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FcαRI (CD89) Alleles Determine the Proinflammatory Potential of Serum IgA

Jianming Wu,* Chuanyi Ji,* Fenglong Xie,* Carl D. Langefeld, † Kun Qian,* Andrew W. Gibson,* Jeffrey C. Edberg,* and Robert P. Kimberly2*

The human IgA FcR (FcαRI; CD89) mediates a variety of immune system functions including degranulation, endocytosis, phagocytosis, cytokine synthesis, and cytokine release. We have identified a common, nonsynonymous, single nucleotide polymorphism (SNP) in the coding region of CD89 (844A→G) (rs16986050), which changes codon 248 from AGC (Ser248) to GGC (Gly248) in the cytoplasmic domain of the receptor. The two different alleles demonstrate significantly different FcαRI-mediated intracellular calcium mobilization and degranulation in rat basophilic leukemia cells and cytokine production (IL-6 and TNF-α) in murine macrophage P388D1 cells. In the absence of FcR γ-chain association in P388D1 cells, the Ser248-FcαRI allele does not mediate cytokine production, but the Gly248-FcαRI allele retains the capacity to mediate a robust production of proinflammatory cytokine. This allele-dependent difference is also seen with FcαRI-mediated IL-6 cytokine release by human neutrophils ex vivo. These findings and the enrichment of the proinflammatory Gly248-FcαRI allele in systemic lupus erythematosus populations in two ethnic groups compared with their respective non-systemic lupus erythematosus controls suggest that FcαRI (CD89) α-chain alleles may affect receptor-mediated signaling and play an important role in the modulation of immune responses in inflammatory diseases. The Journal of Immunology, 2007, 178: 3973–3982.

Immunoglobulin A has a central role in host defense at mucosal surfaces, but its role in systemic immunity is less clear. In the intestinal lamina propria the B lineage cells produce polymeric IgA, which exists almost exclusively as a dimer joined by a J chain polypeptide and linked to the secretory component to form a complex called secretory IgA. Secretory IgA can bind FcαRI (CD89) but cannot trigger phagocytosis (1, 2). Nevertheless, through FcαRI secretory IgA is able to initiate certain inflammatory responses such as a respiratory burst in polymorphonuclear leukocytes and degranulation of eosinophils (2, 3). In human serum, IgA, the second most abundant Ig after IgG, makes up about one-fifth of the total Ig, is primarily of the IgA1 subclass, and exists mainly in monomeric form (>95%) (2). The two human IgA subclasses, IgA1 and IgA2, differ by an additional 13-aa sequence with O-linked glycosylation sites in the hinge region of IgA1 (4). The lack of 13 aa in the hinge confers on IgA2 its resistance to digestion by the bacterial proteases produced by microorganisms such as Streptococcus mutans, Neisseria meningitidis, and Haemophilus influenzae and may be the rationale for the predominance of IgA2 in mucosal secretions (5).

IgA-mediated immune effector responses such as phagocytosis, Ab-dependent cell-mediated cytotoxicity, respiratory burst, and cytokine release are primarily mediated through FcαRI (CD89), an IgA-specific receptor. FcαRI is a type I transmembrane (TM) receptor expressed on cells of the myeloid lineage including neutrophils, monocytes, tissue macrophages, eosinophils, and subpopulations of dendritic cells (6). Both monomeric and dimeric IgA can bind to FcαRI, and IgA immune complexes can activate phagocytosis and other immune responses through the clustering of FcαRI (7).

The gene encoding human FcαRI is located in the leukocyte receptor cluster on human chromosome 19q13.4 along with NK cell inhibitory receptors and leukocyte Ig-like receptors (8, 9). The FcαRI gene consists of five exons and spans ~12 kilobases. The signal peptide is encoded by first two exons, whereas the extracellular (EC) domains (EC1 and EC2) are encoded by exons 3 and 4, respectively. The last exon (exon 5) encodes the TM region and the short cytoplasmic (CYT) tail (10). Several alternatively spliced variants of cDNAs have been described, and a shorter version lacking 66 bp encoding EC2 is expressed on alveolar macrophages in vivo (11–16). A significant decrease in the ratio of the EC2 deletion splice variant to the full-length FcαRI has been found in neutrophils from pneumonia patients compared with those of healthy individuals, suggesting that the alternatively spliced FcαRI isoform might have physiologic relevance in IgA-mediated host defense (16, 17).

FcαRI shares sequence similarity with the leukocyte Ig-like receptors, killer inhibitory receptors, and other members of the leukocyte receptor cluster (8, 9). Unlike the classical low affinity FcγRIII and high affinity FcεRI, which bind their ligands via the membrane proximal EC Ig-like domain (EC2) and form 1:1 receptor:Fc complexes through interactions with hinge-proximal Fc

Abbreviations used in this paper: TM, transmembrane; EC, extracellular; AM, acetoxymethylester; [Ca2+]i, intracellular CA2+ concentration; CYT, cytoplasmic; mIgG, murine IgG; RBL, rat basophilic leukemia; SLE, systemic lupus erythematosus; SNP, single nucleotide polymorphism.

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regions (18, 19), FcαRI has its ligand binding site on the membrane distal EC1 domain and forms a 2:1 complex with an Fcα dimer by binding to each Cγ2–Cγ3 domain interface (20–25). Although FcαRI is only distantly related to FcγRI and FcεRI proteins, it does share some structural similarities and can associate with the Fcγ γ-chain through its TM domain. Unlike FcγRII and FcεRI, however, FcαRI is often expressed in the absence of γ-chain pairing (26, 27) and the function of this nonpaired FcαRI has not been defined. Indeed, the net function of FcαRI per se is unresolved, with some investigators reporting a capacity to initiate a proinflammatory program (28–30) while others report a noninflammatory profile (31–36).

Functionally important single nucleotide polymorphisms (SNPs) in the coding regions of classical FcγRs have provided significant insight into their biological functions (37, 38). As part of an effort to understand the role of naturally occurring polymorphisms in Ig receptors, we sequenced the entire coding region of FcαRI from a large number of donors and confirmed a common nonsynonymous SNP (SNP 844A→G) within the FcαRI CYT domain (39). Using this natural sequence variation as a biological probe, we have demonstrated that the two different alleles of the CYT domain of CD89 α-chain alter receptor signaling and its biological functions. In addition, we found that, in contrast to the Ser248→Thr allele of FcγRIIa, the Gly248→Ser allele of FcγRIIb is able to mediate certain biological functions in the absence of the Fcγ γ-chain. Thus, this functionally important, nonsynonymous SNP in the CYT domain not only plays an important role in the regulation of FcαRI function but may also explain some of the divergent reports on the proinflammatory vs anti-inflammatory potential of FcαRI (6).

Materials and Methods

Donors

Anti-coagulated peripheral blood was obtained from healthy normal volunteers and from SLE patients fulfilling the revised American College of Rheumatology criteria for SLE (40). SLE patients and controls were recruited as part of the University of Alabama at Birmingham DISCOVERY cohort. The human studies were reviewed and approved by the Institutional Review Board of the University of Alabama at Birmingham and all donors were provided written informed consent.

Reagents

All mAbs used in this study were murine in origin. mAbs A59 and A77 are murine IgG1 (mlgG1) specific for FcαRI (CD89; gift from Dr. H. Kubagawa, University of Alabama at Birmingham, Birmingham, AL) and the 32.2 (mlgG1) mAb is specific for the FcγRI (CD64; American Type Culture Collection). The mAbs specific for CD89 and CD64 were produced and purified from the hybridoma cultures at the University of Alabama at Birmingham Epitope Recognition and Immunoreagent Core Facility. The F(ab′)2 of the mAbs A59 and 32.2 were generated at Rockland Immunocytometry, and the purity of these Ab F(ab′)2 preparations was monitored by SDS gel staining and Western blotting assays. Anti-human FcαRI mAb MIP8a (mlgG1) was purchased from Serotec. Anti-FcγRIIa-FITC (mAb 197 (mlgG2a) and mAb 22.2 (mlgG1)) were from Medarex. F(ab′)2 of mlgG and FITC-conjugated and unconjugated goat anti-mouse IgG (H+L) were obtained from Jackson ImmunoResearch. Rabbit anti-Lyn polyclonal Ab was purchased from Santa Cruz Biotechnology. Rabbit anti-γ-chain polyclonal Ab was obtained from Upstate Biotech. An AminoLink kit (cyanoimidazole-activated Sepharose 4B coupling gel and reagents) was purchased from Pierce. Protein G-agarose and a protease inhibitor mixture were from Roche Diagnostics. Cytokine detection ELISA kits and substrates were from BD Pharmingen. Human IgA was obtained from Sigma-Aldrich (catalog no. 1010).

Nucleic acid isolation

Genomic DNA was isolated using the Puregene DNA isolation kit (Gentra Systems). Briefly, 300 μl of blood was lysed in 900 μl of the red blood cell lysis solution. The PBLs were pelleted and lysed with 300 μl of cell lysis solution. The RNase A solution was added to the cell lysate and incubated at 37°C for 15 min. Proteins were precipitated by adding 100 μl of Protein Precipitation solution (Gentra Systems). DNA in the supernatant was precipitated with 300 μl of 100% isopropanol and washed with 70% ethanol once. Total RNA was isolated from 107 PBL using TRIzol total RNA isolation reagent (Invitrogen Life Technologies). Five micrograms of total RNA was used to synthesize cDNA with the SuperScript preamplification system (Invitrogen Life Technologies).

RT-PCR and cDNA sequencing

To facilitate heterozygote detection, a dye primer strategy was adopted for fluorescence-based automated cycle sequencing of PCR products on an ABI 377 sequencer (ABI PRISM dye primer cycle sequencing 211M3 FS and M13REV FS ready reaction kits; Applied Biosystems). Two sets of overlapping primers with either M13 universal or reverse primer sequence tags (underlined letters in the sequences below) at the appropriate 5′ ends were used to amplify the whole coding region starting from nt 40 and ending at nt 903 of the FcαRI cDNA (GenBank accession no. X54150). The first set of primers amplifies from nt 34 to nt 563, yielding a PCR product 585 bp in length including the underlined M13 sequences: 5′-TG TAAAACGCGCCAGTACGATCGACCGACTCAAAAACG-3′ (annealing between nt 34 and nt 54) and 5′-CAGGAAACGCTATGACGGGTTG TCTCCACATTGTTCG-3′ (annealing between nt 542 and nt 563). The second set of primers amplifies from nt 458 to nt 934, yielding a the PCR product 513 bp in length including the underlined M13 sequences: 5′-TG TAAAACGCGCCAGTACGATCGACCGACTCAAAAACG-3′ (annealing between nt 458 and nt 472) and 5′-CAGGAAACGCTATGACGGGTTG TCTCCACATTGTTCG-3′ (annealing between nt 914 and nt 934). The PCR was performed in a 9600 PCR system with 2 μl of cDNA synthesized with the SuperScript preamplification system, 200 nM each primer, 200 μM dNTPs, 1.5 mM MgCl2, and 2.5 U of TaqDNA polymerase in a 50-μl reaction volume starting at 95°C for 5 min, 35 cycles of denaturing at 94°C for 30 s, annealing at 56°C for 45 s, and extension at 72°C for 1 min with a final extension at 72°C for 7 min. The PCR product was purified with the QiAquick gel extraction kit (Qiagen). All PCR products were sequenced in both directions.

Genotyping FcαRI CYT domain SNPs by direct sequencing

To accelerate the genotyping of FcαRI SNPs, a PCR of genomic DNA was designed for the fifth exon encoding for the TM segment and the CYT tail of FcαRI (Fig. 1A). The forward primer 5′-CCATCCACCAAGAGTATA CGA-3′ anneals between nt 692 and nt 712 and the reverse primer 5′-CT GGCCTCCTCCTGCTCACC-3′ anneals between nt 914 and nt 934 (GenBank accession no. X54150). The PCR was performed with 200 ng of DNA, 200 nM each primer, 200 μM dNTPs, 1.5 mM MgCl2, and 2.5 U of TaqDNA polymerase in a 50-μl reaction volume starting at 95°C for 5 min, 35 cycles of denaturing at 94°C for 30 s, annealing at 58°C for 45 s, and extension at 72°C for 1 min with a final extension at 72°C for 7 min. All the PCR reactions were purified from a 2.5% agarose gel with the QiAquick gel extraction kit. The purified genic PCR products from different donors were sequenced from both directions using the BigDye terminator cycle sequencing ready reaction kits on an ABI 377 (ABI PRISM BigDye terminator cycle sequencing ready reaction kits; Applied Biosystems).

Generation of the FcαRI expression constructs

The human FcαRI (CD89) expression constructs were generated by cloning KpnI/EcoRI-flanked RT-PCR products with 864 bp of the FcαRI coding region into the eukaryotic expression vector pcDNA3.1 (Invitrogen Life Technologies). The KpnI/EcoRI-flanked RT-PCR products from a heterozygous SNP 844G→A donor were generated from human mixed mononuclear cell cDNA synthesized using the SuperScript preamplification system (Invitrogen Life Technologies), with the upper primer 5′-TAAATGAGG TACCATGGACCCCAAAACAGACACCCCTC-3′ (underlined and bold nucleotides are FcαRI CD89 3′UTR cutting site) annealing between nucleotide positions 40 and 62 and lower primer 5′-TTATAGAACTCTTACCTGACGACACTTTGGTTTGGC-3′ (underlined and bold nucleotides are EcoRI cutting site) annealing between nucleotide positions 579 and 600. RT-PCR was performed with KOD HIF DNA polymerase (EMD Biosciences, distributed by Novagen). A point mutation at nucleotide position 728, which changes amino acid Arg209 to Leu209 in a TM segment, was made with the QuickChange site-directed mutagenesis kit (Stratagene) following the vendor’s instruction. For Arg209 to Leu209 constructs (nt 728 changes), the sense primer 5′-AGAACTTTGATCCATGGCCTG-3′ and anti-sense primer 5′-GCAACGGCCCATGATGATC-3′ were used on the template plasmids from FcαRI expression constructs with the 844A (Ser248→Thr) allele or the 844G (Gly248→Ser) allele (underlined, bold, and italicized letters are intentional mutations). The sequences of all
the cloned constructs were confirmed by direct sequencing from both directions on an ABI 377 sequencer with the ABI BigDye terminator cycle sequencing kit (Applied Biosystems).

**Generation of the CD64EC-TM/CD89CYT chimeric receptor expression constructs**

The expression constructs for a chimeric receptor comprised of human FcγRI (CD64) EC domains and a TM segment (CD64EC-TM) fused with the cytoplasmic (CY) domain of FcγRII (CD89) CYT domain (CD89CYT) were generated by overlapping PCR to create KpnI/BamHI-flanked RT-PCR products of chimeric cDNA. The resultant CD64EC-TM/CD89CYT coding region was inserted into pCDNA3.1 (Invitrogen Life Technologies). The CD64EC-TM cDNA fragment was amplified from human cDNA with the upper primer 5′-GGAGACAACATGTGGTTC-3′ and the lower primer 5′-ATGGGTCACCCAGAGAAC-3′ (underlined and bold nucleotides are the KpnI cutting site) annealing between nucleotide positions 778 and 797 of the CD64 EC sequences and with the upper primer 5′-GGATCCGGGGTTACCCCGGAGACAACATGTGGTTC-3′ and the lower primer 5′-ATGGGTGCTCAATTTTCTATTTGTTTGACTCACCCGAGACAACATTAGGGTCACCCAGAGAAC-3′. The PCR fragments of CD64EC-TM and CD89CYT were purified with QIAquick gel extraction kit and mixed together with the upper primer 5′-GGGAGACAACATGTGGTTC-3′ and the lower primer 5′-ATGGGTCACCCAGAGAAC-3′ (underlined and bold nucleotides are the KpnI and BamHI cutting sites) to generate RT-PCR products for the chimeric receptor. RT-PCR was performed with KOD HiFi DNA polymerase (EMD Biosciences; distributed by Novagen). The sequences of all cloned constructs were confirmed by direct sequencing from both directions on an ABI 377 sequencer with the ABI BigDye terminator cycle sequencing kit (Applied Biosystems).

**Generation of stable cell lines expressing FcγRI**

The murine macrophage cell line P388D1 and the rat mast cell line rat basophilic leukemia (RBL)-2H3 were obtained from American Type Culture Collection and maintained in DMEM supplemented with 10% FBS (FBS) and 1-glutamine (2 mM) in 5% CO₂. Individual transfections were conducted on a 60-mm cell culture dish with the cell density at 80–100% confluence. FcγRI expression construct plasmid DNA (8 μg), purified with the Wizard PureFecction plasmid DNA purification system (Promega) and the Lipofectamine 2000 reagent (12 μl), were used for transfection according to the vendor’s instructions (Invitrogen Life Technologies). Transfected cells were cultured in DMEM supplemented with 10% FCS and G418 (1 mg/ml) for the selection of stable transfectedants. The G418-resistant cells were sorted on a FACSVantage system (BD Biosciences) for the exclusive expression of all constructs in both the P388D1 and RBL-2H3 cells. At least two independent cell lines derived from independent transfections were prepared to assess receptor function for each of the individual constructs.

**Measurement of change in intracellular Ca²⁺ concentration ([Ca²⁺]i)**

Changes in [Ca²⁺], induced by the cross-linking of FcγRI with anti-FcγRI mAb (clone A59) F(ab')₂, were determined with Indo-1-aceotxymethylster (AM)-loaded ( Molecular Probes), stably transfected cells expressing FcγRI and an SLM 8000 spectrofluorometer. Briefly, cells were washed once on the plate, lifted gently from plate, and washed again with 10 ml of Ca²⁺-,Mg²+-free Hank’s buffer. Washed cells were re-suspended in Iscove’s medium plus 5% FBS at a concentration of 10⁷ cells/ml and incubated with 5 μM Indo-1 AM for 40 min at 37°C. Cell preparations to be opsonized with mAb A59 F(ab')₂ were washed, resuspended in Ca²⁺-, and Mg²+-supplemented Hank’s buffer at concentration of 10⁶ cells/ml, and incubated with saturating concentrations of A59 F(ab')₂ (5 μg/ml) for 40 min. All cells were washed and re-suspended at 5×10⁶ cells/ml in 1 mM Ca²⁺, 1 mM Mg²⁺, 1.0 mg/ml BSA, 10 mM HEPES Hank’s buffer, and then 0.4 ml of cells (2×10⁶ cells) were immediately transferred to a continuously stirring cell cuvette maintained at 37°C in the SLM 8000. With excitation at 355 nm, the simultaneous fluorescence emission at 405 and 490 nm was measured, integrated, and recorded each second. After establishing a baseline for 60 s, goat F(ab')₂, anti-mouse IgG was added at final concentrations of 20 μg/ml, and data acquisition was continued for an additional 3.5 min. Each sample was individually calibrated by lysing cells in 1% Triton X-100 to determine the maximal emission ratio and by adding EDTA (20 mM final concentration) to determine the minimal ratio. The Indo-1 fluorescence emission ratio was converted to [Ca²⁺], as described elsewhere (37).

![Diagram](http://www.jimmunol.org/Downloaded from http://www.jimmunol.org)

**FIGURE 1.** FcγRI coding region polymorphic nucleotide positions and their respective amino acid codon changes. A, The FcγRI coding region was amplified and sequenced from nucleotide position 34 to position 934 with two sets of overlapping primers indicated by arrowheads. The respective SNP sites, nucleotide substitutions, and amino acid codon changes are shown. UTR, untranslated region. B, Chromatograms of SNP 844 in the cytoplasmic (CY) domain of FcγRI showing donors homozygous for SNP 844G (lower row), homozygous for 844A (upper row), and heterozygous for 844G→A (middle row).
Table I. The distribution of FcαRI coding region SNPs in an ethnically diverse group

| Nucleotide Position | nt 363 (n = 73) | nt 376 (n = 73) | nt 679 (n = 68) | nt 836 (n = 238) |
|---------------------|----------------|----------------|----------------|----------------|
| Genotype frequency  | GG: 35 (48%)   | GG: 63 (86%)   | TT: 62 (91%)   | CC: 236 (99%)  |
|                     | GA: 30 (41%)   | GA: 10 (14%)   | TC: 6 (9%)     | CT: 2 (1%)     |
|                     | AA: 8 (11%)    | AA: 0 (0%)     | CC: 0 (0%)     | TT: 0 (0%)     |
| Allele frequency    | G: 0.68        | G: 0.93        | T: 0.96        | C: 0.99        |
|                     | A: 0.32        | A: 0.07        | C: 0.04        | T: 0.01        |

*SNPs at nt 376 and nt 836 (bold) predict a change in encoded amino acid (see Fig. 1). n, The number of donors sequenced.

Degranulation assays
RBL-2H3 cells (10⁵ cells/well) expressing either CD89 (FcαRI) or CD64EC-TM/CD89CYT chimeric receptors were cultured overnight in 24-well culture plates (Corning). The culture medium were removed from plates and the cells were incubated with DMEM containing anti-human FcαRI mAb F(ab')₁ (clone A59; final concentration of 5 μg/ml) or anti-human CD64 mAb F(ab')₂ (clone 32.2; final concentration of 2.5 μg/ml) for 45 min at 4°C. The cells were washed with Tyrode’s buffer (130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 10 mM HEPES, and 0.1% BSA (pH 7.4)) and then stimulated with goat anti-mouse F(ab')₂ (Jackson Immunoresearch Laboratories) at a final concentration of 20 μg/ml in the Tyrode’s buffer. The supernatants were collected at 0, 15, 30, 45, and 60 min for the measurement of β-hexosaminidase activity. Cells from control wells were lysed with the same volume of 0.1% Triton X-100 in Tyrode’s buffer for evaluation of the total β-hexosaminidase activity for each RBL-2H3 stable cell line. Supernatants and cell lysates were incubated with substrate (1.3 mg/ml p-nitrophenyl-N-acetyl β-D-glucosamine) (Sigma-Aldrich) in 0.1 M sodium citrate (pH 4.5) for 1 h at 37°C. The reaction was stopped by 0.2 M sodium carbonate buffer (pH 10.0) and the enzyme reactivity was evaluated by measuring OD at 405 nm. The percentage of specific β-hexosaminidase activity released was calculated as follows: percentage release = 100 × average supernatant activity from 4 wells/average cell lysate activity from four control wells.

Cytokine analysis
Cells were stimulated in 24-well tissue culture plates (Corning) with surface-bound mAb A59 F(ab')₂. Wells were coated with either anti-FcαRI
Arg209Ser248; anti-Fc

F(ab')2 (20 μg/ml) overnight at room temperature. The Fc

pg/ml in Arg209Gly248 cells and 14.66
trifuged at 12000

ml of mAb A59 F(ab')2 coated wells and cultured for 24 h. The level of murine IL-6 or TNF-α in culture supernatants was quantified by ELISA. A. Stably transfected P388D1 cell lines express each of the FcRI (CD89) allele (Ser248 and Gly248), either with (Leu209) or without TM mutations (Arg209) at equivalent levels (Arg209Ser248 = Arg209Ser248, Leu209Ser248, FcRI = Leu209Ser248, Arg209Gly248-FcRI = Arg209Gly248, Leu209Gly248-FcRI = Leu209Gly248, B, FcRI (CD89) with Arg209 to Leu209 mutations within the TM region do not associate with the FcR-γ-chain. 


tCT +/−/H9251

Affinity columns were generated through the coupling of the A77 mAb to 

Biotech). Cells (2×106 cells/ml) were lysed in PBS containing 1% Nonidet P-40 with a protease inhibitor mixture and used for immunoprecipitation and Western blotting.

Column immunoprecipitation of FcRI and detection of γ-chain

Affinity columns were generated through the coupling of the A77 mAb to the cyanoigen bromide-activated Sepharose 4B beads. P388D1 cells (106) expressing FcRI were lysed in 5 ml PBS (pH 7.2) containing 1% digitonin plus a protease inhibitor mixture (lysis buffer). The cell lysates were centrifuged at 12000×g and the supernatants were cleared by passing through an irrelevant mlgG1 isotype control column. The cleared cell lysates were then loaded onto the anti-FcRI (A77) affinity column containing 1 ml of beads and washed with 20 volumes of lysis buffer. FcRI and associated molecules captured on column were eluted with 0.1 M glycine buffer (pH 2.8) containing 0.1% digitonin. The eluted fractions were neutralized with 1 M Tris-HCl buffer (pH 8.0) immediately and analyzed with SDS-PAGE and Western blotting with a rabbit anti-Lyn polyclonal Ab (Santa Cruz Biotechnology) and a mouse anti-CD89 mAb.

Immunoprecipitation of Lyn and detection of FcRI

A rabbit anti-Lyn Ab was used to immunoprecipitate Lyn from P388D1 cells expressing different forms of FcRI. Coimmunoprecipitated FcRI was detected by Western blotting with mAb MIP8a (Serotec). Cells (2×106 cells/ml) were lysed in PBS containing 1% Nonidet P-40 with protease inhibitor mixture and used for immunoprecipitation and Western blotting.

Human neutrophil cytokine production assays

Fresh anti-coagulated blood was diluted 1/1 in HBSS and centrifuged through a two-step Ficoll-Hypaque gradient in 50-ml conical tubes (37). Neutrophils were harvested from the lower Ficoll-Hypaque interface and washed three times with HBSS containing 1% RPMI 1640 medium. The

mAb A59 F(ab')2 (20 μg/ml) or control mlgG F(ab')2 (20 μg/ml) overnight at room temperature. The FcRI-expressing cells were added to coated wells and the culture media were collected after 24 h. The levels of murine IL-6 or TNF-α in culture medium were quantified by ELISA (BD Pharmingen).

Immunoprecipitation of FcRI and detection of γ-chain

FcRI was immunoprecipitated from the transfected cell lines using either mAb MIP8a (Serotec) or mAb A77 prebound to protein G-agarose. The γ-Chain was detected with polyclonal rabbit anti-γ-chain Abs (Upstate Biotech). Cells (2×10⁶ cells/ml) were lysed in PBS containing 1% Nonidet P-40 with a protease inhibitor mixture and used for immunoprecipitation. Immunoprecipitates were analyzed with SDS-PAGE and Western blotting.

Column immunoprecipitation of FcRI and detection of Lyn

Affinity columns were generated through the coupling of the A77 mAb to the cyanogen bromide-activated Sepharose 4B beads. P388D1 cells (10⁶) expressing FcRI were lysed in 5 ml PBS (pH 7.2) containing 1% digitonin plus a protease inhibitor mixture (lysis buffer). The cell lysates were centrifuged at 12000×g and the supernatants were cleared by passing through
residual RBCs were lysed with Paregene red blood lysis solution (Gentra Systems). The resulting neutrophils were washed twice with HBSS. The cells were then counted and resuspended in RPMI 1640 medium supplemented with 10% FBS and 10 mM HEPES (pH 7.2). The neutrophils were stimulated in 12-well tissue culture plates (Corning) previously coated overnight with anti-FcRI mAb A59 F(ab')2 (20 μg/ml), mlgG1 F(ab')2 (20 μg/ml), or human IgA (20 μg/ml). The wells were washed twice with HBSS before use and 2 × 10^6 neutrophils in 1 ml of culture medium were used for each well. The cells were incubated at 37°C in 5% CO2 for 24 h and the culture supernatants were collected for cytokine determination. The levels of human IL-6 in culture medium were quantified by ELISA (BD Pharmingen).

**Flow cytometry**

Aliquots of 5 × 10^6 cells in 0.1 ml PBS were incubated with saturating concentrations of primary mAb for 30 min at 4°C followed by two washes. For indirect immunofluorescence, the cells were then incubated with saturating concentrations of FITC-conjugated goat anti-mouse IgG F(ab')2 at 4°C for another 30 min. After washing, the cells were analyzed immediately for immunofluorescence intensity using a FACScan (BD Biosciences).

**Data analysis**

Student’s t test, unpaired or paired according to experimental design, was used to analyze the data for degranulation, calcium influx, and cytokine production. The paired t test was used to compare means between conditions in matched pair design. The unpaired t test was used to compare means between samples of donors homozgyous for the different alleles. The Mantel-Haenszel χ^2 test was used to test for differences in the genotype and allele frequencies in affected donors and controls both in African Americans and in the combined genetic data from two ethnic groups (Caucasian and African American). The association between genotype and the human lupus phenotype in Caucasians was tested with Fisher’s exact test because of the low frequency number in one cell.

**Results**

**SNP detection in FcαRI coding region**

Two sets of overlapping RT-PCRs were used to amplify the full length of the FcαRI coding region and the purified PCR products were directly sequenced. As shown in Fig. 1A, we identified five SNPs located at nucleotide positions 363 (rs18650996), 376 (rs11666735), 679, 836, and 844 in the coding region of FcαRI. The SNPs 363G→A and 679T→C are silent polymorphisms that change an arginine codon from AGA to AGG and a leucine codon from TTG to CTG, respectively (Table I). Nonsynonymous SNPs at 836 and at 376 change a proline codon (CCG) to a leucine (CTG) and an aspartate (GAC) to asparagine (AAC), respectively, both have minor allele frequencies <0.10. More importantly for the purposes of this study, we confirmed that the SNP 844A→G transition that changes amino acid codon 248 from serine (AGC) to glycine (GGC) is a more common polymorphism located in the CYT domain of CD89 (Fig. 1) (39).

The Gly^{248} and Ser^{248} alleles of FcαRI differ in their capacity for receptor-mediated calcium mobilization

FcαRI can associate with the FcR γ-chain that contains an ITAM (41). Upon Ab-mediated cross-linking of FcαRI or binding of IgA immune complexes to FcαRI, the tyrosines of the γ-chain ITAM are phosphorylated by Src family kinases and the tyrosine-based signaling cascade is initiated (42). Because the CYT domain of FcγRIII (CD16) influences receptor-mediated intracellular calcium mobilization (43), we hypothesized that the two FcαRI CYT domain alleles might also affect the receptor signaling. Using stably transfected RBL cells sorted for the equivalent expression of each of the two FcαRI alleles on the cell surface to ensure equivalent binding of the stimulant (Fig. 2A), we tested comparative receptor-initiated calcium signaling. RBL cells expressing FcαRI were opsonized with anti-FcαRI mAb A59 F(ab')2. When the goat anti-mouse IgG (H+L) F(ab')2 was added to cross-link the FcαRI, cells expressing the Gly^{248}-FcαRI allele were more efficient in mediating intracellular calcium release than Ser^{248}-FcαRI allele (Fig. 2B).

![FIGURE 4. Association of Lyn with FcαRI through the CYT domain.](http://www.jimmunol.org/)

**The Gly^{248} and Ser^{248} alleles of FcαRI differ in their capacity for receptor-mediated degranulation**

FcαRI is expressed on myeloid cells including neutrophils, eosinophils, monocytes, dendritic cells, and tissue macrophages. FcαRI can initiate potent immune responses such as degranulation, endocytosis, phagocytosis, Ab-dependent cellular cytotoxicity, Ag presentation, and the release of inflammatory mediators. Because of the importance of receptor-mediated degranulation, we investigated whether the two alleles of FcαRI mediate comparable degranulation in RBL cells. Despite comparable expression of Ser^{248}-FcαRI and Gly^{248}-FcαRI, cross-linking of the FcαRI on the RBL cell induced significantly more degranulation with the Gly^{248}-FcαRI allele than with the Ser^{248} allele (Fig. 2C). To confirm that this effect was dependent on the alleles of the FcαRI α-chain CYT domain, we created FcγRI/FcαRI chimeric receptors consisting of FcγRIa (CD64) EC and TM domains fused with 41 residues of the FcαRI CYT domain (CD64EC-TM/CD89CYT). Stably transfected RBL cells expressed comparable levels of...
CD64EC-TM/CD89CYT chimeric receptors on the cell surface (data not shown). Remarkably, similar to native FcεRI receptors, cross-linking of the chimeric receptors induced significantly more degranulation in RBL cells expressing the CD64EC-TM/CD89CYT receptor carrying a Gly248-FcεRI allele than in cells expressing CD64EC-TM/CD89CYT carrying a Ser248-FcεRI allele (Fig. 2D). These data demonstrate that the FcεRI (CD89) α-chain CYT domain influences the receptor signaling capacity. Furthermore, the two naturally occurring alleles of the FcεRI α-chain CYT domain significantly alter the receptor’s degranulation function.

The Gly248 and Ser248 alleles of FcεRI alter receptor-mediated cytokine release

Receptor-mediated cytokine release plays an important role in modulation of the immune system. To compare the effects of the FcεRI alleles on cytokine release, we generated stable P388D1 cell lines with comparable expression of the different FcεRI alleles (Ser248-FcεRI or Gly248-FcεRI) with or without mutations within the TM domain to alter γ-chain association (Arg209 or Leu209; Fig. 3A). In contrast to transfecants with the wild-type Arg209 TM sequence of FcεRI, the TM mutants carrying Leu209 lost their association with the endogenous FcεR γ-chain (Fig. 3B). Cross-linking of the FcεRI on cells expressing the Gly248-FcεRI allele induced significantly more IL-6 and TNF-α release than that induced with cells expressing the Ser248-FcεRI allele (Fig. 3C, upper panel for IL-6 and lower panel for TNF-α). Because FcεRI not paired with an FcR γ-chain represents the major fraction of FcεRI molecules expressed on the cell surfaces of human monocytes and neutrophils (26), we also determined whether the “γ-chainless” FcεRI might be able to mediate cytokine production and whether there might be a difference in cytokine release between the Ser248-FcεRI and Gly248-FcεRI alleles in the absence of the FcεR γ-chain. As shown in Fig. 3, A and B, cells expressing the Ser248-FcεRI and Gly248-FcεRI alleles with the TM Leu209 mutation showed comparable receptor expression. In the absence of FcεR γ-chain pairing, stimulation of the Ser248-FcεRI allele receptor was unable to induce IL-6 and TNF-α release (two left bars in Fig. 3D, upper panel for IL-6 and lower panel for TNF-α, respectively). Surprisingly, however, stimulation of the Gly248-FcεRI allele induces significant release of IL-6 and TNF-α in the absence of the FcεR γ-chain pairing (two right bars in Fig. 3D, upper panel for IL-6 and lower panel for TNF-α, respectively). These data demonstrate that the CD89 α-chain, and specifically the Gly248-FcεRI allele of the receptor, maintains signaling capacity in the absence of the γ-chain. The data further demonstrate that the alleles of the FcεRI α-chain CYT domain do not require the presence of the FcεR γ-chain to significantly influence receptor functions and raise the possibility of another molecular partner mediating this function.

Lyn association with FcεRI is allele sensitive

The cross-linking of FcεR activates Src family kinase activity and induces tyrosine phosphorylation of several cellular proteins, including p72Syk, a major target of early protein tyrosine kinase activity. The Src family member Lyn coprecipitates with aggregated FcεRI complexes and is one of the protein tyrosine kinases involved in receptor-mediated signaling (44). These observations prompted us to investigate whether Lyn might associate with FcεRI independent of the γ-chain and provide an explanation for the activity of Gly248-FcεRI. We used three independent assays to assess the association of FcεRI with Lyn. First, we generated a GST-CD89 CYT tail fusion protein to confirm that Lyn is associating with FcεRI. Fig. 4A demonstrates that the FcεRI CYT domain, fused with GST, is able to bind Lyn in P388D1 cell lysates. GST-CD89CYT was also able to pull down Lyn from 293 cells and U937 cells (data not shown), demonstrating that Lyn associates with FcεRI through the FcεRI CYT tail. Second, we used a column-based immunoprecipitation assay to establish the relationship between Lyn and FcεRI. Lynsates from cell lines expressing four different constructs of FcεRI at similar levels (Fig. 3A) were precleared on irrelevant mlgG1 isotype control columns. The precleared cell lysates were then passed over

FIGURE 5. The FcεRI alleles affect receptor mediated IL-6 cytokine release from human neutrophils. A, IL-6 release induced by anti-FcεRI mAb A59 F(ab’)2, on neutrophils from homozygous donors of either Ser248 or Gly248 alleles. The level of IL-6 release was significantly higher in A59 F(ab’)2-treated neutrophils from the Gly248 homozygous donors compared with the Ser248 homozygous donors (8.66 ± 1.85 pg/ml for the Gly248 homozygous donors and 2.34 ± 1.32 pg/ml for the Ser248 homozygous donors respectively; p = 0.032 from t test; n = 4). The IL-6 release was not significantly different between Ser248 homozygous donors and Gly248 homozygous donors in mlgG1 F(ab’)2-treated neutrophils (p = 0.598 from t test; n = 4). B, IL-6 release induced by the treatment of neutrophils with human IgA. The levels of IL-6 release by neutrophils from the Gly248 homozygous donors was significantly higher compared with the Ser248 homozygous donors (14.62 ± 4.81 pg/ml for the Gly248 homozygous donors and 0.2 ± 0.1 pg/ml for the Ser248 allele homozygous donors respectively; p = 0.040 from t test; n = 3). Additionally, human IgA significantly inhibited IL-6 release from neutrophils in all Ser248 homozygous donors (5.08 ± 0.66 pg/ml in the untreated neutrophils to 0.20 ± 0.10 pg/ml in the human IgA treated neutrophils; p = 0.015 from paired t test; n = 3), whereas all Gly248 homozygous donors showed an increase (4.19 ± 0.33 pg/ml in the untreated neutrophils to 14.62 ± 4.81 pg/ml; p = 0.177 from paired t test; n = 3).
columns packed with agarose beads conjugated with the anti-FcRI mAb A77. The proteins eluted from these affinity columns were probed with rabbit anti-Lyn Abs. The bottom panel of Fig. 4B shows that the levels of CD89 (FcRI) eluted from the columns were similar among four different cell lines. However, more Lyn was isolated from lysates with the Gly248-FcRI than with the Ser248-FcRI allele in the presence of γ-chain association (upper panel of Fig. 4B, lanes 1 and 2). This difference was also pronounced in the TM mutants carrying Leu209 that are unable to pair with the FcR γ-chain (upper panel of Fig. 4B, lanes 3 and 4). No CD89 or Lyn could be detected by Western blotting in eluates from the mlgG1 control columns (data not shown). These data establish the specificity of interaction between Lyn and FcRI and show the quantitative differences in the association between Lyn and two alleles of FcRI.

Finally, we immunoprecipitated Lyn using rabbit anti-Lyn Abs and performed anti-FcRI immunoblot analysis to examine the amount of receptor associated with Lyn. Although a comparable amount of Lyn was immunoprecipitated from each of the different cell lines expressing FcRI constructs (Fig. 4C, bottom panel), Lyn was able to pull down significantly more Gly248-FcRI protein (Fig. 4C, lane 2) than Ser248-FcRI protein (lane 1) in the presence of γ-chain association. To further demonstrate the dependence of the association between FcRI and Lyn on the FcRI α-chain, cell lines expressing similar levels of Leu209 TM mutant FcRI were used for coimmunoprecipitation. Although the Leu209 TM mutant receptor of both FcRI alleles did not associate with endogenous γ-chains (Fig. 3B, upper panel; Fig. 4B, middle panel), the Gly248-FcRI readily coimmunoprecipitated with Lyn (Fig. 4C, upper panel, lane 4). In contrast, very little if any of the Ser248-FcRI allele receptor (Fig. 4C, upper panel, lane 3) was evident.

The Ser248 and Gly248 alleles of FcRI affect receptor mediated IL-6 cytokine release by human neutrophils

Because human neutrophils are the primary cell population constitutively expressing high levels of FcRI, we compared the effects of the FcRI alleles on IL-6 release by neutrophils from the genotyped homozygous donors. Neutrophils from donors of both genotypes expressed comparable levels of FcRI (data not shown). Consistent with the results obtained from the transfected P388D1 cell lines, cross-linking of the FcRI with A59 F(ab′)2 induced significantly more IL-6 release by neutrophils from Gly248-FcRI homozygous donors compared with the Ser248-FcRI homozygous donors (Fig. 5A; p < 0.05). Human IgA-mediated cross-linking of the receptors also induced significantly more IL-6 release by neutrophils from Gly248-FcRI allele homozygous donors compared with the Ser248-FcRI allele homozygous donors (Fig. 5B; p < 0.05). Interestingly, the cross-linking of FcRI in the Ser248-FcRI homozygous donors significantly inhibited IL-6 release in human IgA-stimulated neutrophils (p = 0.015; Fig. 5B). Taken together, our data demonstrate that the alleles of FcRI CYT domain significantly affect receptor mediated cytokine release by primary human neutrophils ex vivo.

The Gly248-FcRI allele and SLE

Because FcRI can activate a variety of cell programs in myeloid cells and IgA autoantibodies have received increasing attention (45, 46), we speculated that the proinflammatory FcRI alleles with their impact on receptor signaling might be associated with inflammatory diseases. We compared the genotype frequencies in African American and European American controls and found a significant enrichment of Gly248-FcRI in African Americans compared with European Americans (Table II; p < 0.01 from Fisher’s exact test). Furthermore, in African Americans the Gly248-FcRI allele was significantly enriched in SLE patients compared with corresponding normal controls (Mantel-Haenszel χ2 = 0.0334). A similar tendency toward an increased Gly248-FcRI allele frequency was evident in European American SLE patients compared with controls and was most evident in a dominant genetic model with enrichment of homozygosity of the Gly248-FcRI genotype (p < 0.05 from Fisher’s exact test). In a combined analysis to test the significance of enrichment of the Gly248-FcRI allele in both populations, we found this allele significantly enriched in SLE populations compared with the control populations (Mantel-Haenszel χ2 = 0.0194). These data suggest that the Gly248-FcRI allele may be considered a candidate risk factor for autoimmune disease, such as SLE.

Discussion

The two naturally occurring alleles of human FcRI, Ser248-FcRI and Gly248-FcRI, differ in their quantitative functions and, remarkably, unlike Ser248-FcRI, the Gly248 allele is capable of inducing cytokine release even in the absence of FcR γ-chain association. This capacity is presumably due, at least in part, to its ability to bind Lyn. This association of a Lyn kinase with FcRI may be mediated by an unconventional Src homology 3 domain in the CYT domain of FcRI comprised of the peptide sequence Trp247(Ser248/Gly248)Gln249Gln250, which includes the polymorphic site. This sequence is similar to the unconventional Src homology 3 domain binding motif (WxxQxF/Y) frequently found in peroxisomal membrane proteins (47–49). Thus, it seems that

Table II. The distribution of FcRI SNP 844 (Ser248/Gly248) alleles in SLE patients and non-SLE normal controls

| Genotype frequency | African American | Caucasian |
|-------------------|-----------------|-----------|
| AA                | 40 (40%)        | 63 (69%)  |
| AG                | 47 (48%)        | 22 (24%)  |
| GG                | 12 (12%)        | 6 (8%)    |
| Allele frequency |                 |           |
| A                 | 0.64            | 0.81      |
| G                 | 0.36            | 0.19      |

*The genotype and allele frequencies in African American SLE patients were significantly different from the African American controls (Mantel-Haenszel χ2, p = 0.0334). A similar tendency toward enrichment of the 844G allele in Caucasian SLE patients compared to Caucasian controls is most evident in a dominant model with enrichment of 844G homozygotes in Caucasian SLE patients (Fisher’s exact test, p < 0.05). In a combined analysis, the 844G allele is significantly enriched in SLE populations compared to control populations of both ethnicities (Mantel-Haenszel χ2, p = 0.0194).
Gly<sup>248</sup>-FcαRI may have a constant potential for an activating and perhaps proinflammatory role, whereas the Ser<sup>248</sup>-FcαRI allele requires FcαRI:FcRγ chain pairing for the induction of cytokine release. Given that the net biological activity of FcαRI, therefore, is dependent on both the genotype of the donor and the extent of FcαRI:FcRγ chain pairing, it is not surprising that investigators have drawn divergent conclusions about the activating potential of FcαRI-initiated activity (33, 34).

The difference in the biological potential of the two alleles provides a tool for probing the idea that the balance of FcαRI-initiated activating/inhibitory activities might reflect these naturally occurring alleles and be important in setting thresholds for inflammatory responses (36). Accordingly, we reasoned that FcαRI alleles might play a role in the pathophysiology of disease. Somewhat to our surprise, the Gly<sup>248</sup>-FcαRI allele is over represented in systemic lupus. Although the mean IgA1 and total IgA in samples from SLE patients are significantly higher than those from the normal population (50), IgA Abs have received little attention in SLE. IgA anti-Ro/SSA, anti-La/SSB, anti-cardiolipin, and anti-β2-glycoprotein-I autoantibodies are present in the serum of patients with SLE, Sjogren’s syndrome, and anti-cardiolipin syndrome (45, 51, 52) and, very recently, reports indicate that not only EBV seropositivity but also a specific IgA Ab against EBV are associated with SLE patients (46, 53). The reports of IgA autoantibodies and IgA anti-EBV seropositivity suggest a provocative and perhaps underappreciated link in which a more activating host response to IgA might influence an autoimmune and inflammatory response. The differential biologies of the Ser<sup>248</sup>-FcαRI and Gly<sup>248</sup>-FcαRI alleles could also play a role in other phenotypes involving IgA, including dermatitis herpetiformis, celiac disease, periodontal disease, and possibly IgA nephropathy. Even the uptake and inactivation of virus, demonstrated in epithelial cells with anti-HIV IgA, might be impacted by CD89 alleles (54). Because mice do not have FcαR, the gene encoding CD89, clues will not be forthcoming from studies of spontaneous mouse phenotypes but will need to be done in humans or, possibly, in mice reconstituted with human immune systems (55, 56).

Despite abundant mucosal and serum IgA, the overall role of IgA and FcαRI in host defense and immune regulation remains a matter of some controversy. As noted, some studies have suggested an anti-inflammatory function for IgA and FcαR, as demonstrated in the suppression of neutrophil chemotaxis, down-regulation of the generation of reactive oxygen species, and the induction of the anti-inflammatory cytokine IL-1ra (32, 34). Because IgA in the serum does not fix complement by the classical pathway, IgA is thought to act as a “discrete housekeeper” in which foreign Ags are incorporated into immune complexes by IgA and removed by the phagocytic system, with little or no resultant inflammation (57). However, FcαRI is a multichain immunoreceptor receptor that can associate with the ITAM-containing FcRγ chain subunit. The resultant trimeric FcαRI(FcRγ chain) initiates established phosphorylase-based signaling cascades, and this model is compatible with an activating, proinflammatory receptor (30, 41). The observation that the majority of FcαRI expressed on human cells are not associated with γ chain (26), however, makes FcαRI distinct from FcγRI and FcγRIII, and the apparent association of Gly<sup>248</sup>-FcαRI with Lyn provides an alternative mechanism for initiating cell programs in an allele-sensitive fashion. The intensity of cytokine production stimulated through Gly<sup>248</sup>-FcαRI alone is less than that through the FcγRI: FcRγ chain, and these two signaling assemblies may have qualitative as well as quantitative differences in their capacities. Our data also suggest that IgA may inhibit IL6 production by neutrophils from donors carry the Ser<sup>248</sup> allele of FcαRI. This inhibitory role may be related to the inhibitory functions of the FcRγ chain recently described by Pasquier et al. (36) and by Olas et al. (58). Although the positively charged arginine in the TM domain of FcαRI is required for the pairing with the FcRγ chain, little is known about the mechanisms regulating the pairing of FcαRI:FcRγ chain. This step may be an important element in the understanding of overall FcαRI biology.

Our genotyping data suggest that the proinflammatory 844G allele (Gly<sup>248</sup>-FcαRI) may be associated with SLE in African American and Caucasian populations, raising the possibility that IgA Ab production may play an important role in modulating the immune responses. This association needs to be extended in independent case control and family-based association studies, and linkage disequilibrium with other susceptibility gene(s) in this region must also be evaluated. However, based on the reports that FcγRs contribute to the susceptibility to human autoimmune diseases beyond SLE, genetic associations of FcαRI variants with other human inflammatory diseases, perhaps conditioned on other genetic effects and stratified by different ethnic groups, are likely to be identified.

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Disclosures

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