte antigen assay (MAIGA) was performed with well-defined anti-HNA-1a, -1b, and -1c aabs using MAb LNK16 as a capture antibody. All aabs reacted only with cell lines expressing the homologous receptor (Fig. 1B). Anti-HNA-1a antibodies reacted with HNA-1a-expressing cells but not with the other cell lines. Vice versa, anti-HNA-1b aabs reacted with HNA-1b (HNA-1bb and HNA-1bc)-expressing cells, and anti-HNA-1c aabs recognized HNA-1bc cells but not HNA-1bb cells. Taken together, these results demonstrated that the transfected HEK293 cell lines expressed similar amounts of HNA-1aa, -1bb, and -1bc FcγRIIIb allotypes on their respective cell surfaces. Of note, anti-HNA-1b aabs reacted stronger with HNA-1bc- than with HNA-1bb-transfected cells in the MAIGA, suggesting that the Ala78Asp point mutation enhances anti-HNA-1b aabs binding to HNA-1c (+) cells.

Binding of HNA-1-transfected HEK293 cells and neutrophils to immobilized IgG. The binding affinity of the different FcγRIIIb alloforms toward IgG was determined in an adhesion assay. Cell lines expressing HNA-1aa and HNA-1bb bound immobilized IgG with binding affinities of 1.370 $\pm$ 0.157 and 0.743 $\pm$ 0.094, respectively (Fig. 2A). In

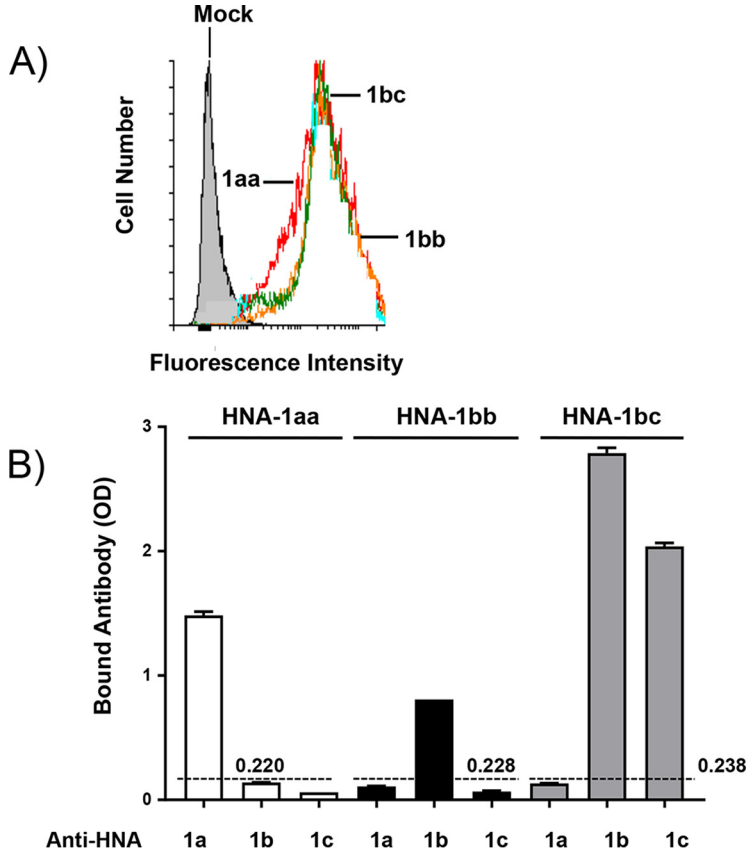

FIG 1 Analysis of allelic forms of FcγRIIIb expressed on the surface of HEK293 transfected cells by flow cytometry and antigen capture assay (MAIGA). (A) Transfected HEK293 cells expressing HNA-1aa, -1bb, and -1bc were incubated with MAb LNK16 against FcγRIIIb as indicated. Mouse IgG was run as an isotype control. After washings, cells were labeled with fluorescence-conjugated donkey anti-mouse IgG and analyzed by flow cytometry. (B) Transfected cells expressing HNA-1aa, -1bb, and -1bc were incubated with anti-HNA-1a, -1b, and -1c aabs together with MAb LNK16. After lysis, a trimolecular complex consisting of the target protein, the capture MAb, and the alloantibody was immobilized onto a microtiter plate. Human antibodies bound to immobilized FcγRIIIb alloform were detected with enzyme-labeled goat anti mouse-IgG and read on an ELISA reader (optical density [OD] at 492/620 nm). Dashed lines are cutoff values for a positive result calculated as twice the OD values obtained with the normal control serum. Data are presented as means ± standard deviations (SD) for triplicates.

contrast, the binding affinity of HNA-1bc-expressing cells was stronger (2.277 ± 0.025), and this difference in binding affinity was significant ($P < 0.01$ in the statistical test), suggesting that HNA-1c has the highest affinity toward IgG. These interactions were abolished by inhibitory MAb 3G8 but not by noninhibitory MAb DJ130c, demonstrating the specificity of the interaction between IgG (Fc-part) and FcγRIIIb in this adhesion assay.

To investigate whether the natural HNA-1c allotype has a similarly high affinity toward IgG, neutrophils derived from German blood donors were investigated in the binding assay. A cohort of blood donors ($n = 440$) were typed for HNA-1 system by a PCR with sequence-specific primer (PCR-SSP) method. Selected HNA-1a-, -1b-, and -1c-positive individuals were phenotyped using well-characterized anti-HNA-1a, -1b, and -1c antibody-containing serum by the standard MAIGA (data not shown). Adhesion experiments were performed with neutrophils derived from HNA-1c-positive (HNA-1abc, -1ac, and -1bc) donors and with neutrophils from HNA-1c-negative (HNA-1ab, -1bb) donors for comparison (Fig. 2B). To control for the impact of variable FcγRIIIb expression levels, data were adjusted for the density of FcγRIIIb as determined by flow cytometry. Neutrophils from HNA-1abc-phenotyped donors bound IgG significantly stronger than did neutrophils from HNA-1ab donors (1.825 ± 0.146 versus 0.903 ±

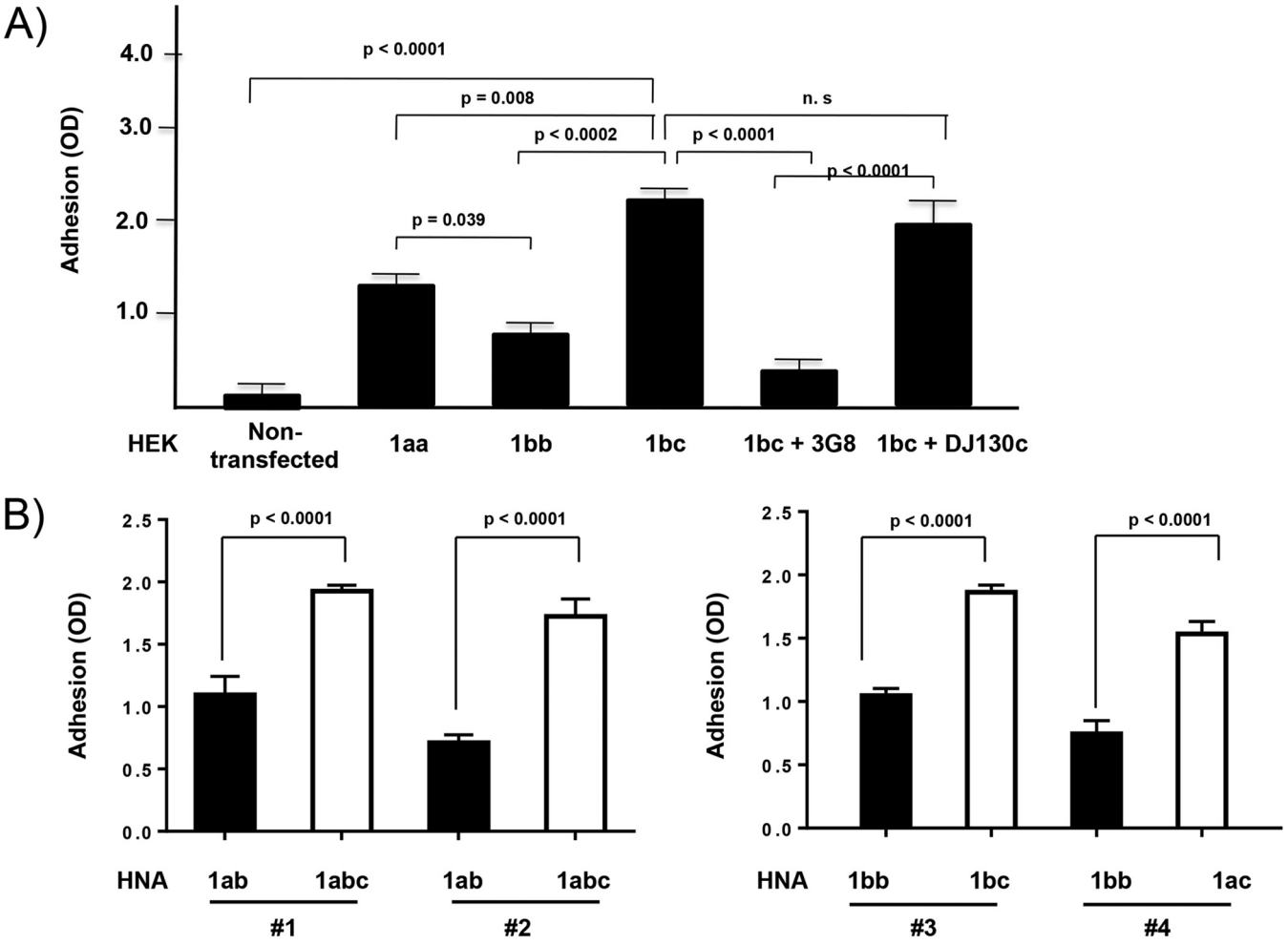

FIG 2 Adhesion of transfected HEK293 cells and neutrophils to immobilized IgG. (A) HEK cells. Adhesion of transfected HEK293 cells expressing HNA-1aa, -1bb, and -1bc antigens to IgGs. Transfected cells (1×10^6 to 4×10^6 cells) were allowed to adhere onto microtiter wells coated either with 5 μg IgG or with bovine serum albumin (BSA) for 1 h at 37°C in the presence of 5% CO_2. In some experiments, 3G8 or DJ130c MAb was added for 30 min at 37°C. After washings, bound cells were stained with crystal violet and their numbers were determined by a microplate reader. Data are normalized with BSA and adjusted to FcγRIIIb expression. Data are presented as means ± SD from five independent experiments. (B) Granulocytes. HNA-phenotyped granulocytes expressing HNA-1ab ($n = 3$), -1ac ($n = 1$), -1bc ($n = 1$), and -1abc ($n = 3$) antigens to IgGs. Neutrophils (1×10^6 to 4×10^6 cells) were allowed to adhere onto microtiter wells coated either with 5 μg IgG or with bovine serum albumin (BSA) for 1 h at 37°C in the presence of 5% CO_2. After washings, bound cells were stained with crystal violet and their numbers were determined in an ELISA reader. Data are normalized with BSA and adjusted to FcγRIIIb expression. Data are presented as means ± SD.

0.233; $P < 0.0001$) (Fig. 2B, left panel). In contrast, neutrophils from HNA-1bc and HNA-1ac donors bound IgG equally well with binding affinities of 1.881 ± 0.038 and 1.554 ± 0.079, respectively ($P < 0.0001$) (Fig. 2B, right panel), suggesting that HNA-1c (+) neutrophils have a stronger affinity toward IgG than do HNA-1c (−) neutrophils.

Binding of allelic recombinant HNA-1 proteins to IgG. The affinity of FcγRIIIb alloforms toward IgG was further investigated using soluble HNA-1aa, -1bb, and -1bc produced on the recombinant form. First, we demonstrated that purified recombinant HNA-1aa and -1bb bound immobilized IgG by enzyme-linked immunosorbent assay (ELISA) similarly, with affinities of 0.118 ± 0.033 and 0.153 ± 0.034, respectively. In contrast, recombinant HNA-1bc bound significantly ($P = 0.010$) stronger (2.125 ± 0.669) to IgG (Fig. 3A). Second, the affinity of the different FcγRIIIb alloforms toward IgG was determined by surface plasmon resonance (SPR) analysis (Fig. 3B). Increasing concentrations of purified IgG was injected on sensor chips with immobilized recombinant HNA-1bb or HNA-1bc. As described above, the FcγRIIIb HNA-1bc alloform interacted stronger with IgG than did the HNA-1bb alloform (K_d, 7.24×10^{-6} M versus

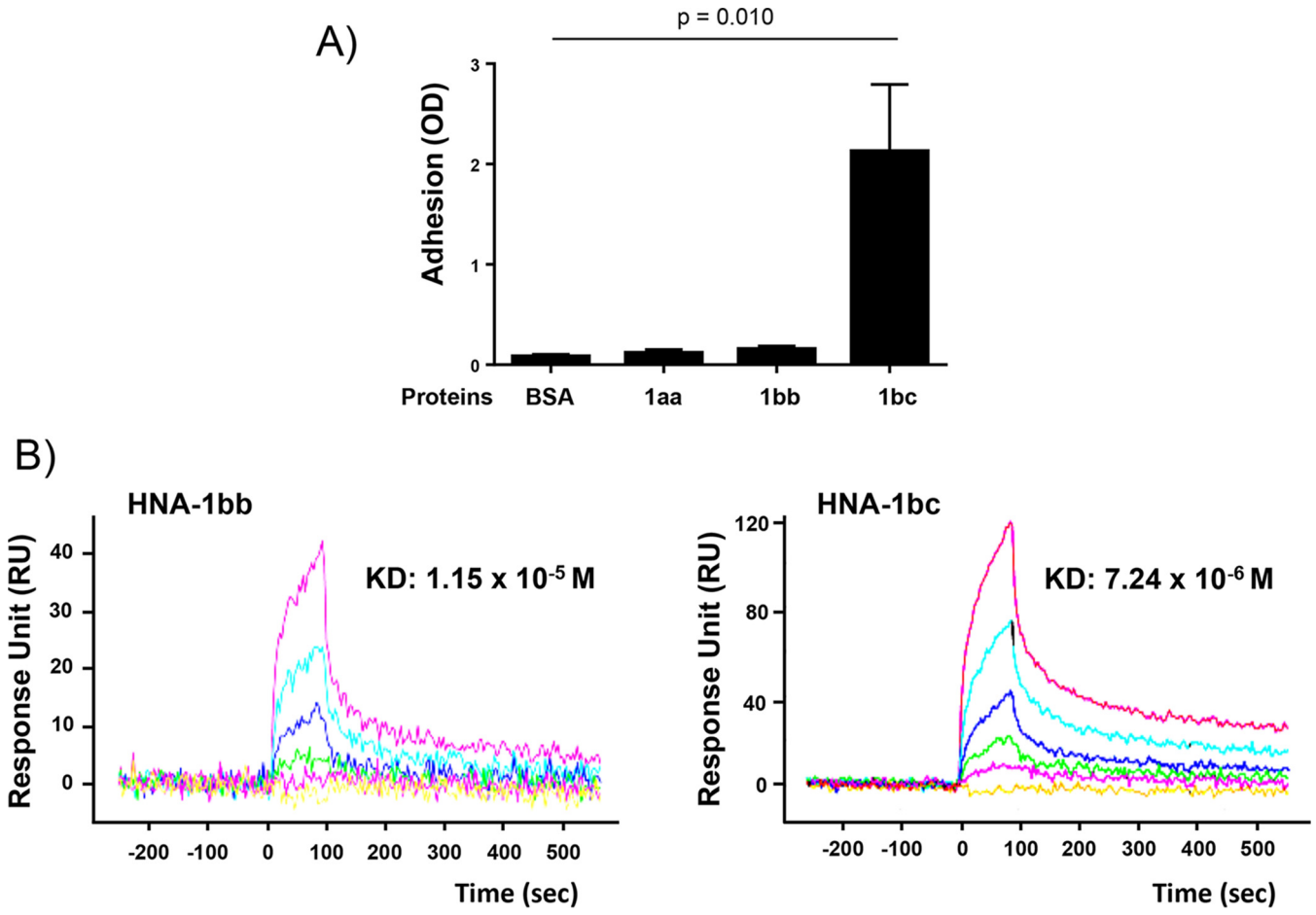

FIG 3 Binding of FcγRIIIb alloforms onto IgG by ELISA and SPR. (A) ELISA. Biotin-labeled recombinant FcγRIIIb alloforms (HNA-1aa, -1bb, and -1bc) were added into microtiter wells coated with 2.5 μg IgG or BSA for 1 h at room temperature. After washings, bound FcγRIIIb protein was detected with HRP-conjugated streptavidin using TMB as the substrate. The color reaction was read on an ELISA reader at 450 nm. Data are presented as means ± SD from three independent experiments. (B) SPR. Different amounts of IgG fractions (50, 100, 200, 400, and 800 nmol/liter) were injected over three flow cells coated with different recombinant FcγRIIIb alloforms (HNA-1aa, -1bb, and -1bc). The binding response in real time was recorded as resonance units (response units) for 500 s. The dissociation constant (K_d) was analyzed using computer software (ProteOn Manager; Bio-Rad).

1.15×10^{-5} M). In contrast, there was no significant difference between the affinities of the HNA-1a and HNA-1b alloforms against IgG (data not shown).

Structural analysis of different FcγRIIIb alloforms' interaction with the Fc part of IgG. Analysis of the three-dimensional (3D) structure of the FcγRIIIb-Fc complex reveals the location of all five polymorphic residues responsible for the formation of HNA-1 alloantigenic determinants on the Ig-like C2-type domain 1 of FcγRIIIb. Since the underlying crystal structure has been determined for the HNA-1b alloform (amino acids Ser36, Ser65, Ala78, Asn82, and Ile106), analysis was generated by virtual mutations of 36Ser>Arg, 65Ser>Asn, 82Asn>Asp, and 106Ile>Val for HNA-1a and 78Ala>Asp for HNA-1c (Fig. 4a and c).

The unique polymorphic residue 78, which controls HNA-1c epitopes, is located in proximity to the positively charged Fc region close to the main contact between FcγRIIIb and Fc. This contact is known as the "proline sandwich" established by Trp108 and Trp131 of FcγRIIIb, which embrace Pro212 of the Fc chain (14). As shown in Fig. 4b, the 78Ala>Asp substitution induces an additional polar molecular interaction between FcγRIIIb and Fc, which leads to the formation of the additional salt bridge between Asp78 (FcγRIIIb) and Lys209 (Fc). In contrast, the introduction of a negatively charged side chain by the 82Asn>Asp mutation (HNA-1b > HNA-1a) does not result into explicit salt bridge formation because position 82 residue is more distant to the Fc region of the IgG than is position 78 (Fig. 4d). Theoretically, the mutation 36Ser>Arg may affect

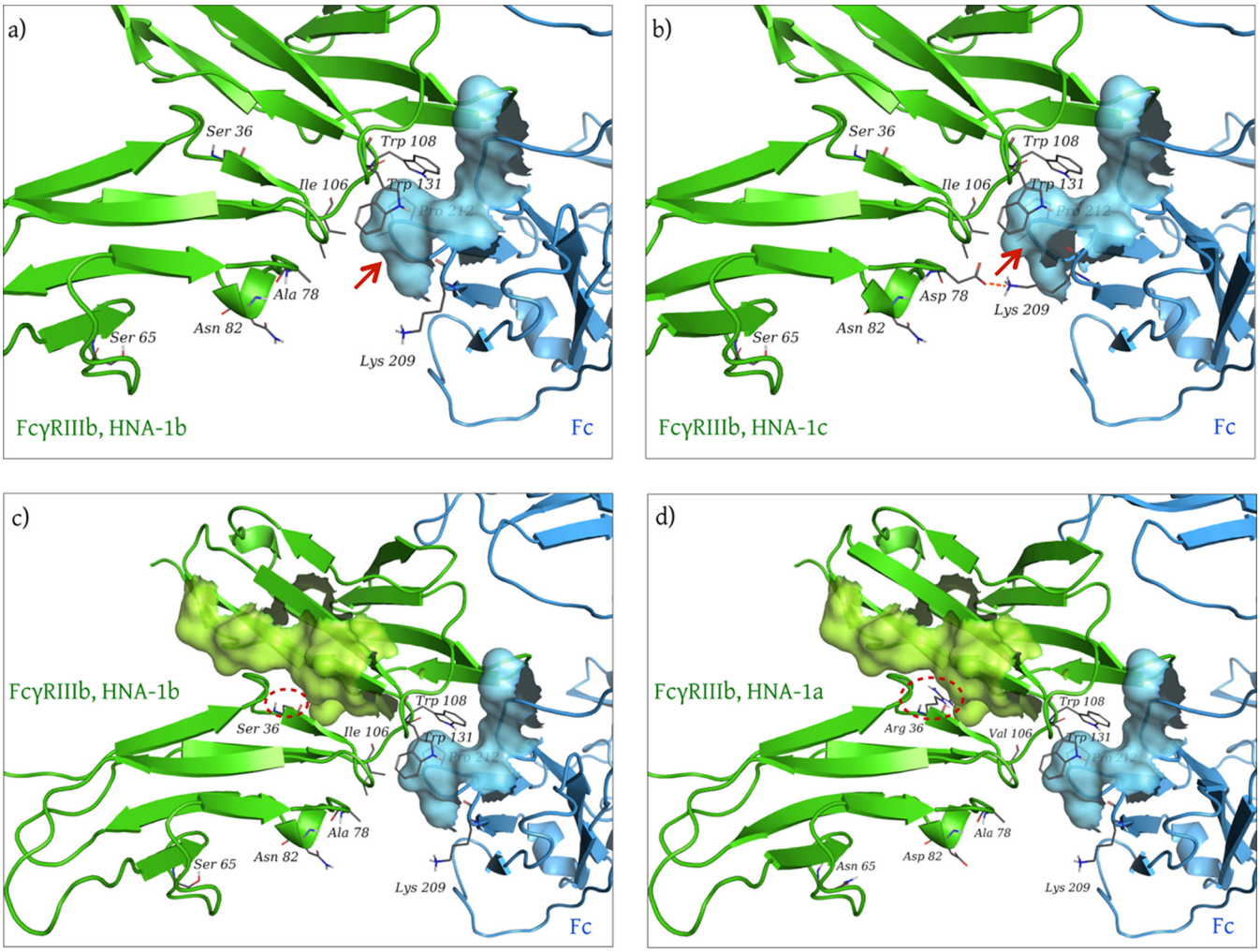

FIG 4 Model of the three-dimensional structure of FcγRIIIb alloforms (green) and the Fc part of IgG (blue) around their interface (blue surface). (a) Structure of the interface between the HNA-1b alloform of soluble FcγRIIIb and Fc ["proline sandwich": Trp108, Pro212 (Fc), and Trp131] as presented by the crystal structure (Protein Data Bank accession code 1E4K [14]). (b) Model of the structure of the HNA-1c alloform, obtained by *in silico* mutation 78Ala>Asp. Additional polar interactions are possible, exemplified by a potential salt bridge between receptor residues Asp78 and Lys209 on the Fc side. (c and d) Model of the geometric arrangement of the two Ig-like C2-type domains of the HNA-1b alloform of FcγRIIIb (c) and of the HNA-1a alloform (d). The contact surface of domain 2 is in green.

allosterically the IgG binding, by altering the hinge region between the two Ig-like C2-type domains of FcγRIIIb. Unfortunately, our model cannot deduce an exact structural prediction for this region. Although Ile106 is located in the main contact region, mutation of this residue into Val results in only a minor distortion of the contact surface in our model. In addition, our model does not allow the analysis of polymorphic residue 65 located on the domain 1 distant to the Fc region.

This model facilitates our experiments explaining the high-affinity nature of HNA-1c compared to HNA-1a and HNA-1b, which show similar affinity levels. Thus, amino acid Asp(D)78 not only is important for determining the allospecificity but also seems to be important in regulating FcγRIIIb affinity.

ADRB with HNA-1-phenotyped neutrophils. Finally, we asked whether opsonized *Plasmodium falciparum* merozoites might enhance cross-linking of FcγRIIIb on HNA-1c-positive neutrophils leading to neutrophil degranulation and generation of reactive oxygen species (ROS), which are known to inhibit intraerythrocytic malaria parasite development (9, 15). To test this hypothesis, an antibody-dependent respiratory burst (ADRB) assay was performed with HNA-1abc- and HNA-1ab-typed neutrophils in the presence of affinity-purified GLURP-specific IgG antibodies and *P. falciparum* merozo-

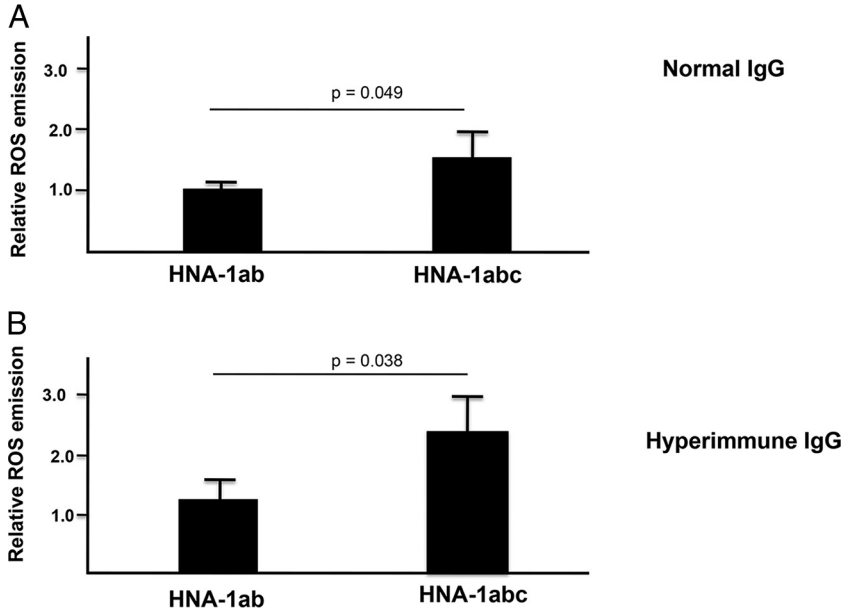

FIG 5 ROS emission of HNA-1 phenotypes neutrophils primed with antibodies, in the presence of merozoites. HNA-1-phenotyped neutrophils derived from HNA-1ab ($n = 4$) and HNA-1abc ($n = 4$) donors were incubated with IgG (A) or specific IgG antibodies against malaria (B) in the presence of merozoites (see Materials and Methods). ROS emission derived from activated neutrophils was measured with DHR fluorescence probe by flow cytometry. The ratio between ROS with IgG mediation and without IgG (DHR alone) was calculated. Data are presented as means ± SD.

ites. ROS emission was measured by flow cytometry using dihydrorhodamine 123 (DHR) as a fluorochrome (Fig. 5). Since most of the HNA-1c-positive individuals in our cohort are phenotypically HNA-1abc (3.86%) and rarely HNA-1ac (0.68%) or HNA-1bc (0.23%), only HNA-1abc-typed neutrophils were used. Significantly, different amounts of ROS emission were observed for neutrophils from HNA-1abc- and HNA-1ab-typed individuals when primed with normal IgG purified from a healthy blood donor. Similarly, priming of HNA-1abc neutrophils with GLURP-specific IgG antibodies resulted in significantly ($P = 0.038$) greater amounts of ROS than those resulting from HNA-1ab neutrophils (Fig. 5). Importantly, the presence of *P. falciparum* merozoites increased overall ROS production, indicating the higher efficacy of malaria-specific antibodies. However, the level of ROS in HNA-1abc neutrophils primed with GLURP-specific antibodies in the presence of *P. falciparum* merozoites was not significant compared to the level observed when normal IgG was used ($P = 0.143$).

DISCUSSION

The low-affinity FcγRIIIb receptor is expressed solely on neutrophils and carries polymorphic residues responsible for the formation of HNA-1a, -1b, -1c, and -1d alloantigenic determinants. HNA-1c represents a low-frequency antigen among Caucasians (around 5%) (16) and Asians (3% to 16%) (17, 18), but a high prevalence of HNA-1c (34.9%) is observed in some African populations (16). However, little is known about the clinical impact of HNA-1c polymorphism and diseases. Here, we studied the relevance of HNA-1c (+) neutrophil-mediated immune mechanisms and malaria.

To approach this question, we developed stable transfected HEK293 mammalian cells expressing HNA-1aa, -1bb, and -1bc on the cell surface as well as soluble recombinant HNA-1aa, -1bb, and -1bc proteins. These reagents were first used to study the affinity of different HNA alloforms toward IgG. Here, we show that the HNA-1c alloform of FcγRIIIb bound significantly stronger to IgG than did the HNA-1a and HNA-1b alloforms. This result demonstrates that the HNA-1c alloform can function as a high-affinity FcγRIIIb receptor for IgG (the HNA-1c K_d is 60 times higher than that of HNA-1a and HNA-1b alloforms). This increase in affinity seems to be regulated solely by

amino acid substitution Ala78Asp, but not by higher copy number, since all experimental conditions were based on equal amounts of FcγRIIIb (19). Indeed, analysis based on the three-dimensional crystal structure of the FcγRIIIb/Fc complex showed the importance of the hydrophobic amino acid alanine (A) replacement by the negatively charged aspartic acid (D) at position 78 for the molecular interaction between FcγRIIIb and the Fc portion of IgG.

In a previous study, the binding affinities of FcγRIIIb alloforms toward different IgG subclasses did not differ significantly (20). In this study, however, FcγRIIIb proteins from Chinese hamster ovary (CHO) cells were used. It is known that glycosylation patterns differ between glycoproteins synthesized in nonhuman cells (such as CHO) and in human cells (such as HEK). In comparison to CHO cells, HEK293 cells are able to synthesize glycoprotein structures with higher carbohydrate complexity (21). In this regard, Drescher and coworkers demonstrated that N-glycosylation of FcγRIIIb at position N163 is important for regulating the affinity of FcγRIIIb to its ligand IgG. Other asparagine residues (N39, N75, and N170), however, did not seem to alter the IgG binding affinity (22). Since all FcγRIIIb alloforms used in the present study were produced in HEK293 cells, we are convinced that the high affinity of the HNA-1c alloform arises from the A78D mutation rather than N163 glycosylation.

Recent studies have shown that the inheritance of the HNA-1c allele might contribute to reducing the risk of clinical malaria (12, 13). In contrast, individuals carrying the HNA-1b allotype have an increased risk of developing severe malaria (23). This phenomenon could be caused by different antibody binding affinities toward the Fcγ receptor(s) triggering neutrophil activation. It is known that activation of neutrophils can cause degranulation and generation of ROS (also referred to as oxidative burst). ROS are highly toxic to intraerythrocytic malaria parasites, and high ROS production correlates with accelerated clearance of *P. falciparum* and, subsequently, protection from clinical malaria (9, 10). Furthermore, Joos and coworkers showed that this correlation depends on the presence of antibodies against malaria (24).

In accordance with these observations, we found that the interaction between antimalaria antibodies and HNA-1c (+) neutrophils leads to significantly higher ROS emission than that obtained with HNA-1c (−) neutrophils. It is plausible that this observation explains the protective effect of HNA-1c against clinical malaria. Human neutrophils also constitutively express FcγRIIa, mediating antibody-dependent phagocytosis rather than respiratory burst reactions (25). A polymorphism in FcγRIIa has been associated with protection against clinical malaria in some studies (26, 27) but not in others (12).

Malaria is considered to have exerted strong selective pressure on the recent history of the human genome (28). A higher prevalence of HNA-1c in areas where malaria is endemic than in areas of nonendemicity has been reported (12). In Asian populations, the highest frequency (16%) of HNA-1c (+) individuals has been recorded among Asian Indians (18) living in areas where malaria still is a burden. Recently, a low to medium frequency of HNA-1c was found in Thai (0.9%) and Burmese (3.1%) individuals living in a region where malaria is endemic (17). These findings support the notion that malaria has exerted a strong selection pressure on the inheritance of the otherwise-rare HNA-1c allotype.

In conclusion, we have demonstrated that the FcγRIIIb HNA-1c allotype has a higher affinity toward IgG than other known allotypes, thus substantiating the possible involvement of this allotype in protection against clinical malaria. Further research on the clinical consequences of HNA-1c inheritance and diseases (such as immune complex diseases) may highlight the clinical importance of neutrophil antigens and antibodies.

MATERIALS AND METHODS

Genotyping of HNA-1a, -1b, and -1c. Genomic DNA was isolated from healthy blood donors and was typed for the HNA-1 system by PCR-SSP using the BAGene SSP kit as recommended by the manufacturer (BAG Health Care, Lich, Germany). The ethics committee of the University of Giessen approved the study, and written informed consent was obtained from all donors.

Serum samples and antibodies. Serum samples containing anti-HNA-1a, anti-HNA-1b, and anti-HNA-1c aabs were obtained from mothers of children with NAIN. In some cases, neutrophils were phenotyped for the HNA-1 system using well-characterized HNA-1a, -1b, and -1c aabs by the standard MAIGA as previously described (29). Normal serum collected from a healthy blood donor without white blood cell-specific antibodies was used as a control. MAbs 3G8, DJ130c, and LNK16 reacted against different epitopes on FcγRIIIb (Immunotech, Marseille, France; Santa Cruz Biotechnology, Heidelberg, Germany) were used in this study. Mouse IgG, used as a control, was from Biolegend, Fell, Germany. Soluble recombinant HNA-1a, -1b, and -1c antigens were produced in our laboratory by stable transfection of High Five insect cells (30). Malaria-specific IgG antibodies were affinity-purified antibodies against *P. falciparum* GLURP (31).

Analysis of FcγRIIIb expressed on the surface of HEK293 cells by flow cytometry. Allele-specific FcγRIIIb constructs encoding HNA-1aa, -1bb, and -1bc were used to generate stable transfected cell lines (32). Aliquots of 3×10^5 transfected HEK293 cells in phosphate-buffered saline (PBS) containing 0.2% bovine serum albumin (BSA) were incubated with MAbs against FcγRIIIb at 4°C and subsequently stained with 50 μl Alexa Fluor 488-conjugated donkey anti-mouse IgG (1:50; Life Technologies, USA) for flow cytometry analysis as recently described (32).

Analysis of HNA-1 allospecificities of FcγRIIIb expressed on the surface of HEK293 cells by MAIGA. MAIGA with transfected HEK293 cells expressing HNA-1aa, -1bb, and -1bc was established using LNK16 MAbs. In brief, aliquots of 100 μl of paraformaldehyde-fixed transfected HEK293 cells (3×10^5 cells) in PBS containing 0.2% BSA (PBS-BSA) were incubated with 50 μl of serum in the presence of 10 μl MAb (20 μg/ml). Subsequently, cells were lysed with 100 μl of lysis buffer. Cell lysates were centrifuged for 20 min at $712 \times g$ at room temperature. Aliquots of 70 μl supernatants were removed and added into microtiter wells containing 180 μl of TBS buffer (10 mM Tris, 0.5% Triton X-100, 0.05% Tween 20, 0.5 mM $CaCl_2$, 140 mM $NaCl_2$, pH 7.4). After mixing, aliquots of 100 μl diluted lysates (duplicate) were added into F-bottom microtiter plates precoated with goat anti-mouse IgG (3 μg/ml in coating buffer overnight at 4°C; Dianova, Hamburg, Germany). Finally, bound antigen-antibody complex was detected with horseradish peroxidase (HRP)-labeled goat anti-human IgG (dilution, 1:4,000 in TBS; Dianova), followed by incubation with the enzyme substrate (*o*-phenylenediamine; Kem-En-Tec Diagnostics A/S, Taastrup, Denmark) and colorimetric reading (492/620 nm) on an ELISA reader (Sunrise; Tecan, Männedorf, Switzerland) as recently described (32).

Cell adhesion assay. Cell adhesion onto IgG was performed according to a previously published protocol (33). Briefly, 96-well plates (Greiner Bio-One, Frickenhausen, Germany) were coated overnight at 4°C with 5 μg purified human IgG (Sigma) or 0.3% BSA (Serva, Heidelberg, Germany) in 20 mM HEPES buffer (Thermo Fisher, Darmstadt, Germany). After washings, wells were blocked with 100 μl 3% BSA for 1 h at 4°C and washed twice. Aliquots (1×10^6 to 4×10^6) of neutrophils or transfected HEK293 cells were added to either purified IgG- or BSA-coated wells. Plates were incubated for 1 h at 37°C in the presence of 5% CO_2, and wells were washed once. Adherent cells were stained with crystal violet (Sigma) and quantified by a microtiter reader at 592 nm (Tecan). In some experiments, cells were treated with 50 μl MAb (3G8 or DJ130c) against FcγRIIIb (20 μg/ml) for 30 min prior to the cell adhesion assay.

Enzyme-linked immunosorbent assay. Microtiter wells (Greiner Bio-One) were coated with 5 μg of purified IgG (Sigma) or BSA overnight at 4°C. Wells were blocked with 100 μl 3% PBS-BSA for 1 h at room temperature and washed three times with 200 μl PBS-BSA containing 0.05% Tween 20. Aliquots of 100 μl of biotinylated recombinant proteins or BSA in PBS buffer (2.5 μg/ml) were incubated for 1 h at room temperature (30). Wells were washed twice, 100 μl of horseradish peroxidase-conjugated streptavidin (1:2,000 in PBS; GE Healthcare UK Limited, Buckinghamshire, UK) was added, and the mixture was incubated for 1 h at room temperature. Wells were then washed three times, and 100 μl of 3,3′,5,5′-tetramethyl benzidine substrate solution (TMB; Sigma) was added. After 15 min of incubation at room temperature in the dark, the reaction was stopped with 100 μl of 1.0 M HCl and was read at 450 nm on an ELISA reader (Tecan).

Surface plasmon resonance. SPR analysis was performed on a protein interaction array system (ProteOn XPR36; Bio-Rad, Munich, Germany) as previously described (34). Recombinant HNA-1aa, -1bb, -1bc antigens or BSA (as control) were immobilized on different flow cells of a GLM-sensor chip (25 μg in 250 μl ProteOn acetate buffer). Aliquots of 50 μl purified IgG (Sigma) were diluted in 200 μl reaction buffer (50 to 800 nmol/liter) and injected at a flow rate of 30 μl/min over the flow cells. The binding response was recorded as resonance units (RU). Data were analyzed with computer software (ProteOn Manager; Bio-Rad).

***In silico* experiments.** *In silico* experiments were performed based on the crystal structure of a soluble Fcγ receptor in complex with an Fc fragment of human IgG1 (Protein Data Bank accession code 1E4K [14]). Virtual mutation(s) was executed by replacing the respective amino acid side chain by a cascade of energy minimization approaches according to AMBER parm99 (35) and MMFF94s force field computation (36). Visualizations were prepared with the software Pymol 1.7 (37). For the analysis of the polymorphic residue 78 of the FcγRIIIb, the amino acids 207 to 210 of the opposing Fc chain are considered to be flexible, allowing the formation of additional contact(s) between Fc and FcγRIIIb.

Antibody-dependent respiratory burst assay. An assay measuring the ability of antibodies to trigger the release of reactive oxygen species from neutrophils in response to *P. falciparum* merozoites was performed as previously described with some modifications (38, 39). Merozoites were isolated from *P. falciparum* line NF54 as described previously (32). Briefly, aliquots of 10 ml heparinized blood were mixed with 3 ml of 5% dextran (in Hanks balanced salt solution [HBSS] buffer; PAN-Biotech, Aidenbach, Germany) for 35 min at room temperature. Subsequently, the cells in the supernatant were resuspended in HBSS and centrifuged at $1,500 \times g$ for 10 min. Cells were resuspended in 3 ml HBSS, carefully layered

onto 8 ml Percoll gradient (GE Healthcare, Uppsala, Sweden), and centrifuged at 400 × *g* for 20 min. Neutrophils were washed twice and adjusted to a concentration of 10^7 cells/ml with HBSS. Aliquots of 200 μl paraformaldehyde-fixed merozoites (2×10^5) were incubated with 50 μl of affinity-purified GLURP-specific IgG (final concentration, 0.4 mg/ml) purified from malaria-immune Liberian adults (29) or 50 μl normal IgG as a control for 30 min at room temperature. Antibody-pretreated merozoites were then added into a 100-μl neutrophil suspension (see above) in the presence of 150 μl HBSS buffer and 3 μl dihydrorhodamine 123 (DHR; final concentration, 3 mM; Enzo Life Sciences, Farmingdale, NY, USA) for 5 min at 37°C in a total volume of 500 μl. The reaction mixture was chilled for 10 min on ice. After centrifugation (425 × *g*, 5 min, 4°C), cells were washed once with HBSS, treated with 300 μl fixative solution (Becton Dickinson), and measured immediately by flow cytometry (488-nm laser; FACSCanto II).

Statistics. Statistical comparisons were made using an unpaired, 2-tailed Student's *t* test or 1-way analysis of variance (ANOVA), as appropriate. A *P* value of <0.05 was assumed to represent statistical significance. All statistical analyses were performed using GraphPad Prism (GraphPad, Inc., La Jolla, CA, USA).

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We declare that we have no conflicts of interest.

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