FcRn: an IgG receptor on phagocytes with a novel role in phagocytosis

Gestur Vidarsson, Annette M. Stermerding, Nigel M. Stapleton, Suzanne E. Splethoff, Hans Janssen, Frank E. Rebers, Masja de Haas, and Jan G. van de Winkel

Here, we report that the MHC class I–related neonatal Fc receptor (FcRn) is expressed within azurophilic and specific granules of neutrophils and relocates to phagolysosomes on phagocytosis of IgG-opsonized bacteria. We found FcRn to enhance phagocytosis in a pH–dependent manner which was independent of IgG recycling. IgG-opsonized bacteria were inefficiently phagocytosed by neutrophils from β2M−/− or FcRn α-chain−/− mice, which both lack expression of FcRn. Similarly, low phagocytic activity was also observed with mutated IgG (H435A), which is incapable of binding to FcRn, while retaining normal binding to classical leukocyte Fcγ receptors. Finally, a TAT peptide representing intracellular endocytosis and transport motifs within FcRn strongly inhibited IgG-mediated phagocytosis. These findings support a novel concept in which FcRn fulfills a major role in IgG-mediated phagocytosis.

© 2006 by The American Society of Hematology

Materials and methods

Recombinant antipneumococcal 6A/B antibodies

The generation and functional characterization in vitro and in vivo of human antipneumococcal serotype 6A/B GDb1 antibodies used in this study have been described in detail before in Saeland et al.14 The H435A IgG1 variant was generated by mutating the γ1 heavy chain with a Quickchange Site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s guidelines, using the CH3-specific primer gaggctctgcacaacGCctacacgcagaagagcc (mutated bases underlined and capitalized) and its complementary primer. After verification of the expected incorporation of the mutated bases by sequencing (ABI 373 Stretch automated sequencing machine; Applied Biosystems, Foster City, CA), resulting in the corresponding amino-acid change from a histidine to an alanine in position 435, the heavy chain was transfected together with the corresponding light chain, produced, and purified as described in Saeland et al.14 and Vidarsson et al.15

Detection antibodies

Mouse IgG1 MAC-1 (CR3)–specific mAb was purchased from Becton Dickinson (San Jose, CA), and Cy3-labeled goat anti–mouse IgG F(ab’)2

The authors declare no competing financial interests.

G.V. and A.M.S. contributed equally to this study.

An Inside Blood analysis of this article appears at the front of this issue.

Reprints: Gestur Vidarsson, Department of Experimental Immunohematology, Sanquin Research, and Landsteiner Laboratory, Plesmanlaan 125, 1066 CX Amsterdam, The Netherlands; e-mail: G.Vidarsson@sanquin.nl.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked “advertisement” in accordance with 18 USC section 1734.

© 2006 by The American Society of Hematology

Introduction

Phagocytic cells express members of 3 classes of leukocyte IgG-Fc receptors, FcyRI, FcyRII, and FcyRIII. All share considerable structural and functional homology and recognize similar residues within the CH2 region of IgG.1 FcyR activates phagocytes on interaction with IgG-opsonized particles, involving immunoreceptor tyrosine-based activation motifs (ITAMs). This activation signal may possibly be downregulated by the immunoreceptor tyrosine-based inhibitory motif (ITIM)–containing FcγRIIB receptor on polymorphonuclear neutrophils (PMNs) and monocytes.2 No other signaling motifs have been implicated in FcyR-mediated phagocytosis. Crosslinking of ITAM-bearing receptors (eg, T-cell receptor, FceRI) does not initiate phagocytosis, but it generally triggers fusion and release of granule contents into sealed immunologic synapses between effector cells and targets. In phagocytes these granules contain various components, including enzyme complexes that initiate the respiratory burst and phagosome acidification, as well as antimicrobial peptides and enzymes that serve to kill invading pathogens.3,4

A distinct IgG receptor, the neonatal FcγR (FcRn), consisting of a unique α-chain and β2-microglobulin (β2M), is a major histocompatibility class I (MHC-I) homolog.5 FcRn is present in epithelial cells, placental syncytiotrophoblasts, as well as endothelial cells. In these cells, FcRn has been implicated in transport of IgG across mucosal cells,6,7 from mother to fetus,7 and regulation of IgG half-life,8,11 respectively. This receptor has been found in human monocytes,12 albeit that no function has been attributed to monocyte FcRn. FcRn does not bind IgG at physiologic pH (7.4). Only in the acidic environment of endocytic vacuoles (pH ≈ 6.5), where histidine residues in the Fc-tail of IgG become protonated, can FcRn bind IgG with high affinity. Both β2M and the FcRn α-chain participate in IgG binding within the CH2–CH3 interface.13

In this study we document expression of FcRn within PMNs. Furthermore, we observed FcRn translocation to nascent phagosomes, where FcRn facilitates IgG-mediated bacterial phagocytosis through signaling motifs found within the cytoplasmic tail. These results point to a novel role for FcRn in phagocyte biology.

Materials and methods

Recombinant antipneumococcal 6A/B antibodies

The generation and functional characterization in vitro and in vivo of human antipneumococcal serotype 6A/B GDb1 antibodies used in this study have been described in detail before in Saeland et al.14 The H435A IgG1 variant was generated by mutating the γ1 heavy chain with a Quickchange Site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s guidelines, using the CH3-specific primer gaggctctgcacaacGCctacacgcagaagagcc (mutated bases underlined and capitalized) and its complementary primer. After verification of the expected incorporation of the mutated bases by sequencing (ABI 373 Stretch automated sequencing machine; Applied Biosystems, Foster City, CA), resulting in the corresponding amino-acid change from a histidine to an alanine in position 435, the heavy chain was transfected together with the corresponding light chain, produced, and purified as described in Saeland et al.14 and Vidarsson et al.15

Detection antibodies

Mouse IgG1 MAC-1 (CR3)–specific mAb was purchased from Becton Dickinson (San Jose, CA), and Cy3-labeled goat anti–mouse IgG F(ab’)2

The authors declare no competing financial interests.

G.V. and A.M.S. contributed equally to this study.

An Inside Blood analysis of this article appears at the front of this issue.

Reprints: Gestur Vidarsson, Department of Experimental Immunohematology, Sanquin Research, and Landsteiner Laboratory, Plesmanlaan 125, 1066 CX Amsterdam, The Netherlands; e-mail: G.Vidarsson@sanquin.nl.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked “advertisement” in accordance with 18 USC section 1734.

© 2006 by The American Society of Hematology
fragments from Jackson (West Grove, PA). Protein G-isolated mouse IgG1 directed against the α-chain of FcRn (mAb 1G3) was obtained from ATCC (Manassas, VA). Mouse IgM mAb 22H4C11 was raised by immunizing balb/c mice with a peptide stretch comprising the FcRn α-chain intracellular tail. Rabbit antisera against human FcRn was a generous gift from Dr Neil Simister (Brandeis University, Waltham, MA). Mouse IgG1 was detected in fluorescence-activated cell scanner (FACS) experiments by means of FITC-labeled goat F(ab’2) fragments of anti-mouse IgG (Protos, Burlingame, CA). Rabbit antisera was detected with biotin-labeled swine anti-rabbit IgG F(ab’2) fragments (DAKO, Glostrup, Denmark), followed by streptavidin-Alexa555 (Molecular Probes, Leiden, The Netherlands). Human recombinant IgG1 concentrations were quantified by a sandwich enzyme-linked immunosorbent assay (ELISA), capturing the recombinant antibodies by a rabbit anti-human-kappa antiserum, with alkaline phosphatase-conjugated rabbit anti-IgG (Fc specific) antisera for detection as described in Vidarsson et al. Antibody titers were obtained by calculating FcRn expression levels as a ratio of ABL (Abelson) gene expression. Expression levels were distinguished by PE-labeled Rat IgG anti-Gr-1/Ly-6; Becton Dickinson). In microscopy experiments, vital cells were allowed to attach to poly-L-lysine–coated superfrost (Sigma) microscopy slides for 30 minutes at 37°C in a humidified chamber. FACS phagocytosis experiments were carried out as described for FACS phagocytosis experiments (see “Phagocytosis experiments”) but on microscopy slides. Before all stainings, cells were fixed with paraformaldehyde (3.7%), washed with saponin buffer (PBS with 0.5% saponin and 1% bovine serum albumin; saponin was omitted for extracellular staining), and incubated for 60 minutes at room temperature with either mouse IgG1 MAC-1 (CR3) or rabbit anti-FcRn antisera, followed by appropriate conjugates (see “Detection antibodies”). All detection antibodies and conjugates were diluted in saponin buffer to allow intracellular staining. To detect the primary antibody, cells were incubated with streptavidin-Alexa555 (Molecular Probes). Mouse PMNs were isolated as described in Vidarsson et al using freshly isolated human PMNs. All phagocytosis experiments were carried out at 37°C for 15 minutes by mixing IgG, 10^5 PMNs, and 5 × 10^9 FITC-labeled bacteria, except where indicated otherwise. PMN-adhered or ingested bacteria were evaluated by confocal microscopy and FACS. IgG recycling

PMNs were allowed to ingest recombinant antipneumococcal IgG1 (10 μg/mL), either alone or in the presence of 6B pneumococci for 5 minutes at 37°C.
followed by 2 washes with medium (RPMI 1640 medium with 2% FCS, adjusted to pH 2.5) to wash away extracellularly bound IgG (confirmed by FACS; data not shown). Cells were then incubated for an additional 30 minutes, and supernatants were harvested and analyzed for the presence of human anti–6B pneumococcal antibodies by antigen-specific ELISA.

Statistical analyses
One-way ANOVA or 2-tailed Student t tests were used to compare differences in phagocytosis and binding indexes after testing for normal distribution using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). Significance was set at \( P < 0.05 \).

Results
FcRn is expressed in PMNs
We observed FcRn to be highly expressed in freshly isolated human and mouse neutrophils (PMNs), as analyzed by real-time quantitative RT-PCR analyses and flow cytometry (Figure 1A).

FcRn was exclusively localized intracellularly in resting PMNs (Figure 1B). By electron microscopy, FcRn was found in granular structures but not on the plasma membrane (Figure 2A). The most prominent staining was observed within myeloperoxidase-positive azurophil granules (33% of myeloperoxidase-positive granules also stained FcRn positive). A lower level was also detected in specific granules (18% of lactoferrin-positive granules; Figure 2B). FcRn could be detected transiently on the surface by FACS analysis on stimulation with the degranulation agent phorbol 12-myristate 13-acetate but not with N-formyl-methionyl-leucyl-phenylalanine (n/H110053, data not shown). This is in agreement with previously published data on intracellular trafficking of monocyte FcRn.

Furthermore, we observed that FcRn enveloped target pathogens following human IgG1-mediated phagocytosis (Figure 3A), suggesting that FcRn-containing granules fuse with developing phagosomes and/or phagolysosomes during or after internalization. On phagocytosis, the bulk of FcRn expression was observed around phagosomes (Figure 3B-C).
Ingestion of FITC-labeled pneumococci incubated at 37°C with PMNs from WT, H435A IgG1 and WT IgG1, relative to WT IgG1, with approximately 4-fold more PMNs with bacteria on the outside when bacteria were opsonized with H435A IgG1 (Figure 4B). After quenching of noningested bacteria and quantification of internalized bacteria by FACS, we also observed significantly less phagocytosis of FITC-labeled pneumococci incubated at 37°C with PMNs from WT, β2M- and FcRn-knockout mice in the presence of IgG1. Fluorescence of bound but not ingested bacteria were quenched as described in "Phagocytosis experiments." NS indicates not significant; *P < .05 when compared with WT. Data are presented as means plus standard deviations from 2 (A) or 4 (D) experiments, respectively. Experiments (B-C) were performed 3 times, yielding similar results.

**Fcrn-mediated IgG recycling**

If FcRn-mediated IgG recycling was responsible for enhancing phagocytosis of IgG-opsonized bacteria, this effect would be expected to be more prominent at lower IgG concentrations, when IgG is a limiting factor. We noted that the lower level of phagocytosis observed in the absence of FcRn engagement was consistently more pronounced at saturating IgG concentrations and to be lower (or absent) at limiting IgG concentrations (Figure 6A). This was observed when IgG1 was compared with H453A IgG1 with human PMNs and when comparing knock-out with wild-type mouse PMNs. We next tested whether FcRn-dependent IgG recycling may underlie the lowered phagocytic activity of PMNs in the absence of FcRn engagement. However, both WT and β2M knock-out PMNs mediated similar low-level recycling of IgG, with less than 1% of IgG1 offered to the cells being recycled to culture medium following IgG1-mediated phagocytosis (Figure 6B). At this IgG1 concentration no significant phagocytosis was observed (Figure 6A). No IgG recycling was observed in the absence of bacteria (Figure 6B). In conclusion, our data do not support the hypothesis that FcRn mediates recycling of IgG after IgG-mediated phagocytosis.

**Involvement of FcRn signaling motifs in phagocytosis**

Next, we studied whether FcRn was actively involved with the enhanced internalization of IgG-opsonized bacteria. Peptides consisting of a TAT sequence and previously recognized internalization and transport motifs within the FcRn intracellular tail25-27 (Figure 7A) readily diffused over the plasma membrane (Figure 7B).
Figure 6. Recycling of IgG is not responsible for enhanced phagocytosis mediated by FcRn. (A) Effect of IgG level on phagocytosis of pneumococci by wild-type and β2M−/− mouse PMNs. Note that phagocytosis by β2M−/− PMNs is more impaired at higher concentrations of human IgG1. (B) Recycling of human IgG1 by wild-type and β2M−/− mouse PMNs. Wild-type mouse PMNs and β2M−/− PMNs were allowed to take up human IgG1 antiserotype 6B mAb (10 μg/mL) for 5 minutes at 37°C in the presence of serotype 6B pneumococci. After extensive washing, cells were incubated for an additional 30 minutes in medium to allow for exocytosis/recycling of IgG, and supernatants were subsequently analyzed for the presence of human anti-6B pneumococcal antibodies by ELISA. Control samples in which mouse PMNs were incubated with IgG1 only (without bacteria) did not result in significant recycling. Experiments were repeated 4 times, with essentially identical results. Data represent means plus or minus standard deviation.

Discussion

The central initiator of IgG-mediated phagocytosis on human PMNs, FcγRIIa (CD32), is expressed primarily on the cell surface and is quickly internalized together with IgG-coated particles on receptor engagement.14 Unlike the classical leukocyte FcyRs, which are the only IgG receptors described so far on PMNs, we found FcRn to be exclusively localized in intracellular compartments in freshly isolated PMNs. The intracellular localization to both azurophilic and specific granules corroborates previous studies showing the presence of β2M in specific granules and secretory vesicles in PMNs30 and with studies documenting FcRn expression within human macrophages31 and in human and porcine monocytes.12 Our data are also in agreement with previous work showing that degranulation mediates translocation of FcRn from intracellular compartments to the cell surface on monocytes.12

To investigate FcRn’s involvement in IgG-mediated phagocytosis by PMNs, we performed a series of confocal microscopic and FACS analyses, in which we observed a prominent FcRn staining around phagolysosomes on internalization of IgG-opsonized bacteria (Figure 3A), indicating FcRn to be selectively concentrated to phagolysosomes on IgG-mediated phagocytosis (Figure 3B-C).

We observed phagocytosis of both human and mouse PMNs to be severely impaired under experimental conditions preventing FcRn engagement. For experiments with mouse PMNs, we used human IgG1 which has been documented to have high affinity at pH 6.0 to mouse and human FcRn31 and to mediate efficient FcγRIII-dependent phagocytosis of pneumococci by mouse PMNs.14 By blocking acidification of phagolysosomes, IgG1-mediated phagocytosis was inhibited. Conversely, phagocytosis was found to be enhanced on adjustment of the extracellular pH to pH 6.0 (Figure 5). PMNs from β2M knock-out mice, lacking functional FcRn expression,9,11 were unable to efficiently phagocytose IgG1-opsonized pneumococci. PMNs from FcRn α-chain knock-out mice8 showed a similar phenotype (Figure 4D). Likewise, lowered phagocytic activity was observed by human PMNs when allowed to internalize pneumococci opsonized with a mutated IgG derivative (H435A) incapable of binding FcRn (Figure 4B-C).23 These studies show that a functional pH-dependent interaction between FcRn and IgG-opsonized pneumococci is required for optimal phagocytosis.

It has been established in various models that FcRn binds IgG at low pH and recycles or transports IgG through cells.8,14 IgG recycling by FcRn might, therefore, theoretically underlie the observed differences in phagocytosis. However, the differences in phagocytosis with and without FcRn engagement were more extreme at saturating IgG concentrations (Figure 6A). Although low levels of IgG recycling were observed, these were not due to FcRn expression (Figure 6B), suggesting that other mechanisms underlie the observed IgG recycling.32 Importantly, the level of IgG recycling was too low to account for significant phagocytosis...
(Figure 6A-B). IgG recycling is therefore unlikely to explain the apparent role of FcRn in phagocytosis.

Furthermore, we assessed the possibility that FcRn contributed to phagocytosis by actively mediating signals or links to the cellular IgG-transport machinery. We constructed 2 peptides; one containing a recently described tryptophan-based endocytosis motif, a serine within this motif important for apical to basolateral transport, and a separate endocytosis signal consisting of a dileucine motif which apparently functions in the context of 2 aspartic acids, and another peptide with these key amino acids scrambled (Figure 7A). Both peptides were constructed in the context of the HIV-derived TAT peptide, which mediates translocation over eukaryotic cellular membranes. The wild-type peptide, but not the scrambled control peptide, inhibited IgG-mediated phagocytosis of PMNs and monocytes, providing evidence that the intracellular FcRn tail is important for phagocytosis.

Our present data are consistent with the current paradigm within FcγR biology, where FcγR bind IgG-opsonized targets, resulting in degranulation and translocation of FcRn to nascent phagosomes. Subsequently, additional binding of FcRn to the IgG-opsonized targets may occur after phagosome acidification, which approaches pH 6.5 in around 8 minutes, and facilitate phagocytosis (summarized in Figure 8). How exactly FcRn mediates the observed effects in PMNs, monocytes, and macrophages is at present unclear. One attractive potential mechanism is that FcRn provides a direct link to molecular components of both endocytic and phagocytic machineries through association of the tryptophan motif with adaptor protein complex 2 (AP-2), and AP-2 is known to associate with clathrin (and associated molecules), both of which have been implicated in IgG-mediated phagocytosis. The nature of the molecular components and signals involved in these processes warrants more detailed studies.

The MHC class I–like family of proteins arose early in vertebrate evolution, and its members have been implicated in a variety of biologic pathways, ranging from antigen presentation of peptides and phospholipids (MHC-I, CD1), coagulant and inflammatory responses (EPCR), fat (ZAG) and iron metabolism (HFE), phenome perception, and IgG and albumin catabolism (FcRn). FcRn has been found in mammals and marsupials and is evolutionarily highly conserved, with mouse and human FcRn overall sharing 65% identical amino acid sequences. This degree of conservation includes the intracellular tail, suggesting a strong selection pressure on FcRn-encoded functions.

In summary, we observed that FcRn envelops pneumococci-containing phagosomes in PMNs in the presence of pneumococcal-specific IgG and found phagocytosis to be impaired under conditions when FcRn-binding to IgG was abrogated. We evaluated this by introducing specific mutations in IgG and by knocking out either β2M or FcRn. By interfering with select FcRn intracellular signaling motifs, a similar reduction in phagocytosis was observed. These findings implicate FcRn as directly involved in a cellular mechanism that has not been described for this molecule before, namely IgG-mediated phagocytosis. Our observations may explain why monocytes efficiently mediate phagocytosis through the ITAM-bearing FcγRIIA (CD16), whereas NK cells (that do not express FcRn, Figure 1A) are incapable of phagocytosis on triggering of the same receptor. Furthermore, the present data may well provide a rationale for the relative inability of the phagocyte IgA receptor (FcαR1, CD89) to mediate phagocytosis by IgA (that does not bind FcRn), despite its potent capacity to trigger PMN degranulation.

In conclusion, IgG-mediated phagocytosis, pathogen elimination, and the role of FcγR in phagocyte biology in particular need to be re-evaluated in light of these data.

Acknowledgments

We thank Dr Derry Roopenian for supplying FcRn knock-out mice for this study; Drs Jeffrey Beekman, Eirikur Saeland, Nina van Sorge, Frank Miedema, Ellen van der Schoot, Frank A. Redegeld, and Maurice W. van der Heijden for helpful discussions; Marc Jansen, Marco Jansen, and Henriëtte Vilé for technical assistance; and Dr Dirk Roos for critically reviewing the manuscript.

References


FcRn: an IgG receptor on phagocytes with a novel role in phagocytosis

Gestur Vidarsson, Annette M. Stemerding, Nigel M. Stapleton, Suzanne E. Spliethoff, Hans Janssen, Frank E. Rebers, Masja de Haas and Jan G. van de Winkel