

# Role of Novel Rat-specific Fc Receptor in Macrophage Activation Associated with Crescentic Glomerulonephritis\*

Received for publication, May 13, 2011, and in revised form, October 27, 2011. Published, JBC Papers in Press, December 19, 2011, DOI 10.1074/jbc.M111.260695

Theresa H. Page<sup>‡</sup>, Zelpha D'Souza<sup>§</sup>, Satoshi Nakanishi<sup>¶</sup>, Tadao Serikawa<sup>¶</sup>, Charles D. Pusey<sup>||</sup>, Timothy J. Aitman<sup>§</sup>, H. Terence Cook<sup>\*\*</sup>, and Jacques Behmoaras<sup>\*\*1</sup>

From the <sup>‡</sup>Kennedy Institute of Rheumatology Division, Imperial College London, London W6 8LH, United Kingdom, the <sup>§</sup>Medical Research Council Clinical Sciences Centre, the <sup>||</sup>Renal Department, and the <sup>\*\*</sup>Centre for Complement and Inflammation Research Imperial College London, Hammersmith Hospital, W12 0NN London, United Kingdom, and the <sup>¶</sup>Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, Yoshidakano-cho, Sakyo-ku, Kyoto 606-8501, Japan

**Background:** Fc receptor-mediated macrophage activation is a major cause of glomerular damage in crescentic glomerulonephritis.

**Results:** We investigated the role of a novel rat Fc receptor, Fcgr3-rs, in human and rat macrophage activation.

**Conclusion:** We showed that this receptor prevents the cell signaling of its paralogue (Fcgr3).

**Significance:** These results provide a novel way to inhibit Fc receptor-mediated cell activation in macrophages.

Crescentic glomerulonephritis (Crgn) is a complex disease where the initial insult is often the glomerular deposition of antibodies against intrinsic or deposited antigens in the glomerulus. The role of Fc receptors in the induction and progression of Crgn is increasingly recognized, and our previous studies have shown that copy number variation in Fcgr3 partially explains the genetic susceptibility of the Wistar-Kyoto (WKY) rat to nephrotoxic nephritis, a rat model of Crgn. The Fcgr3-related sequence (Fcgr3-rs) is a novel rat-specific Fc receptor with a cytoplasmic domain 6 amino acids longer than its paralogue, Fcgr3. The Fcgr3-rs gene is deleted from the WKY rat genome, and this deletion is associated with enhanced macrophage activity in this strain. Here, we investigated the mechanism by which the deletion of Fcgr3-rs in the WKY strain leads to increased macrophage activation. By lentivirus-mediated gene delivery, we generated stably transduced U937 cells expressing either Fcgr3-rs or Fcgr3. In these cells, which lack endogenous Fcgr3 receptors, we show that Fcgr3-rs interacts with the common Fc- $\gamma$  chain but that Fc receptor-mediated phagocytosis and signaling are defective. Furthermore, in primary macrophages, expression of Fcgr3-rs inhibits Fc receptor-mediated functions, because WKY bone marrow-derived macrophages transduced with Fcgr3-rs had significantly reduced phagocytic activity. This inhibitory effect on phagocytosis was mediated by the novel cytoplasmic domain of Fcgr3-rs. These results suggest that Fcgr3-rs may act to inhibit Fcgr3-mediated signaling and phagocytosis and could be considered as a novel mechanism in the modulation of Fc receptor-mediated cell activation in autoimmune diseases.

In crescentic glomerulonephritis, leukocytes, especially monocytes/macrophages, infiltrating the glomerulus play a critical role in the development of the disease by recognizing immunoglobulins and complement factors via cell surface receptors (1). One of the checkpoints regulating the immune response in glomerular inflammation is through leukocyte cell surface receptors for the Fc (fragment crystallizable) portion of IgG. The family of Fc receptors can regulate the fine balance between pro-inflammatory *versus* anti-inflammatory states of cell activation, and there is increasing evidence suggesting that genetic susceptibility to autoimmune diseases is partly dependent on Fc receptor-mediated activating and inhibitory signaling pathways determining the net signal (pro- or anti-inflammatory) in the progression of the disease (2, 3). In systemic lupus erythematosus, aberrant expression or the presence of allelic variants of Fc $\gamma$ R with altered functionality have been reported to contribute to the pathogenesis of the disease (4).

There are two distinct classes of Fc receptors: the activatory and the inhibitory receptors. Most activatory receptors serve as the ligand-binding component of a receptor that also includes a signal transducing molecule containing an immunoreceptor tyrosine-based activation motif (ITAM)<sup>2</sup> (5). In monocytes and macrophages, this role is fulfilled by the common  $\gamma$  chain. Signaling via its ITAM motif triggers the activation of multiple downstream signaling pathways including the activation of Src and Syk kinases, calcium mobilization, and NF- $\kappa$ B activation, leading ultimately to cellular responses such as phagocytosis and cytokine production. The importance of activatory Fc $\gamma$  receptors in glomerulonephritis has been shown using mice engineered to lack the ITAM-bearing Fc receptor  $\gamma$  chain. We and others have shown that these mice are protected from glomerulonephritis induced by antibodies to glomerular basement membrane (6–8). In contrast, the inhibitory Fc receptor Fc $\gamma$ RIIB has an immunoreceptor tyrosine-based inhibitory

\* This work was supported by intramural funding from the Wellcome Trust, Clinical Sciences Centre, and the United Kingdom Medical Research Council. Recipient of an Imperial College Junior Research Fellowship.

⌘ Author's Choice—Final version full access.

<sup>1</sup> To whom correspondence should be addressed: Centre for Complement and Inflammation Research, Imperial College London, Hammersmith Hospital, Du Cane Rd., W12 0NN London, UK. Tel.: 44-20-8383-2339; Fax: 44-20-8383-8577; E-mail: Jacquesb@imperial.ac.uk.

<sup>2</sup> The abbreviations used are: ITAM, immunoreceptor tyrosine-based activation motif; FcR, Fc receptor; BMDC, bone marrow-derived macrophage; NTN, nephrotoxic nephritis; Crgn, Crescentic glomerulonephritis; WKY, Wistar-Kyoto; qRT, quantitative RT; PMA, phorbol 12-myristate 13-acetate.

motif and does not use the common  $\gamma$  chain (3). Activatory and inhibitory Fc $\gamma$  receptors are often expressed on the same cell, and when co-aggregated by IgG antibody, the net signal to the cell depends on the sum total of the activator and inhibitor signals, thus setting thresholds for effector cell responses (3).

In our previous studies of nephrotoxic nephritis (NTN), a model of crescentic glomerulonephritis in the Wistar-Kyoto (WKY) rat, we performed genome-wide linkage analysis for glomerular crescents, macrophage infiltration, and proteinuria in an F2 population derived from NTN-susceptible WKY and NTN-resistant Lewis rats (9). The most significant linkage (logarithm of odds > 8) was obtained with a quantitative trait locus mapping to chromosome 13 (*Crgn1*) accounting, respectively, for 21.8, 16.7, and 12.9% of the genetic variance in crescent formation, proteinuria, and macrophage infiltration. Positional cloning of *Crgn1* led to the identification and functional characterization of the *Fcgr3*-related sequence (*Fcgr3-rs*), a novel rat-specific activatory Fc receptor arising from copy number variation in the *Fcgr3* gene (9). The genomic rearrangement in the *Fcgr3* gene is such that there is a duplication of exon 5 in the NTN-resistant Lewis genomic DNA with the presence of a shorter exon lacking a sequence of 226 bp in its 3'-untranslated region. This shorter exon 5 was deleted in the WKY genome, suggesting a copy number variation in the *Fcgr3* gene. Sequence analysis of this shorter copy of exon 5 revealed deletion of a single guanine nucleotide at position 129 ( $\Delta$ G129) in the coding sequence of the cytoplasmic domain, resulting in a frameshift and generating a novel cytoplasmic domain 6 amino acids longer than that encoded by *Fcgr3*. We designated this copy number variant *Fcgr3*-related sequence (*Fcgr3-rs*) and showed that this novel variant is transcribed and translated in the Lewis strain, whereas it is deleted from the WKY genome (9).

Given the increased macrophage activity and associated NTN susceptibility in the WKY rat that lacks the *Fcgr3-rs* molecule, it is important to understand how the presence of this molecule might be influencing macrophage activity. Thus, we have studied the role of *Fcgr3-rs* in macrophage activation. We first investigated whether the genomic duplication/deletion event giving rise to *Fcgr3-rs* occurred during the derivation of inbred Wistar-related colonies. We have then generated U937 cells stably expressing either *Fcgr3* or *Fcgr3-rs* and have studied the role of *Fcgr3-rs* in macrophage activation both in U937 cells and WKY bone marrow-derived macrophages (BMDMs) transduced with *Fcgr3-rs*. Our results show that the absence of *Fcgr3-rs* is not specific to the WKY strain but is widely distributed throughout the rat phylogenetic tree. Furthermore, in both human monocyte cell lines and primary rat macrophages, expression of *Fcgr3-rs* inhibits *Fcgr3*-mediated signaling and phagocytosis, suggesting that the rat possesses a unique mechanism by which Fc receptor-mediated cell activation can be modulated.

## EXPERIMENTAL PROCEDURES

**Animals**—WKY (WKY/NCr1) rats were purchased from Charles River. All of the procedures were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act.

**Reagents**—RPMI 1640 containing 25 mM L-glutamine was purchased from Biowhittaker (Berkshire, UK). FCS was from PAA (Yeovil, UK) or Labtech (East Sussex, UK). All of the growth media were supplemented with penicillin (100 units/ml) and streptomycin (100  $\mu$ g/ml), supplied as a 100 $\times$  sterile solution from Biowhittaker. LPS was obtained from Sigma. Antibodies were from Millipore (anti-Myc), Santa Cruz (F(ab')<sub>2</sub>, anti-Syk, anti-CD16), GE Healthcare (HRP-conjugated antibodies for Western blot), or Upstate Biotech (anti- $\gamma$  chain). pCSGW vector was a gift from Richard Jenner (University College London, London, UK).

***Fcgr3* Exon 5 Genotyping**—*Fcgr3* exon 5 genotype was determined by PCR and agarose gel electrophoresis by using primers to amplify exon 5 genomic DNA from 134 laboratory rat strains and substrains available in the National BioResource project for the rat in Japan. The primer sequence used is as follows: 5'-GTCCCTAAATTCTGAATTC-3' and 5'-AAAGAAGT-CACAGAAAGGAG-3'.

**Lentiviral Constructs**—*Fcgr3* and *Fcgr3-rs* were amplified from Lewis spleen cDNA with forward primer 5'-AAGCTTGCCACCATGACTTTGGAG-3' and reverse primer 5'-TCTAGATTATTAGCCATACGATGGGAT-3'. Clones were reamplified with forward primer 5'-CGGAAGCTTGCCACCATGAGCAGAAACTCATCTCTGAAGAGGATCTGACTTTGAGACCCAGATGTTTCAG-3' and reverse primer 5'-CCTTGAGCACCTGGATCCATGGGG-3' and recloned. Site-directed mutagenesis was performed on the *Fcgr3* construct for  $\Delta$ G129 with primers 5'-GAGAAATCTTCAAACCTCGGGGAGGACTGGAGGAAATCCC-3' (forward) and 5'-GGGATTTCTCCAGTCCCTCCCCGAGGTTTGAAGATTTCTC-3' (reverse). All  $\alpha$  subunit constructs were cloned into the pcDNA3.1/Hygro(+) expression vector (Invitrogen). For inserting an N-terminal Myc tag into the *Fcgr3* constructs, 1.0  $\mu$ g of the *Fcgr3*, *Fcgr3-rs*, and *Fcgr3*- $\Delta$ G constructs were amplified with forward primer 5'-CGGAAGCTTGCCACCATGAGCAGAAACTCATCTCTGAAGAGGATCTGACTTTGGAGACCCAGATGTTTCAG-3' and reverse primer 5'-CCTTGAGCACCTGGATCCATGGGG-3'. Constructs and PCR products were cut with HindIII and BamHI and ligated to each other. Lentivirus constructs were prepared as follows: DNA was excised from pcDNA3.1 by digestion with HindIII/XbaI, and blunt ends were created using *Pfu* DNA polymerase. This was ligated into a SmaI cut pENTR4.3F. This "in house" modified version of the pENTR4 gateway vector contains a multiple cloning site downstream of a CMV promoter and upstream of an internal ribosome entry sequence, which mediates expression of an enhanced GFP sequence. Inserts in the correct orientation were identified by diagnostic restriction enzyme digests. Subsequent digestion of this vector with SalI and NotI released a DNA "cassette" containing the "gene of interest" internal ribosome entry sequence-EGP sequences. This was then cloned into the pCSGW lentiviral transfer vector (10) following removal of the vectors own GFP sequence.

**Lentiviral Production and Titration**—Transfer vector (pCSGW), lentiviral mammalian expression vector (envelope, pMD2.G), and packaging vector (psPAX2) were mixed at a ratio of 4:3:1 and used to infect confluent monolayers of 293T17 cells by calcium phosphate precipitation. The medium was replaced

## Rat *Fcgr3*-rs Inhibits FcR Signaling and Cytokine Production

after 16 h. Media containing virus were collected after a subsequent 24 and 48 h of culture. Insoluble debris was removed by centrifugation and sterilized by filtration through 0.2- $\mu$  filters. This crude viral supernatant was stored at  $-80^{\circ}\text{C}$  until use. Titers of viral preparations produced in this way were established by infection of U937 cells as follows. Briefly, U937 cells were mixed with varying dilutions of viral supernatant (typically 1/5 to 1/500) in the presence of 8 mg/ml polybrene (hexadimethrine bromide; Sigma). The cells were "spinoculated" by spinning at 2200 rpm for 2 h at  $30^{\circ}\text{C}$ . After 72 h, the percentage of GFP-positive cells (FITC channel) was determined by FACS analysis (using a LSRII FACS). The lowest dilution factor with GFP+ score of  $<20\%$  was used to calculate viral titer.

**U937 Lentiviral Line Production**—Human monoblastic leukemia U937 cells were infected with lentivirus carrying the gene of interest at a ratio of 1:1 (cells:virus) as described above. The cells were then cultured overnight before removal of virus containing media. Cell surface Myc-positive cells (Myc-Fcgr3 or Myc-Fcgr3RS) were isolated by panning on 10-cm Petri dishes coated with anti-Myc antibody (Millipore). Adherent cells were expanded in culture and used in subsequent experiments. GFP-positive cells expressing pCSGW (no surface Myc) were isolated by cell sorting based on FITC fluorescence. Subsequent analysis of cell lines by FACS analysis confirmed 90–100% of cells to be FITC-positive.

**FcR Stimulation**—The cells were resuspended in ice-cold serum-free RPMI and anti-Myc antibody (Millipore) added to a final concentration of 130  $\mu\text{g}/\text{ml}$ . After 60 min of incubation on ice, the cells were washed three times and resuspended in serum free RPMI with 8 mg/ml goat anti-mouse IgG(ab')<sub>2</sub>. The cells were then incubated at  $37^{\circ}\text{C}$  for the indicated times. The reaction was stopped by the addition of gel sample buffer (2% SDS, 10% glycerol, 0.5%  $\beta$ -mercaptoethanol, 50 mM Tris, pH 6.8).

**Immunoprecipitations and Western Blot Analysis**—The cells were lysed in ice-cold lysis buffer (25 mM Tris, pH 7.6, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, pH 7.8, 1% digitonin), containing protease inhibitor mixture (Sigma) with 1 mM DTT, 10 mM NaF, 100 mM sodium orthovanadate). The nuclei and cell debris were removed by centrifugation, and supernatants were precleared with protein G-Sepharose. Myc-labeled proteins were then precipitated with anti-Myc antibody (Millipore) or isotype matched control antibody and protein G-Sepharose for 1.5 h. Immunoprecipitated complexes were washed in lysis buffer and were resolved on 4–20% SDS-PAGE gels (Criterion).

After electrotransfer on to nitrocellulose membrane, the membranes were blocked in 2% BSA/TBS/Tween (0.05%) and probed with the appropriate antibodies at  $4^{\circ}\text{C}$  overnight followed by HRP-conjugated secondary antibody. Blots were developed using ECL (Amersham Biosciences).

**ELISAs**—At 24 h after stimulation, the supernatants were harvested. The concentrations of TNF $\alpha$  and IL-10 were determined by ELISA (PharMingen) according to the manufacturer's instructions. Absorbance was read and analyzed at 450 nm on a spectrophotometric ELISA plate reader (Labsystems Multiskan Biochromic) using the Ascent software program.

**BMDM Preparation and Phagocytosis Assay**—Femurs from adult WKY rats were isolated and flushed with Hanks' balanced salt solution. Total BM-derived cells were plated and infected with lentivirus carrying the gene of interest at a ratio of 5:1 (cells: virus). 48 h following the infection, the medium was replaced with DMEM that contained 25 mM HEPES (Sigma), 25% L929 conditioned medium, 25% deplete complemented FBS (Biosera), penicillin (100 units/ml; Invitrogen), streptomycin (100  $\mu\text{g}/\text{ml}$ ; Invitrogen), and cultured for 5 days. These cells were characterized as macrophages by ED-1 staining. To assess phagocytosis, latex polystyrene 6.0- $\mu\text{m}$  microspheres (50 beads/macrophage; Polysciences Inc.) were opsonized with BSA-anti-BSA IgG (Sigma) and added to transduced WKY macrophages cultured in 8-well glass chamber slides. Following staining with Diff-Quick fix (Dade Behring), 200 macrophages were counted to determine the number of beads ingested per cell.

**Expression Analysis by qRT-PCR**—Stably transduced U937 cells were differentiated in macrophages after adding PMA (100 nM) for 48 h and stimulated with LPS (Sigma; 1  $\mu\text{g}/\text{ml}$ ) for 3 h. Total RNA was extracted using TRIzol (Invitrogen), and one-step qRT-PCR was performed using 100 ng of total RNA and Brilliant II SYBR<sup>®</sup> Green QRT-PCR Master Mix Kit (Agilent) with specific primers for *Tnfa* and *IL1b1* in an ABI 7900 sequence detection system (Applied Biosystems, Warrington, UK). In rat BMDMs, *Fcgr3* levels relative to total *Fcgr3* (*Fcgr3* + *Fcgr3*-rs) were assessed by selecting a forward primer where *Fcgr3* differs from *Fcgr3*-rs by two single nucleotide polymorphisms at the end of the 3'-end. The selected primers were *Fcgr3*-Forward: 5'TCTAGTGTGGTTCCATGCCG-3' and *Fcgr3*-Reverse: 5'-TGTCCTGTGGAGCCTTGACT-3'; total *Fcgr3* (*Fcgr3*+*Fcgr3*-rs)-Forward: 5'-CTCCAGACCCCTCAACTGGT-3' and total *Fcgr3* (*Fcgr3*+*Fcgr3*-rs)-Reverse: 5'-GCAGTAGTAGTCCCCTGT-3'.

**Statistical Analysis**—Statistical differences in mean values were compared using one-way analysis of variance followed by Dunnett's multiple comparison post-test;  $p < 0.05$  was considered statistically significant.

## RESULTS

**The Phylogeny of Genomic Deletion of *Fcgr3*-rs in Laboratory Rat**—Exon 5 of *Fcgr3*-rs contains a deletion of a single guanine nucleotide at position 129 ( $\Delta\text{G129}$ ) in the coding sequence of the cytoplasmic domain (9). When compared with *Fcgr3*, this  $\Delta\text{G129}$  variant results in a frameshift in the *Fcgr3*-rs coding sequence and generates a novel cytoplasmic domain 6 amino acids longer than that encoded by *Fcgr3* that was not found in any other species (Fig. 1). We have previously shown that *Fcgr3*-rs was deleted from NTN-susceptible Kyoto-derived strains (9), but to investigate the distribution of this deletion throughout the rat genealogical tree, we carried out a phylogenetic tree analysis of 94 inbred laboratory strains and 40 of their substrains (Fig. 2). The results show that the deletion of *Fcgr3*-rs is widespread throughout the rat phylogeny (32 of 94 strains), suggesting that the origin of the deletion event is earlier than the derivation of these strains. We also investigated the relative levels of *Fcgr3* and *Fcgr3*-rs mRNA levels in Lewis rat BMDMs by qRT-PCR and showed that although there is relatively

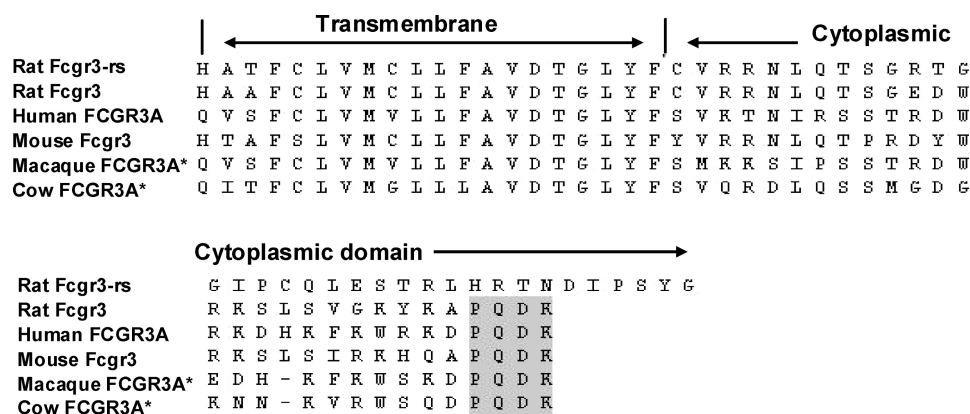


FIGURE 1. **Rat-specific *Fcgr3-rs* is a novel Fc receptor with a unique cytoplasmic domain 6 amino acids longer than *Fcgr3*.** Shown is a comparison of amino acid sequences of transmembrane and cytoplasmic domains of rat *Fcgr3* and *Fcgr3-rs*, human *Fcgr3*, and FCGR3 in other species. *Fcgr3-rs* has lost the terminal PDK sequence (highlighted) that is highly conserved across species. \*, amino acid prediction based on human FCGR3A using Ensembl Genome Browser.

increased *Fcgr3* expression when compared with *Fcgr3-rs*, LPS stimulation did not change the relative levels of both receptors (Table 1).

**U937 Cells Expressing Either *Fcgr3* or *Fcgr3-rs*: Surface Expression and Interaction with  $\gamma$  Chain**—The U937 cell line does not express any endogenous Fc $\gamma$ RIIIA (11) and is therefore an ideal tool for the analysis of Fc $\gamma$ RIII receptors, allowing direct functional comparison between the rat orthologues *Fcgr3* and *Fcgr3-rs*. Thus, to study the role of *Fcgr3-rs* in macrophage activation, we generated U937 cells stably transduced with lentivirus constructs expressing the protein of interest. U937 cells were infected with either Myc-tagged *Fcgr3* (Myc-*Fcgr3*), Myc tagged-*Fcgr3-rs* (Myc-*Fcgr3RS*), or empty (pCSGW) lentiviral vectors. All of the lentiviral vectors contained GFP. Expression of Myc-*Fcgr3* and Myc-*Fcgr3-rs* was confirmed by fluorescence microscopy, detecting GFP+ cells and also by a Myc Western blot on lysates from stably transduced U937 cells (Fig. 3A). We then investigated whether Myc-*Fcgr3* and Myc-*Fcgr3-rs* are expressed on the surface of U937 cells. Flow cytometry analysis using an anti-Myc-APC mouse antibody (mAb) showed that both Myc-*Fcgr3* and Myc-*Fcgr3-rs* are expressed on the cell surface at similar levels in these cells (Fig. 3B). Because *Fcgr3* transport to and expression at the cell surface is known to be dependent on its association with the common  $\gamma$  chain (12), these findings implied that both the *Fcgr3* and *Fcgr3-rs* molecules were associated with  $\gamma$  chain at the cell surface. Co-immunoprecipitation analysis of U937 cells expressing either *Fcgr3* or *Fcgr3-rs* demonstrated that this was indeed the case (Fig. 3C). Thus, although *Fcgr3-rs* has an additional cytoplasmic domain 6 amino acids longer than its paralogue *Fcgr3*, it binds the  $\gamma$ -chain on the surface of stably transduced and PMA-differentiated U937 macrophages.

***Fcgr3-rs*-mediated Signaling and Phagocytosis in U937 Cells**—In most macrophage Fc receptor complexes (with the exception of Fc $\gamma$ RII), the ITAM-containing common  $\gamma$ -chain is necessary for downstream signaling (13, 14). Thus, the dimeric  $\gamma$ -chain forms multichain complexes with the ligand binding  $\alpha$ -chain of *Fcgr3* to allow signal transduction (3). This signaling starts with tyrosine phosphorylation of the ITAMs by kinases of the SRC family (15, 16) and leads to the recruitment and subsequent phosphorylation of the Syk

kinase, followed by the activation of various downstream targets such as PI3K and NF- $\kappa$ B. Subsequent changes in gene expression led to the induction of inflammatory cytokines such as TNF $\alpha$ . We were therefore keen to understand whether the *Fcgr3-rs* is able to transduce signals similar to those described for *Fcgr3*. However, despite its interaction with the  $\gamma$ -chain, cross-linking of *Fcgr3-rs* with anti-Myc antibody does not lead to phosphorylation of Syk or the degradation of I $\kappa$ B $\alpha$  (Fig. 4A). This was not the case for the U937 macrophages stably transduced with *Fcgr3* (Fig. 4A).

Furthermore, Fc receptor-mediated TNF $\alpha$  production and IgG-opsonized bead phagocytosis were also significantly less in U937 macrophages expressing *Fcgr3-rs* when compared with U937 macrophages stably transduced with *Fcgr3* (Fig. 4B). Taken together, these results suggest that the rat-specific *Fcgr3-rs* is unable to mediate the same intracellular signals as the wild type receptor.

***Fcgr3-rs*-and TLR4-mediated TNF $\alpha$  and IL-1 $\beta$  Expression Levels**—To test whether the relative reduction in TNF $\alpha$  production in U937 cells transduced with Myc-*Fcgr3RS* is specific to receptor cross-linking, we have stimulated the stably transduced PMA-differentiated U937 cells with LPS for 3 h and measured TNF $\alpha$  and IL-1 $\beta$  expression levels by qRT-PCR (Fig. 5). Although the results have confirmed the previously shown up-regulation of these genes in U937 cells (17), there was no significant difference in the up-regulation of TNF $\alpha$  and IL-1 $\beta$  between U937 + Myc-*Fcgr3* and U937 + Myc-*Fcgr3RS*, demonstrating that the inability of these cells to produce TNF $\alpha$  is not an intrinsic property of these cells and is specific to *Fcgr3* stimulation (Fig. 5).

**The Role of the Novel Cytoplasmic Domain of *Fcgr3-rs* in Rat and Human Macrophage Activation**—To study the effect of *Fcgr3-rs* on the phagocytic activity of primary WKY BMDMs where other endogenous, FcRs (*Fcgr3* and others) are expressed, we transduced these cells with lentivirus encoding either Myc-*Fcgr3rs* or Myc-*Fcgr3* and used cells transduced with the empty vector (pCSGW) as controls.

Because the *Fcgr3-rs* also contains a number of single-base pair substitutions in its extracellular domain, we were also keen to establish whether these might explain the differences between the related sequence and wild type *Fcgr3* molecules, possibly as a

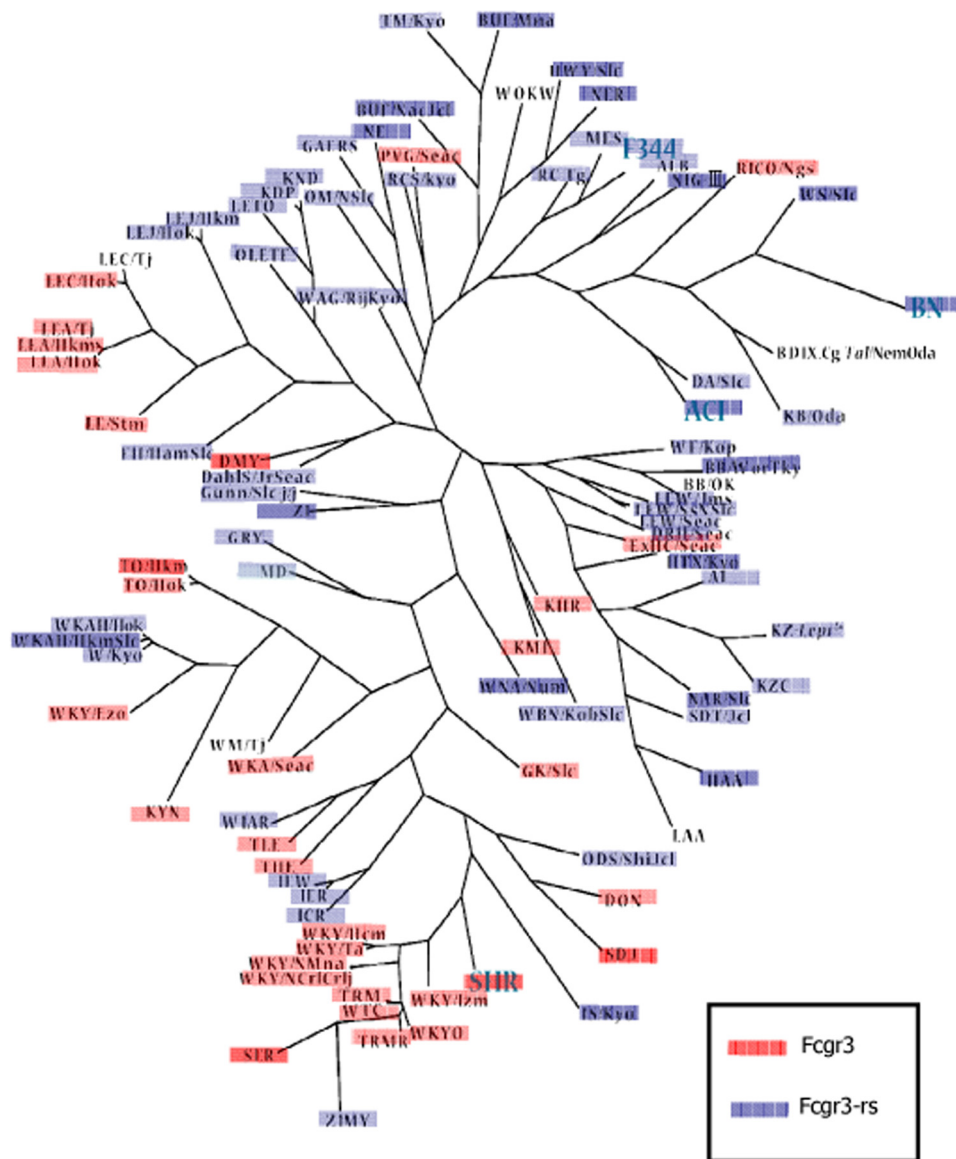


FIGURE 2. **Phylogenetic tree of 134 laboratory rat strains according to the presence or absence of *Fcgr3*-rs in the genome.** A phylogenetic tree of 94 strains is shown. Strains having numerous substrains (i.e. SHR, F344) are shown in capital letters, and all of the substrains showed the same genotype as the founder strain. The tree was developed through a heuristic search for maximum parsimony implemented in PAUP 4.0b10. TreeView was used to display the radial tree. For BD IX, LEC/Tj, and WM/Tj, genotypes could not be determined.

**TABLE 1**  
Relative mRNA levels of *Fcgr3* and *Fcgr3*-rs in Lewis rat BMDMs

Expression was assessed by qRT-PCR with specific primers that differentiate between *Fcgr3* and *Fcgr3*-rs in basal and LPS-stimulated (100 ng/ml, for 5 h) BMDMs. *Fcgr3* and *Fcgr3*-rs expression levels were normalized to total *Fcgr3* (*Fcgr3* + *Fcgr3*-rs) levels.

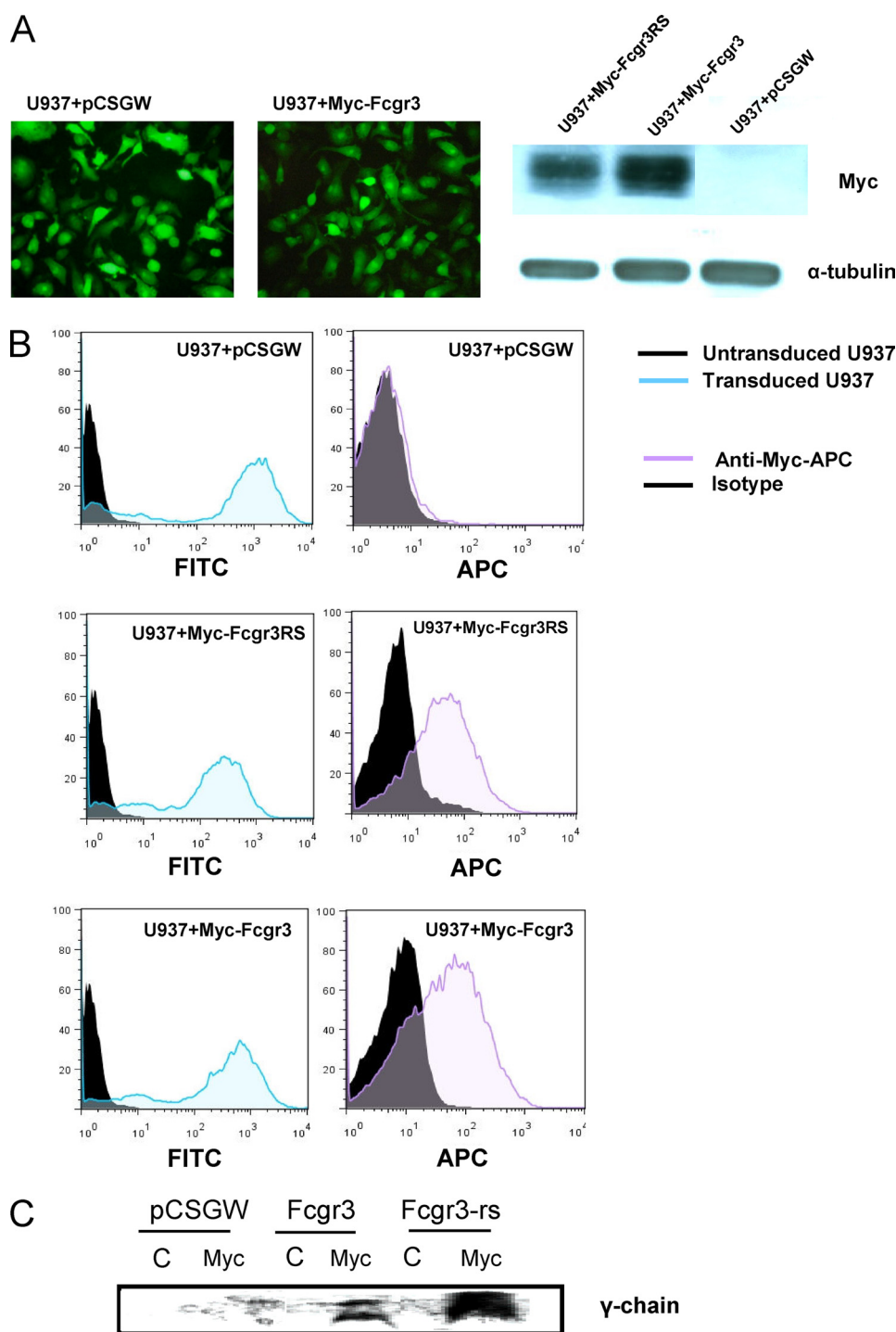
Expression	BMDM	
	Basal	LPS
<i>Fcgr3</i> /( <i>Fcgr3</i> + <i>Fcgr3</i> -rs)	0.73 ± 0.02	0.67 ± 0.01
<i>Fcgr3</i> -rs/( <i>Fcgr3</i> + <i>Fcgr3</i> -rs)	0.27 ± 0.02	0.33 ± 0.01

result of altered FcR binding. Thus, we also generated an additional lentiviral construct encoding a chimeric protein to specifically test the effect of the novel cytoplasmic domain of *Fcgr3*-rs on Fc receptor-mediated phagocytosis. *Fcgr3*-ΔG contains the wild type *Fcgr3* extracellular, transmembrane, and cytoplasmic domains with the presence of the *Fcgr3*-rs-specific novel cytoplasmic domain (9) (Fig. 6A).

We first measured the expression of *Fcgr3* by using primers specific for the transmembrane domain, thereby allowing differential amplification between *Fcgr3* and *Fcgr3*-rs transcripts (Fig. 6B). We then measured the number of opsonized beads phagocytosed by 200 WKY BMDMs transduced with each different lentiviral construct.

Phagocytosis was significantly reduced in *Fcgr3*-rs transduced WKY BMDMs when compared with those transduced with pCSGW (Fig. 6C). This inhibitory effect is clearly mediated by the novel cytoplasmic domain of *Fcgr3*-rs, because similar reductions in phagocytosis activity were seen in WKY BMDMs transduced either with *Fcgr3*-ΔG or with *Fcgr3*-rs (Fig. 6C). Taken together, these data suggest that the presence of FcRs containing the related sequence cytoplasmic domain is able to reduce phagocytosis mediated by endogenous FcRs in the WKY BMDMs.

To further understand the mechanism of action of the *Fcgr3*-rs, we then investigated how the *Fcgr3* and *Fcgr3*-rs



**FIGURE 3. Surface expression of Fcgr3-rs and Fcgr3 and assessment of their interaction with the  $\gamma$  chain in stably transduced U937 cells.** A, illustration of GFP expression in U937 + pCSGW and U937 + Myc-Fcgr3 by fluorescent microscopy ( $\times 40$ ). Myc and  $\alpha$ -tubulin (loading control) Western blots of whole cell lysates from stably transduced U937 cells are shown. B, the human monoclastic leukemia cell line U937 was stably transduced to express either Myc-Fcgr3 (U937 + Myc-Fcgr3) or Myc-Fcgr3RS (U937 + Myc-Fcgr3RS) lentiviral constructs co-expressing GFP together with transgene. Untransduced cells (U937) and cells transduced with lentiviral construct expressing GFP alone (without any transgene, U937 + pCSGW) were used as controls. Surface expression was measured by using anti-Myc-APC mAb. All of the transduced cells were GFP+ as shown by FITC emission. C, stably transduced U937 cells were differentiated into macrophages after adding PMA (100 nM) for 48 h, and cell lysates were precipitated with anti-Myc antibody. Immunoprecipitated proteins were blotted with anti- $\gamma$  chain antibody.

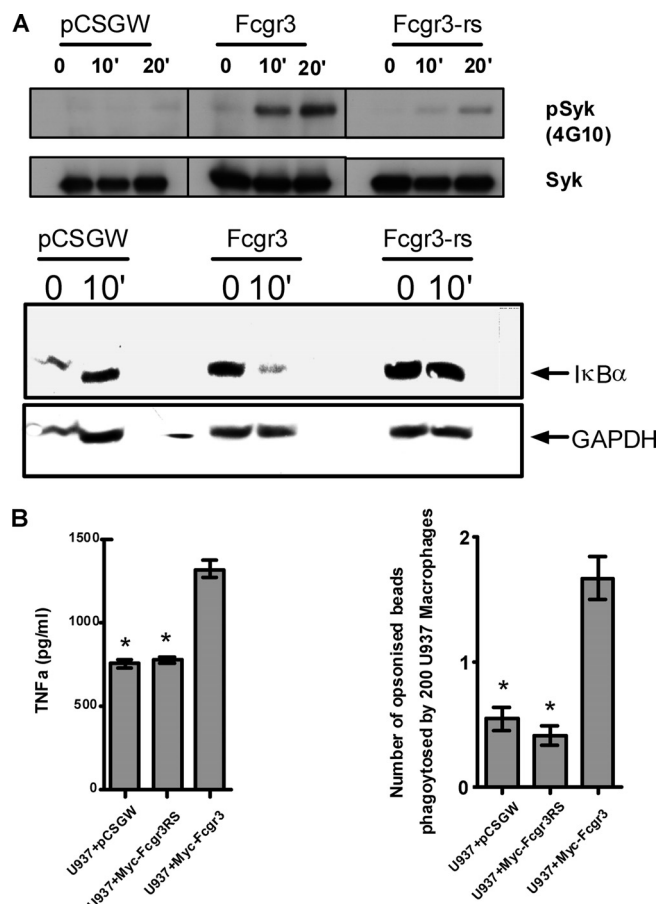
interact with TLR4-mediated TNF $\alpha$  production following LPS stimulation. U937 cells transduced with FcRs (U937 + Myc-Fcgr3, U937 + MycFcgr3RS, and U937 + Myc-Fcgr3 $\Delta$ G) were cross-linked with anti-Myc antibodies and IgG F(ab')<sub>2</sub>. Following washing, the cells were stimulated

with LPS for 5 h. TNF $\alpha$  and IL-10 protein levels were measured by sandwich ELISA.

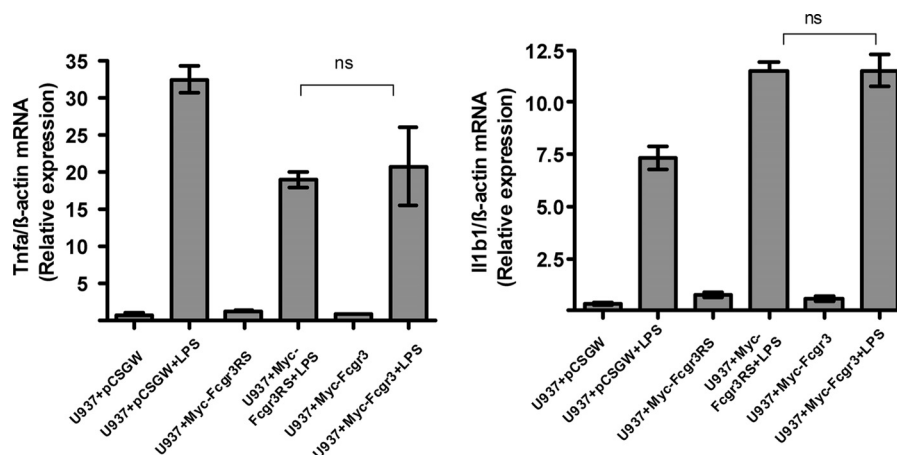
In the absence of any additional FcR expression in these cells, treatment with Ab-Fab complexes resulted in a 38% decrease in TNF production. This was further decreased in

## Rat *Fcgr3-rs* Inhibits FcR Signaling and Cytokine Production

the presence of the Fcgr3 molecule to 50% of that seen in cells that are not cross-linked, revealing that signals from Fcgr3 act to reduce TLR4-induced TNF production in U937



**FIGURE 4. Fc receptor-mediated signaling and phagocytosis in U937 cells stably transduced with Myc-Fcgr3RS.** A, Fc receptor stimulation after cross-linking with anti-Myc Ab for a maximum of 20 min and analysis of Syk phosphorylation and IκBα degradation in stably transduced U937-derived macrophages. B, TNFα levels were assessed by sandwich ELISA after cross-linking stably transduced U937 macrophages by anti-Myc mAb. Supernatants were collected after 48 h of incubation. Fc receptor-mediated bead phagocytosis was assayed by incubating macrophages with IgG opsonized latex beads. Nonopsonized beads did not show any phagocytosis. \*,  $p < 0.001$  when compared with U937 + Myc-Fcgr3.



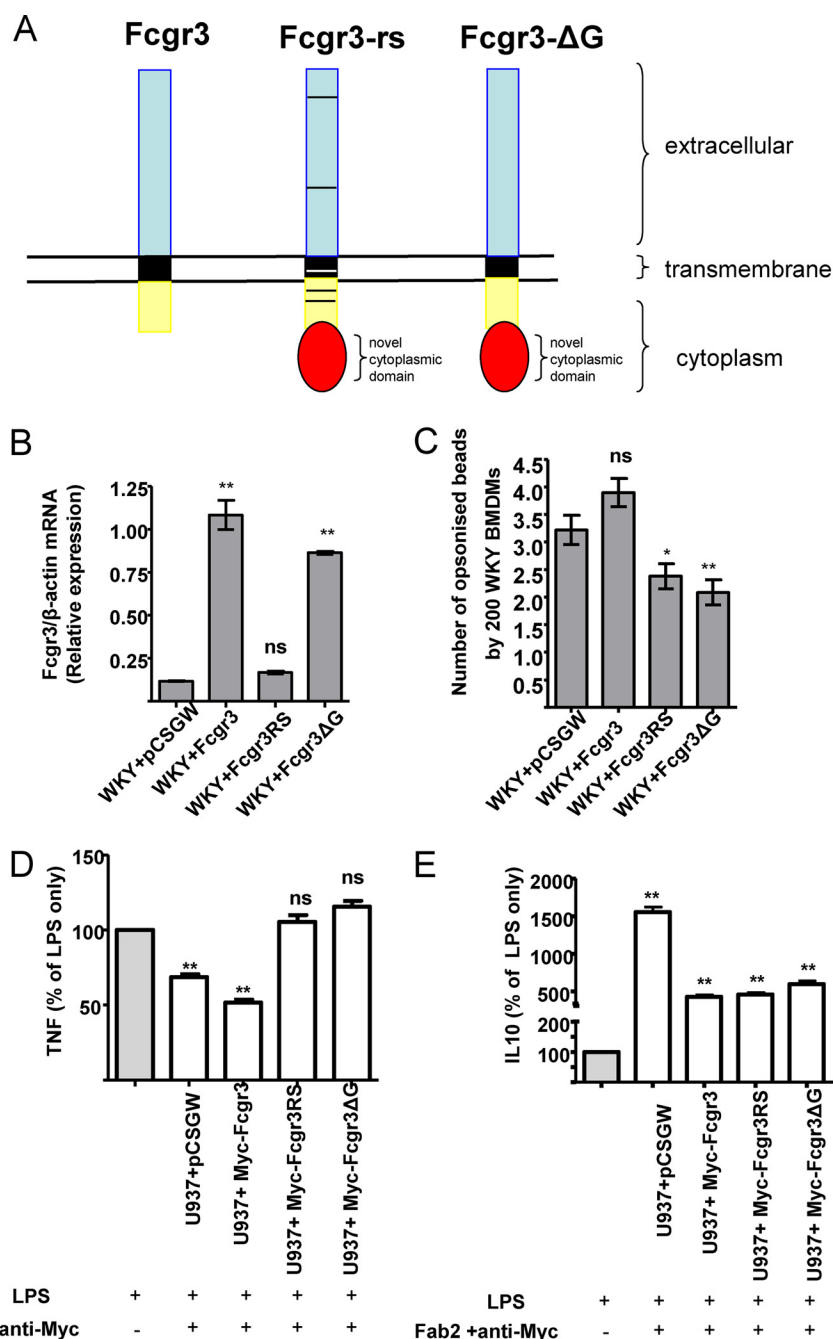
**FIGURE 5. Fcgr3-rs does not affect TLR4-mediated *Tnfa* and *Il1b1* expressions in stably transduced U937 cells.** Stably transduced U937 cells were differentiated in macrophages after adding PMA (100 nM) for 48 h and stimulated with LPS (1 μg/ml) for 3 h. *Tnfa* and *Il1b1* gene expression were measured by qRT-PCR. ns, not significant.

cells (Fig. 6D). In contrast, the presence of either the Fcgr3 or Fcgr3ΔG did not result in any changes in TNFα production compared with cells that are not cross-linked. Levels of IL-10 produced in these cells did not differ significantly between Fcgr3 and Fcgr3-rs or Fcgr3-ΔG cells (Fig. 6E), revealing that the Fcgr3-rs is not functioning in these cells by increasing levels of this anti-inflammatory cytokine.

## DISCUSSION

There is considerable evidence from experimental models of crescentic glomerulonephritis that Fcγ receptors play a central role in disease progression. In mice, the FcR chain-deficient mice (FcRγ<sup>-/-</sup>) lack surface expression of both murine activator Fc receptors I, III, and IV, and the high affinity IgE receptor (FcRI) (18). Several studies have shown that FcRγ<sup>-/-</sup> mice are protected from disease in accelerated nephrotoxic nephritis (6, 7, 19), and we have shown that the disease is mediated by Fcγ receptors on circulating leukocytes and not intrinsic renal cells (8). Our previous studies in the NTN model in the Wistar Kyoto rat showed that the disease architecture is complex and highly heritable, with seven quantitative trait loci (*Crgn1-7*) linked to the percentage of glomerular crescents and controlling mainly macrophage activation through distinct transcriptome profiles (9, 20–22). In this model, we showed that copy number variation in rat *Fcgr3* locus led to the deletion of *Fcgr3-rs* in the WKY rat, predisposing this strain to enhanced macrophage activity (9).

The purpose of the current study was to understand the mechanism by which the genomic deletion of *Fcgr3-rs* leads to macrophage overactivation and whether the novel cytoplasmic domain binds to the common γ chain to trigger downstream signaling pathways. We first investigated the distribution of *Fcgr3-rs* deletion throughout the rat phylogeny and found that the deletion was widespread. It was previously reported that five outbred Wistar and one Long-Evans stocks have *Fcgr3-rs* (23), suggesting that the deletion has originated from the ancestor outbred Wistar colony in the Wistar institute in the United States. We now confirm and extend these findings because all of the laboratory rat strains that we genotyped for Fcgr3 copy



**FIGURE 6. Fcgr3-rs inhibits Fc receptor-mediated phagocytosis in WKY BMDMs.** *A*, schematic representation of the functional differences between Fcgr3 and Fcgr3-rs. Although there are amino acid changes in the Fcgr3-rs extracellular, transmembrane, and cytoplasmic domains (indicated by bars) when compared with Fcgr3, the major structural difference is the presence of a novel cytoplasmic domain. The chimeric Fcgr3-ΔG has the extracellular transmembrane and cytoplasmic domains identical to Fcgr3, the only difference being the presence of the novel cytoplasmic domain. *B*, *Fcgr3* expression was assessed by qRT-PCR in stably transduced U937 cells by using specific primers designed based on polymorphisms in the transmembrane domain differentiating between *Fcgr3* and *Fcgr3*-rs. The expression of Myc-Fcgr3 ΔG was also assessed and showed similar expression levels as Fcgr3. This is because Fcgr3 and Fcgr3-ΔG share the same sequence in the transmembrane domain. *C*, WKY bone marrow-derived cells were transduced with lentiviral constructs co-expressing GFP with either Myc-tagged Fcgr3-rs (Myc-Fcgr3RS) or Myc-tagged wild type Fcgr3 (Myc-Fcgr3) or Myc-tagged Fcgr3-ΔG. The lentiviral construct expressing GFP alone (without any transgene) was used as control (pCSGW). The cells were then differentiated into BM-derived macrophages by culturing in DMEM containing L929, and Fc receptor-mediated phagocytosis was performed. \*\*,  $p < 0.01$ ; \*,  $p < 0.05$ ; ns, not significant when compared with pCSGW. *D* and *E*, LPS-induced TNF $\alpha$  (*D*) and IL-10 (*E*) levels were assessed by sandwich ELISA after cross-linking stably transduced U937 macrophages by anti-Myc mAb and F(ab')<sub>2</sub>. Following cross-linking, the cells were washed, and supernatants were collected after 5 h of incubation with LPS (1  $\mu$ g/ml). TNF $\alpha$  and IL-10 production were expressed as a percentage of that produced in LPS-stimulated cells without FcR cross-linking (100% of cytokine production). \*\*,  $p < 0.001$  when compared with LPS-stimulated cells without FcR cross-linking (100% of cytokine production; histogram in gray)

number variation are derived from outbred Wistar and Long-Evans stocks.

Previously, to investigate how Fcgr3-rs exerts its inhibitory effect on Fc $\gamma$  receptor signaling, we have transfected COS7 cells

and have shown that although Fcgr3-rs is expressed on the cell surface, it is less efficient than Fcgr3 in mediating phagocytosis (9). It also had an inhibitory effect on Fcgr3-mediated phagocytosis when both receptors were co-transfected in COS7 cells



## Rat Fcgr3-rs Inhibits FcR Signaling and Cytokine Production

(9). However, the COS7 cells are not primarily phagocytic cells and do not endogenously express the  $\gamma$  chain, making it difficult to draw mechanistic conclusions concerning the role of Fcgr3-rs in Fc receptor-mediated signaling and activation. Here we have examined the signaling function of Fcgr3-rs in a human monocyte cell line U937, a well characterized cell line previously used to study Fc receptor function (11). U937 cells constitutively express only Fcgr1 and Fcgr2a but not Fcgr3 (11). We have used lentivirus-mediated gene transfer to generate stably transduced U937 cell lines. Our results showed that Fcgr3-rs associates with the common  $\gamma$ -chain in these cells, but Fc receptor-mediated signaling and phagocytosis are defective. Importantly, WKY BMDMs transduced with the Fcgr3-rs also showed reduced bead phagocytosis, and this was mediated through the rat-specific novel cytoplasmic domain.

It is well established that the transmembrane domain is required for association with the  $\gamma$  chain and consequently cell surface expression of Fcgr3 (24). Because of the amino acid changes between Fcgr3 and Fcgr3-rs in the transmembrane domains, it could therefore be argued that the amino acid changes simply prevented *Fcgr3-rs* expression. This, however, was not the case, and in both U937 cells and primary rat macrophages, Fcgr3-rs was expressed at the cell surface at similar levels to Fcgr3. The use of the  $\Delta G$  chimeric molecule, which contains the extracellular and transmembrane domains of Fcgr3 combined with the cytoplasmic domain of Fcgr3-rs, also demonstrates that the differences seen between Fcgr3 and Fcgr3-rs functions are mediated only by the novel cytoplasmic domain of Fcgr3-rs and cannot be explained by point mutations in the extracellular domain of Fcgr3-rs, which might simply have prevented Fc binding of IgG.

The majority of signaling cascades emanating from the Fcgr3 receptor complex are thought to be derived from the  $\gamma$  chain via its ITAM motifs. Thus, removal of the  $\gamma$  chain cytoplasmic domain or mutations of tyrosine residues within the ITAM motifs affects intracellular  $Ca^{+}$  levels, PI3K activity and receptor-induced phosphorylation events (25). In particular there is compelling evidence that activation of Syk is essential for subsequent events such as receptor-induced phagocytosis (26, 27). However, the cytoplasmic tail of the Fcgr3 molecule is unlikely to be completely redundant in this process, and Hou *et al.* (28) have suggested that sequences within the cytoplasmic domain of Fcgr3, in particular those close to the transmembrane domain, are required for signaling. Our findings demonstrate that signaling from the Fcgr3-rs is clearly different from that mediated by Fcgr3, because Fcgr3-rs $\cdot\gamma$  chain complexes do not generate phosphorylated Syk molecules or activate the NF- $\kappa$ B transcription factor that is required for transcription of Fc-responsive genes such as TNF $\alpha$ . Accordingly, IgG-induced TNF $\alpha$  production and phagocytosis are both significantly lower in U937 cells expressing Fcgr3-rs compared with Fcgr3. Moreover, expressing Fcgr3-rs in primary rat WKY BMDMs resulted in lower levels of phagocytosis than cells expressing Fcgr3 alone, suggesting that Fcgr3-rs has an inhibitory effect on Fcgr3-mediated phagocytosis in these cells.

Although the Fcgr3-rs cytoplasmic domain includes a novel sequence with a single tyrosine molecule, this is not an immunoreceptor tyrosine-based inhibitory motif in the same way as

that found in the FcgrIIb receptor. Elegant studies by Kim *et al.* (29) have shown that specific regions of the transmembrane region of Fc $\gamma$  receptors are important for signaling leading to phagocytosis. The same group has provided further evidence on differences in the signaling properties of Fc $\gamma$ R1/ $\gamma$  and Fc $\gamma$ RIIA such as their interaction with Syk and Src-related tyrosine kinases (30). The region of the cytoplasmic domain shown by Hou *et al.* (28) to be important in signaling is present in the Fcgr3-rs; however, it is possible that the altered sequence of the remainder of the cytoplasmic domain may render this region inaccessible to essential downstream signaling molecules, thereby rendering it inactive. The question of whether the Fcgr3-rs exerts its effects by acting as a decoy receptor, binding immunoglobulins, but not signaling, or whether its novel cytoplasmic domain induces distinct signaling pathways that act to inhibit those from the wt Fcgr3 is therefore an interesting one.

Our demonstration herein that the -rs does not induce Syk or NF- $\kappa$ B activation suggests that this molecule may not signal, and this is underscored by the demonstration that LPS-induced TNF and IL-10 production is unaffected in U937 cells by the presence of the -rs. Taken together, these data add further weight to the argument that Fcgr3-rs is a decoy receptor that is able to bind Fc but unable to signal such that its expression inhibits co-expressed Fc receptor-mediated signaling and cell activation. However, we cannot completely rule out a potential additional role of this receptor, which will lead to inhibition by a novel negative signaling pathway. Investigations into this question are continuing in this laboratory.

In conclusion, our results suggest that the rat-specific *Fcgr3-rs* acts to inhibit *Fcgr3*-mediated signaling and phagocytosis in both rodent and human cells and could be considered as a novel therapeutic mechanism for the modulation of Fc receptor-mediated cell activation in macrophage-mediated diseases such as crescentic glomerulonephritis.

---

*Acknowledgments*—We thank Sunil Modi for technical assistance. We are thankful to the National BioResource Project for providing rat strains.

---

## REFERENCES

1. Mason, J. C., and Pusey, C. D. (2008) *The Kidney in Systemic Autoimmune Diseases*, 1st Ed., Elsevier, Amsterdam
2. Nimmerjahn, F., and Ravetch, J. V. (2007) Fc-receptors as regulators of immunity. *Adv. Immunol.* **96**, 179–204
3. Nimmerjahn, F., and Ravetch, J. V. (2008) Fc $\gamma$  receptors as regulators of immune responses. *Nat. Rev. Immunol.* **8**, 34–47
4. Brown, E. E., Edberg, J. C., and Kimberly, R. P. (2007) Fc receptor genes and the systemic lupus erythematosus diathesis. *Autoimmunity* **40**, 567–581
5. Strzelecka, A., Kwiatkowska, K., and Sobota, A. (1997) Tyrosine phosphorylation and Fc $\gamma$  receptor-mediated phagocytosis. *FEBS Lett.* **400**, 11–14
6. Park, S. Y., Ueda, S., Ohno, H., Hamano, Y., Tanaka, M., Shiratori, T., Yamazaki, T., Arase, H., Arase, N., Karasawa, A., Sato, S., Ledermann, B., Kondo, Y., Okumura, K., Ra, C., and Saito, T. (1998) Resistance of Fc receptor-deficient mice to fatal glomerulonephritis. *J. Clin. Invest.* **102**, 1229–1238
7. Suzuki, Y., Shirato, I., Okumura, K., Ravetch, J. V., Takai, T., Tomino, Y., and Ra, C. (1998) Distinct contribution of Fc receptors and angiotensin II-dependent pathways in anti-GBM glomerulonephritis. *Kidney Int.* **54**, 1166–1174
8. Tarzi, R. M., Davies, K. A., Robson, M. G., Fossati-Jimack, L., Saito, T.,

- Walport, M. J., and Cook, H. T. (2002) Nephrotoxic nephritis is mediated by Fc $\gamma$  receptors on circulating leukocytes and not intrinsic renal cells. *Kidney Int.* **62**, 2087–2096
9. Aitman, T. J., Dong, R., Vyse, T. J., Norsworthy, P. J., Johnson, M. D., Smith, J., Mangion, J., Robertson-Lowe, C., Marshall, A. J., Petretto, E., Hodges, M. D., Bhargal, G., Patel, S. G., Sheehan-Rooney, K., Duda, M., Cook, P. R., Evans, D. J., Domin, J., Flint, J., Boyle, J. J., Pusey, C. D., and Cook, H. T. (2006) Copy number polymorphism in *Fcgr3* predisposes to glomerulonephritis in rats and humans. *Nature* **439**, 851–855
  10. Bainbridge, J. W., Stephens, C., Parsley, K., Demaison, C., Halfyard, A., Thrasher, A. J., and Ali, R. R. (2001) *In vivo* gene transfer to the mouse eye using an HIV-based lentiviral vector. Efficient long-term transduction of corneal endothelium and retinal pigment epithelium. *Gene Ther.* **8**, 1665–1668
  11. Floto, R. A., Clatworthy, M. R., Heilbronn, K. R., Rosner, D. R., MacAry, P. A., Rankin, A., Lehner, P. J., Ouweland, W. H., Allen, J. M., Watkins, N. A., and Smith, K. G. (2005) Loss of function of a lupus-associated Fc $\gamma$ RIIb polymorphism through exclusion from lipid rafts. *Nat. Med.* **11**, 1056–1058
  12. Park, J. G., Isaacs, R. E., Chien, P., and Schreiber, A. D. (1993) In the absence of other Fc receptors, Fc  $\gamma$  RIIIA transmits a phagocytic signal that requires the cytoplasmic domain of its  $\gamma$  subunit. *J. Clin. Invest.* **92**, 1967–1973
  13. Lowry, M. B., Duchemin, A. M., Robinson, J. M., and Anderson, C. L. (1998) Functional separation of pseudopod extension and particle internalization during Fc  $\gamma$  receptor-mediated phagocytosis. *J. Exp. Med.* **187**, 161–176
  14. Davis, W., Harrison, P. T., Hutchinson, M. J., and Allen, J. M. (1995) Two distinct regions of FC  $\gamma$ RI initiate separate signalling pathways involved in endocytosis and phagocytosis. *EMBO J.* **14**, 432–441
  15. Ghazizadeh, S., Bolen, J. B., and Fleit, H. B. (1994) Physical and functional association of Src-related protein tyrosine kinases with Fc  $\gamma$ RII in monocytic THP-1 cells. *J. Biol. Chem.* **269**, 8878–8884
  16. Wang, A. V., Scholl, P. R., and Geha, R. S. (1994) Physical and functional association of the high affinity immunoglobulin G receptor (Fc  $\gamma$ RI) with the kinases Hck and Lyn. *J. Exp. Med.* **180**, 1165–1170
  17. Sharif, O., Bolshakov, V. N., Raines, S., Newham, P., and Perkins, N. D. (2007) Transcriptional profiling of the LPS induced NF- $\kappa$ B response in macrophages. *BMC Immunol.* **8**, 1
  18. Takai, T., Li, M., Sylvestre, D., Clynes, R., and Ravetch, J. V. (1994) FcR  $\gamma$  chain deletion results in pleiotropic effector cell defects. *Cell* **76**, 519–529
  19. Wakayama, H., Hasegawa, Y., Kawabe, T., Hara, T., Matsuo, S., Mizuno, M., Takai, T., Kikutani, H., and Shimokata, K. (2000) Abolition of anti-glomerular basement membrane antibody-mediated glomerulonephritis in FcR $\gamma$ -deficient mice. *Eur. J. Immunol.* **30**, 1182–1190
  20. Behmoaras, J., Bhargal, G., Smith, J., McDonald, K., Mutch, B., Lai, P. C., Domin, J., Game, L., Salama, A., Foxwell, B. M., Pusey, C. D., Cook, H. T., and Aitman, T. J. (2008) *Jund* is a determinant of macrophage activation and is associated with glomerulonephritis susceptibility. *Nat. Genet.* **40**, 553–559
  21. Behmoaras, J., Smith, J., D'Souza, Z., Bhargal, G., Chawanasantoropoj, R., Tam, F. W., Pusey, C. D., Aitman, T. J., and Cook, H. T. (2010) Genetic loci modulate macrophage activity and glomerular damage in experimental glomerulonephritis. *J. Am. Soc. Nephrol.* **21**, 1136–1144
  22. Maratou, K., Behmoaras, J., Fewings, C., Srivastava, P., D'Souza, Z., Smith, J., Game, L., Cook, T., and Aitman, T. (2011) Characterization of the macrophage transcriptome in glomerulonephritis-susceptible and -resistant rat strains. *Genes Immun.* **12**, 78–89
  23. Kuramoto, T., Nakanishi, S., and Serikawa, T. (2008) Functional polymorphisms in inbred rat strains and their allele frequencies in commercially available outbred stocks. *Physiol. Genomics* **33**, 205–211
  24. Lanier, L. L., Yu, G., and Phillips, J. H. (1991) Analysis of Fc  $\gamma$  RIII (CD16) membrane expression and association with CD3  $\zeta$  and Fc epsilon RI- $\gamma$  by site-directed mutation. *J. Immunol.* **146**, 1571–1576
  25. Indik, Z. K., Park, J. G., Hunter, S., and Schreiber, A. D. (1995) The molecular dissection of Fc  $\gamma$  receptor mediated phagocytosis. *Blood* **86**, 4389–4399
  26. Crowley, M. T., Costello, P. S., Fitzer-Attas, C. J., Turner, M., Meng, F., Lowell, C., Tybulewicz, V. L., and DeFranco, A. L. (1997) A critical role for Syk in signal transduction and phagocytosis mediated by Fc $\gamma$  receptors on macrophages. *J. Exp. Med.* **186**, 1027–1039
  27. Wirthmueller, U., Kurosaki, T., Murakami, M. S., and Ravetch, J. V. (1992) Signal transduction by Fc  $\gamma$  RIII (CD16) is mediated through the  $\gamma$  chain. *J. Exp. Med.* **175**, 1381–1390
  28. Hou, X., Dietrich, J., and Geisler, N. O. (1996) The cytoplasmic tail of Fc $\gamma$ RIIIA $\alpha$  is involved in signaling by the low affinity receptor for immunoglobulin G. *J. Biol. Chem.* **271**, 22815–22822
  29. Kim, M. K., Huang, Z. Y., Hwang, P. H., Jones, B. A., Sato, N., Hunter, S., Kim-Han, T. H., Worth, R. G., Indik, Z. K., and Schreiber, A. D. (2003) Fc $\gamma$  receptor transmembrane domains: role in cell surface expression,  $\gamma$  chain interaction, and phagocytosis. *Blood* **101**, 4479–4484
  30. Huang, Z. Y., Hunter, S., Kim, M. K., Chien, P., Worth, R. G., Indik, Z. K., and Schreiber, A. D. (2004) The monocyte Fc $\gamma$  receptors Fc $\gamma$ RI/ $\gamma$  and Fc $\gamma$ RIIA differ in their interaction with Syk and with Src-related tyrosine kinases. *J. Leukoc. Biol.* **76**, 491–499