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Inhibitory ITAM Signaling by FcαRI-FcRγ Chain Controls Multiple Activating Responses and Prevents Renal Inflammation

Yutaka Kanamaru,*† Séverine Pfirsch,*† Meryem Aloulou,*† François Vrtovský,*†‡ Marie Essig,*†‡ Chantal Loirat,*†‡ Georges Deschênes,§ Claudine Guérin-Marchand,§† Ulrich Blank,2,3† and Renato C. Monteiro2,3†

Inhibitory signaling is an emerging function of ITAM-bearing immunoreceptors in the maintenance of homeostasis. Monovalent targeting of the IgA Fc receptor (FcαRI or CD89) by anti-FcαRI Fab triggers potent inhibitory ITAM (ITAMI) signaling through the associated FcγRI chain (FcαRI-FcγRI ITAMI) that prevents IgG phagocytosis and IgG-mediated asthma. It is not known whether FcαRI-FcγRI ITAMI signaling controls receptors that do not function through an ITAM and whether this inhibition requires Src homology protein 1 phosphatase. We show in this study that FcαRI-FcγRI ITAMI signals depend on Src homology protein 1 phosphatase to target multiple non-ITAM-bearing receptors such as chemotactically activated receptors, cytokine receptors, and TLRs. We found that anti-FcαRI Fab treatment in vivo reduced kidney inflammation in models of immune-mediated glomerulonephritis and nonimmune obstructive nephropathy by a mechanism that involved decreased inflammatory cell infiltration and fibrosis development. This treatment also prevented ex vivo LPS activation of monocytes from patients with lupus nephritis or vasculitis, as well as receptor activation through serum IgA complexes from IgA nephropathy patients. These findings point to a crucial role of FcαRI-FcγRI ITAMI signaling in the control of multiple heterologous or autologous inflammatory responses. They also identify anti-FcαRI Fab as a new potential therapeutic tool for preventing progression of renal inflammatory diseases. The Journal of Immunology, 2008, 180: 2669–2678.

Many kidney diseases progress to end-stage renal disease (ESRD),1,2 and represent a major public health problem worldwide (1). Disease progression is characterized by a persistent inflammatory response that causes irreversible renal glomerulosclerosis and tubulointerstitial fibrosis eventually leading to ESRD. Human nephropathies are frequently associated with leukocyte infiltration, a feature of poor prognosis (2–6). Mice that spontaneously develop lupus-like renal inflammation are protected when they lack FcγRI, the common subunit of activating Fc receptors on myeloid cells (7). Immune complex glomerulonephritis induced by anti-glomerular basement membrane (GBM) Abs, a disorder that involves leukocyte infiltration, is also largely attenuated in mice lacking activating Fc receptors (8, 9). Likewise, cross-linking of myeloid IgA Fc receptors (FcαRI or CD89) by anti-IgA complexes from IgA nephropathy patients. These findings point to a crucial role of FcαRI-FcγRI ITAMI signaling in the control of multiple heterologous or autologous inflammatory responses. They also identify anti-FcαRI Fab as a new potential therapeutic tool for preventing progression of renal inflammatory diseases. The Journal of Immunology, 2008, 180: 2669–2678.
(20). It remained to be evaluated whether FccRI-mediated ITAM inhibition has a broader action that could target non-ITAM-mediated inflammatory responses. We describe in this study that preincubation with the anti-FccRI Fab clone A77 inhibited proinflammatory responses in human monocytes and human mononuclear cell lines triggered by various heterologous receptors that use different signaling effectors. The inhibitory mechanism involved activation of Src homology protein (SHP)-1 phosphatase, which neutralizes receptor-activated phosphorylation responses. Moreover, monovalent FccRI targeting desensitized autologous responses triggered by IgA immune complexes in human monocytes. We obtained evidence that triggering of the inhibitory pathway was beneficial in experimental models of renal disease and prevented inflammation in both ITAM- and non-ITAM-mediated models. Suppression involved inhibition of renal leukocyte infiltration. These data support the important role of infiltrating leukocytes in kidney disease and suggest that ITAM-mediated inhibition of myeloid cell activation might be beneficial in inflammatory renal disorders.

Materials and Methods

Animals
C57Bl/6 and BALB/c mice transgenic for the human FccRI (CD89, line 83) and FccRI+/-/+/FEC (line 1) were used (10, 24). Genotyping was done by PCR. Mice were bred and maintained at the mouse facilities of IRM2 (Faculté de Médecine, Xavier Bichat, Paris, France). All experiments were done in accordance with national guidelines and were approved by a local ethics committee.

Subjects

Heparinized blood and serum were obtained from 49 patients with biopsy-proven IgA nephropathy and no on-going steroid treatment, and from six healthy individuals. Samples from nine patients with other inflammatory renal diseases (antineutrophil cytoplasmic Abs-associated glomerulonephritis, lupus nephritis) and 10 patients with noninflammatory renal disease (membranous nephropathy, minimal change, diabetic nephropathy) were also included. A local ethics committee approved this part of the study, and all the patients gave their informed consent.

Cells and cell lines

Human PBMC were isolated from healthy volunteers by Ficoll-Hypaque density gradient centrifugation. Enriched (70–80%) monocyte populations were obtained by adherence to plastic as described (10). Mouse peritoneal macrophages were prepared as previously described (10). The RBL-2H3 mast cell transfectants FcRIg7OG/FcRI and FcRIg7OG/FcRIg7OG/FcRIg7OG/FcRIg7OG were maintained as described (20). The human monocytic cell line THP-1 (American Type Culture Collection) was cultured in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C with 5% CO2 in a humidified incubator. The MonoMac 6 cell line was cultured in the same medium with bovine insulin (9 μg/ml; Sigma-Aldrich).

Igs and Abs

BALB/c-derived (IgG1) mouse mAbs specific for FccRI (clone A77) and the irrelevant control mAb (clone 320.1) were used as Fab fragments (20). Fab was prepared by pepsin digestion followed by reduction with 0.01 M cysteine and alkylation with 0.15 M iodoacetamide at pH 7.5. Complete digestion and purity were controlled by SDS-PAGE. The following Abs were also used: rabbit monoclonal anti-CCR2 mAb (Epicom), rat anti-type I collagen (Southern Biotechnology Associates), rat anti-Mac1 (Santa Cruz Biotechnology), mouse anti-β-actin mAb (Sigma-Aldrich) and rat anti-mouse CD4 Ab (L3T4; Southern Biotechnology Associates). The developing Abs were rat anti-mouse IgG-biotin, goat anti-rabbit HRP and donkey anti-rabbit HRP, and goat anti-mouse FITC (Southern Biotechnology Associates).

Immune complex preparation

Immune complexes were precipitated from serum by incubation with an equal volume of 3.8% polyethylene glycol (PEG) 6000 (Merck) in PBS (pH 7.4). The precipitate was collected by centrifugation at 3000 × g for 20 min at 4°C and washed twice with 3.5% PEG; each PEG precipitate was re-dissolved in DMEM (volume equal to the serum starting volume).

Cytokine and chemotaxis assays

Human and rat TNF-α levels were measured by ELISA (R&D Systems). Monocyte chemotaxis was measured in 24-well Transwell plates (Corning; Costar). THP-1 cells or human PBMC (1.5 × 106/ml) were placed in the upper chamber, separated from the lower chamber by a polycarbonate membrane (5 μm pore size). MCP-1 (10 ng/ml in RPMI 1640 medium containing 1 mg/ml BSA; R&D Systems) was added to the lower chamber, and cells were allowed to transmigrate for 2 h at 37°C in humidified air with 5% CO2. Migrated cells in the lower chamber were counted directly by light microscopy.

Small interfering RNA (siRNA) knock down of SHP-1

Human SHP-1 in MonoMac 6 cells was targeted with a mixture of the following three siRNAs (Eurogentec): siRNA1 (sense strand) CACGCCGCAUCAUGUGCAU; siRNA2 GAACCGCGCAAAAAGAAACAU; and siRNA3 CAGAGCAGGUGGAGUGAUCUA. A universal scramble-negative siRNA (GCCCGCGCAUAACGUAG) was used as control. MonoMac 6 cells were transfected with siRNA by two successive electroporations 24 h apart. For each transfection, cell density was adjusted to 1 × 106/ml in electroporation medium, and electroporation with annealed siRNAs (0.05 μM) using an Easyjet transfection apparatus (Eurogentec) at 250 V and 2100 μF. Two successive transfections were preferred over a single one, to improve the knock down of SHP-1. After each electroporation, cells were cultured in complete medium. Sixteen hours after the second electroporation, cells were incubated with anti-FccRI (clone A77) and control Ab (clone 320) for 3 h, before stimulation with MCP-1 and TNF-α, respectively, followed by cell lysis. The effectiveness of siRNA treatment was tested by SHP-1 immunoblotting.

Tyrosine phosphorylation assay, immunoprecipitation, and Western blotting

Cells were cultured in 6-well plates at 3 × 106 cells/well in 3 ml overnight at 37°C and were then treated with either PBS or the indicated Fab fragments (10 μg/ml) for 2 h unless otherwise indicated. Cells were washed in DMEM and stimulated with various agents as indicated. After stimulation, cells were solubilized in lysis buffer (50 mM HEPES (pH 7.4), 0.3% Triton X-100, 50 mM NaF, 50 mM NaCl, 1 mM Na3VO4, 30 mM Na3P04, 50 μM aprotinin, 10 μg/ml leupeptin, and postnuclear supernatants were prepared. Lysates were resolved by SDS-PAGE, transferred to PVDF membranes and immunoblotted with rabbit anti-phospho-p38 or anti-phospho-ERK MAPK Abs (Cell Signaling Technology), anti-phospho-INK (Santa Cruz Biotechnology), anti-phospho-ink (Sigma-Aldrich) followed by goat anti-rabbit or goat anti-mouse Ig, both coupled to HRP. Filters were developed by ECL (GE Healthcare).

Anti-GBM glomerulonephritis and unilateral ureteral obstruction (UUO) nephritis models

Immune-mediated glomerulonephritis was induced by rabbit anti-GBM in BALB/c FccRI transgenic mice (6- to 9-wk-old) using an accelerated model of glomerulonephritis as described (10). Nonimmune mediated nephritis was induced in C57Bl/6, FccRI transgenic mice, or FccRIg7OG, transgenic mice (10-12 wk-old) by UUO as described (25). For immunotherapy, animals were treated i.v. with either 100 μg/20 g body weight of A77 mAb Fab in 200 μl PBS or 100 μg/20 g body weight of 320 mAB Fab in 200 μl of PBS, for 14 days at 24-h intervals. The first dose was administered 24 h before anti-GBM Ab injection or UUO. On the indicated days, blood samples were collected, animals were sacrificed, and kidneys were processed as described (10). Renal function parameters (proteinuria, serum creatinine, and blood urea nitrogen), and histological and immunohistochemical parameters were studied as previously described (10). Macroscopic infiltration was studied in vivo following injection of Dil-labeled thioglycolate-derived FccRI macrophages obtained from transgenic mice as described (10).

Data analysis was completed by ANOVA for statistical calculation as indicated. Data are reported as mean ± SD, and values for p < 0.05 were considered to represent significant differences.
Results

FceRI-FcγRI-mediated inhibitory signaling inhibits non-ITAM-activated responses

FceRI-FcγRI ITAM function can be triggered in the absence of coaggregation (20). We therefore postulated that monovalent targeting in addition to inhibiting coexpressed ITAM-bearing receptors might more generally affect responses of receptors that use different signaling pathways. We analyzed the effect of anti-FcεRI Fab A77 pretreatment on the chemotactic response to MCP-1 in human monocytes and in THP-1 human monocytic cells that express CCR2, the high-affinity receptor for MCP-1 (data not shown). Fab A77, but not an irrelevant Fab (clone 320), markedly inhibited the MCP-1-induced chemotactic response by both cell types (Fig. 1A). This inhibition was concentration- and time-dependent, with an IC₅₀ around 3 μg/ml and a maximal effect after 3 h of incubation (Fig. 1B). Key events in MCP-1-mediated chemotaxis, such as p38 and p42–44 ERK MAPK phosphorylation (26), were strongly inhibited in CCR2-expressing MonoMac 6 monocytic cells (Fig. 1C and data not shown). Next, we examined the effect of Fab A77 on TNF-α-initiated activation of p38, ERK and JNK in MonoMac 6 cells, which can also be activated through TNF receptors (27). TNF-α readily induced phosphorylation of p38, ERK and JNK MAPK (Fig. 1D). All responses were inhibited by preincubation with Fab A77 but not with the irrelevant Fab 320. TNF-α-mediated p38 and ERK phosphorylation responses were also inhibited in peritoneal macrophages isolated from FcεRI transgenic mice (Fig. 1E), but not in peritoneal macrophages from FcεRI-FcRγ₂₀₀L transgenic mice expressing a FcγRI-less receptor, and hence, ITAM-deficient receptor (data not shown).

The FceRI-FcγRI inhibitory mechanism requires SHP-1 phosphatase

Our previous data have shown recruitment of SHP-1 to FcεRI following monovalent targeting by Fab A77, suggesting that this phosphatase could play a role in inhibitory mechanism (20). To further demonstrate this association, we immunoprecipitated SHP-1 phosphatase and found that phosphorylated FcγRI is coimmunoprecipitated in activated macrophages following treatment with Fab A77 (Fig. 2A). No association between SHP-1 and FcγRI was found after multivalent cross-linking of FcεRI (data not shown).
shown), confirming data previously described for FcγRI pull downs (20). We therefore directly tested the role of this phosphatase using siRNA knock down in MCP-1- or TNF-α-stimulated MonoMac 6 cells. We focused on ERK activation for MCP-1-induced signaling and on p38 for TNF-α-induced signaling. Both kinases have been implicated in signal transduction leading to inflammatory responses through these cytokines/chemokines (28, 29). As shown in Fig. 2A, siRNA inhibited expression of SHP-1 by >80% in MonoMac 6 cells. Moreover, SHP-1 knock down significantly reversed Fab A77 inhibition of MCP-1-induced responses (Fig. 2B) and of TNF-α-mediated responses (Fig. 2C), supporting SHP-1 involvement in ITAM-mediated inhibition of different receptor systems.

**FcaRI monovalent targeting diminishes inflammation in both immunological and nonimmunological models of kidney disease**

Our previous study (20) and these findings demonstrate that monovalent targeting of FcaRI broadly inhibits responses that involve ITAM- and non-ITAM bearing receptors. To determine whether this might have therapeutic implications for renal inflammatory diseases, we analyzed the effect of Fab A77 treatment in immunological and nonimmunological mouse models of kidney disease.

In a first set of experiments, FcaRI transgenic mice were injected with anti-GBM Abs (30) to induce an IgG immune complex glomerulonephritis. Anti-GBM glomerulonephritis involves activation of inflammatory responses by myeloid cells expressing activating IgG Fc receptors (31) and multiple chemokines/cytokines, including TNF-α (32). Mice treated with PBS or irrelevant Fab 320 developed elevated proteinuria, as well as blood urea nitrogen, and creatinine levels (Fig. 3, A and B). All signs of renal disease were significantly attenuated in mice treated with Fab A77. Histological analysis of Fab A77-treated animals also revealed markedly less glomerular expansion and hypercellular changes such as crescent formation associated with aneurysm, sclerosis, and necrosis (Fig. 3C). Renal injury was more severe in mice treated with...
PBS or Fab 320. Fab A77-treated animals had significantly fewer CD11b+/F4/80+ macrophages and CD4+ T cells in glomeruli and interstitial tissue (Fig. 3D), but anti-GBM deposition was similar in glomeruli of the three groups of mice (data not shown). Thus, Fab A77 treatment showed remarkable efficacy in IgG immune complex-mediated glomerulonephritis.

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FIGURE 3. Treatment of FcαRI transgenic mice with anti-FcαRI Fab A77 improves renal disease parameters in anti-GBM-induced glomerulonephritis. A, Evaluation of proteinuria over 14 days in mice after induction of anti-GBM glomerulonephritis and treatment with PBS, Fab A77, or Fab 320 as described in Materials and Methods. B, Evaluation of blood urea nitrogen (BUN) and creatinine levels 7 and 14 days after induction of anti-GBM glomerulonephritis in mice treated with PBS, Fab A77, or Fab 320. C, Histologic analysis (PAS staining) after induction of anti-GBM glomerulonephritis and following treatment with PBS, Fab A77, or Fab 320. Corresponding quantitative evaluation (below) of histologic disease parameters by disease severity score is shown. *, p < 0.05. D, Immunohistological analysis of CD4-, Mac-1-, and F4/80-positive cells following treatment with PBS, Fab A77, or Fab 320, and corresponding quantitative analysis (below) of intraglomerular and periglomerular infiltrating cells per glomerular cross-sections (gcs) is shown below the staining. *, p < 0.05.

FIGURE 4. Treatment of FcαRI transgenic (Tg) mice with anti-FcαRI Fab A77 inhibits renal inflammation after UUO. Histological analysis on day 14 of UUO left kidney sections (PAS staining) from FcαRI Tg mice (A) and from FcαRI_R209L Tg mice (B) treated with PBS, Fab A77, or Fab 320. The corresponding quantitative evaluation of histological disease parameters for both analyses is also shown (below) by disease severity score. *, p < 0.05.
We next examined the effect of Fab A77 treatment in a nonimmunological model of obstructive nephropathy induced by UUO, which involves MCP-1-induced macrophage recruitment (33–35). On day 14 after left-sided UUO in Fc/H9251 RI transgenic mice, macroscopic examination showed strongly increased kidney size in control Fab 320-treated animals as compared with the nonobstructed right kidneys (data not shown). This increase was diminished in Fab A77-treated animals, indicating that inflammatory response was reduced. Further histological analysis confirmed that Fab A77-treated transgenic mice had significantly less inflammation, tubular dilatation, tubular atrophy, and mononuclear cell infiltration than control animals (Fig. 4A). Immunohistochemical analysis showed that F4/80+ interstitial macrophage and CD4+ T cell numbers in the obstructed kidneys were lower in Fab A77-treated animals than in controls (Fig. 5A). To determine whether these effects involved active inhibitory signaling through the ITAM-containing FcRγ chain, UUO was also induced in FcRI/H209L transgenic mice expressing a FcRγ-less receptor (10). A77 treatment of these mice had no effect on UUO-associated inflammation, which was similar to that in control groups (Fig. 4B). Similarly, Fab A77 had no effect on macrophage or T cell infiltration (Fig. 5B). These results confirmed our previous in vitro results (20) and further supported the importance of ITAM-bearing FcRγ in Fab A77-mediated inhibition.

**FIGURE 5.** Treatment of FcαRI transgenic (Tg) mice with anti-FcαRI Fab A77 inhibits inflammatory cell infiltration after UUO. A, Immunohistological analysis of Mac-1-, F4/80-, and CD4-positive cells in FcαRI transgenic mice after UUO (day 14) following treatment with either PBS, Fab A77, or control Fab 320, as described in Materials and Methods. The corresponding quantitative analysis of infiltrating cells. *, p < 0.05. B, Immunohistological analysis of Mac-1-, F4/80-, and CD4-positive cells in FcαRI/H209L transgenic mice after UUO (day 14), following treatment with PBS, Fab A77, or control Fab 320. Corresponding quantitative analysis of infiltrating cells per high-power field (HPF) is shown below each staining.

**FIGURE 6.** Treatment of FcαRI transgenic (Tg) mice with anti-FcαRI Fab A77 inhibits fibrosis development after UUO. A, Evaluation of fibrosis in UUO kidneys (day 14) after treatment of FcαRI transgenic mice with PBS, Fab A77, or control Fab 320 as described in Materials and Methods using staining with Sirius red. B, Treated mice after immunofluorescence staining with anti-type 1 collagen. The corresponding quantitative evaluation of disease severity score is shown on the right. *, p < 0.05.
The UUO model is characterized by progressive fibrosis, fibroblast proliferation, increased accumulation of extracellular matrix proteins, and tubule atrophy (12). We therefore analyzed Sirius red-stained kidney sections (Fig. 6A) and type I collagen deposition (Fig. 6B) as markers of fibrosis in FcRI transgenic mice treated with Fab A77 or Fab 320. Both parameters were substantially attenuated by Fab A77 but not by Fab 320, and no such reduction was observed in FcRIR209L transgenic mice (data not shown). Together, these results indicate that anti-FcRI Fab treatment has remarkable anti-inflammatory efficacy in nonimmune obstructive kidney disease.

**FcRI monovalent targeting directly inhibits transgenic macrophage accumulation in inflamed kidney**

One of the main features observed after Fab A77 treatment was the strong reduction in the inflammatory cell infiltrate in both the immunological and nonimmunological disease models. To determine whether this feature was due to a direct effect of Fab A77 on the homing of myeloid cells to the inflamed kidney, we examined the effect of Fab A77 treatment on macrophage recruitment in the UUO model by conducting adoptive transfer experiments. Syngenic Dil-labeled peritoneal human FcRI transgenic macrophages were injected i.p. into nontransgenic littermate recipients 1 day before UUO. On day 14, obstructed kidneys in mice treated with Fab 320 or Fab A77 were analyzed for the presence of fluorescent infiltrating macrophages. The number of macrophages was significantly lower in Fab A77-treated animals than in controls (Fig. 7), supporting a direct effect on macrophage homing.

**FcRI monovalent targeting diminishes inflammatory signaling in human monocytes, independently of disease status**

To analyze the therapeutic potential in humans we examined the effect of Fab A77 treatment on TNF-α production by human blood monocytes isolated from healthy subjects and patients with inflammatory and noninflammatory kidney diseases. LPS was chosen as stimulant, as it induces strong TNF-α production and because bacterial infection of the upper respiratory tract is often associated with nephropathies contributing to disease aggravation (36). As shown in Fig. 8A, unstimulated monocytes from healthy subjects, patients with lupus nephritis, and patients with antineutrophil cytoplasmic Ab-associated glomerulonephritis produced similar baseline levels of TNF-α. LPS strongly induced TNF-α secretion, to a similar degree in all groups, ruling out priming or unresponsiveness of patients’ monocytes. Pretreatment with Fab A77, but not with Fab 320, led to a similar marked reduction in TNF-α production in both healthy subjects and patients. The anti-inflammatory effect of Fab A77 after LPS stimulation was confirmed in experiments showing a decrease in p38 and ERK MAPK phosphorylation in MonoMac 6 cells (Fig. 8B).

**FIGURE 7.** Anti-FcRI Fab A77 treatment directly inhibits influx of FcRI transgenic macrophages after UUO. Macrophages isolated from the peritoneal cavity of FcRI transgenic (Tg) mice were labeled with the fluorescent dye Dil and injected i.p. into FcRI-negative littermate (Lt) recipients 1 day before UUO. Infiltrating macrophages in UUO kidneys (day 14) were counted by fluorescence microscopy after treatment of mice with PBS, Fab A77, or control Fab 320 as described in Materials and Methods. A representative photograph and data obtained by analysis of 50 high-power fields (HPF) are shown. *, p < 0.05.

**FIGURE 8.** Treatment with anti-FcRI Fab A77 inhibits heterologous LPS-induced TNF-α production and autologous IgA immune complex-induced downregulation of FcRI RBL mast cell transfectants. A, Blood monocytes obtained from normal subjects (n = 6), patients with inflammatory renal disease (n = 10; including four antineutrophil cytoplasmic Abs (ANCA)-associated glomerulonephritis (GN) and 5 lupus nephritis), and patients with noninflammatory renal disease (n = 10, including four membranous nephropathy, three minimal change, and three diabetic nephropathy) were stimulated with LPS (100 ng/ml). TNF-α production was measured by ELISA and compared with unstimulated cells after preincubation (24 h) with PBS, Fab A77 (10 μg/ml), or control Fab 320 (10 μg/ml). *, p < 0.05. B, Representative phosphorylation response of p42–44 ERK and p38 MAPKs after LPS stimulation (100 ng/ml) of MonoMac 6 cells, as revealed by immunoblotting with the indicated phospho-specific Abs. Reprobing with anti-β-actin is shown as a control for equal loading. C, PBS, Fab 320 (10 μg/ml), or Fab A77 (10 μg/ml) treated (2 h) FcRIK209A/FcγRI (left) and untreated ITAM-mutated FcRIK209A/FcγRIβ267/278F (right) mast cell transfectants were stimulated (3 h) with either control buffer (PBS) or PEG-purified immune complex (IC) from 10 patients (shown be each symbol) with IgA nephropathy. TNF-α release was then determined.
This extends the ITAM function of FcαRI to signaling induced by TLR4.

IgA nephropathy patients have pathologic IgA immune complexes in their serum, which contribute to disease development (37). Our recent evidence points to the involvement of FcαRI, as specific cross-linking of this receptor aggravated symptoms in a mouse model of spontaneous IgA nephropathy. Given the fact that Fab A77 binds to FcαRI at a site different from IgA and therefore does not block IgA immune complex binding to the receptor (38, 39), we examined whether Fab A77 could inhibit responses induced by its own receptor stimulated through IgA immune complex. We used FcαRI-humanized RBL mast cells transfected with a chimeric FcαRI2m09/FcεRIγ that can be activated by IgA immune complex purified by precipitation with PEG from serum of an IgA nephropathy patient (24). As shown in Fig. 8C (left), incubation of cells with buffer alone did not induce significant TNF-α production. Of 49 patients’ sera tested, IgA immune complex from 10 patients induced significant TNF-α production when transfectants had been preincubated with control Fab 320 (Fig. 8C, left). In contrast, preincubation with Fab A77 strongly inhibited the TNF-α production triggered by these patients’ IgA immune complex. To test the specificity of stimulation through FcαRI, IgA immune complexes were added to transfectants expressing an ITAM-mutant chimeric receptor FcαRIγC209L/FcεRIγY258E/S278R. As shown in Fig. 8C (right), PEG precipitates from 6 of the 10 responder patients did not stimulate mutant receptor transfectants, suggesting that these six patients had indeed stimulatory FcαRI-targeting IgA immune complex, whereas the other four patients appeared to contain an unidentified stimulatory factor in their precipitates. However, all responses were inhibited by A77 Fab, further supporting the broad efficacy of Fab A77 to inhibit multiple responses (Fig. 8, left). These results also demonstrate that monomeric targeting of FcαRI inhibits autologous and heterologous receptor-activated responses.

Discussion

Myeloid cell-expressed FcαRI and its associated ITAM-bearing FcεRI subunit have recently emerged as new actors in immune homeostasis (19, 20, 40). They act as a dual-function module that can either activate cells or attenuate cell activation through heterologous ITAM-bearing receptors, depending on the interaction with its ligand. Contrary to receptors bearing a bona fide inhibitory ITIM, ITAM-mediated inhibition does not require coaggregation of activating and inhibitory receptors. We therefore postulated that monovalent targeting of FcαRI might not only act on other ITAM-bearing receptors but control cell activation in a more general manner. We provide evidence that induction of ITAM signaling through FcαRI induces a broadly effective inhibitory signal toward responses induced by multiple inflammatory receptors and ligands. Besides inhibiting other ITAM-bearing receptors, as previously shown (20), we found that responses induced by a highly diverse set of inflammatory mediators such as MCP-1, TNF-α, and LPS were inhibited. The homologous ITAM-bearing adaptor DAP12, when associated with the isoform TREM-2, has also been found to down-regulate activating responses triggered via TLRs and Fc receptor (21, 22). Interestingly, incubation with anti-FcαRI Fab before stimulation with IgA immune complex present in serum of IgA nephropathy patients also attenuated activation induced by its own receptor. Such autologous ITAM-dependent desensitization has also been reported to occur during T cell Ag receptor activation in response to weakly binding ligands (41). Our data support the view that monovalent targeting of FcαRI induces a general desensitized state that interferes with activation through a whole variety of receptors, including autologous FcαRI.

Previous studies pointed to several distinct mechanisms responsible for ITAM-mediated inhibitory functions (reviewed in Refs. 19, 42). They included interference of PI3K and phospholipase Cγ with TLR activation, intrinsic negative regulation by ITAM phosphotyrosines, sequestration of signaling effectors, differential vesicular targeting to intracellular signaling compartments, and production of anti-inflammatory cytokines. In the case of FcαRI, we have proposed a role for SHP-1 phosphatase, based on preferential recruitment of SHP-1 over Syk to FcαRI following inhibition of FcαRI-stimulated degranulation responses. We confirm an enhanced recruitment to the phosphorylated FcαRI-associated FcεRI chain in TNF-α-stimulated FcαRI transgenic macrophages after incubation with Fab A77. The role of SHP-1 was directly shown by means of siRNA experiments. Specific knock down of SHP-1 markedly reduced the capacity of FcαRI to inhibit both MCP-1- and TNF-α-induced signals identifying SHP-1 as a major factor in the FcαRI inhibitory mechanism.

Our described broad inhibitory action of anti-FcαRI Fab suggests its potential for a general immunotherapeutic agent for inflammatory diseases in addition to IgE-mediated asthma (20). Given its capacity to inhibit responses mediated both by immune receptors and by a variety of other receptors, we tested the therapeutic potential of FcαRI targeting in immunological (anti-GBM) and nonimmunological (UUO) models of inflammatory kidney diseases. Treatment was clearly beneficial in both models. In the anti-GBM model, renal function parameters such as proteinuria, blood urea nitrogen, and creatinine levels were considerably improved by Fab A77 pretreatment, and histological signs of disease activity were attenuated. Similarly, in the UUO model, inflammatory signs were reduced, both macroscopically and histologically. Fab A77 treatment also prevented fibrosis development, as shown histologically and immunohistologically (type I collagen staining). The effectiveness of Fab A77 in UUO was abolished in FcαRIγC209L transgenic mice showing that the FcεRI inhibitory mechanism.

The protective effect of Fab A77 treatment involves a major inhibition of leukocyte accumulation in the kidneys. In both disease models the number of infiltrating macrophages and T cells was strongly reduced by Fab A77 treatment. Most leukocytes found in diseased kidneys are recruited from the circulation (43, 44). In the UUO model, we showed by means of adoptive transfer experiments with FcαRI transgenic macrophages that Fab A77 treatment directly inhibited the influx of these cells. These data indicate that FcαRI targeting directly affects macrophage chemotaxis in vivo and are in keeping with our in vitro findings showing significantly decreased chemotaxis of THP-1 monocytic cells and purified blood monocytes in response to MCP-1 after Fab A77 treatment. As anti-inflammatory FcαRI is mainly expressed by macrophages, our data demonstrate a deleterious role of macrophage infiltration in kidney disease and fibrosis associated with UUO. It has been reported that T cells can directly interact with renal tubular epithelial cells leading to increased production of proinflammatory proteins (45). However, as T lymphocytes do not express FcαRI (46) our results suggest that infiltration by T lymphocytes may be secondary to activation of myeloid cells such as macrophages. Indeed, the various T cell subsets express a multitude of receptors that could respond to chemokines produced by macrophages in inflamed kidney tissue (47, 48).
We also showed the anti-inflammatory effect of monovalent targeting of FcγRI on human monocytes ex vivo. Preincubation with Fab A77 inhibited LPS-induced TNF-α production by monocytes from healthy subjects and patients with both inflammatory and noninflammatory kidney diseases. These results are in keeping with the previously reported inhibition of FcγR-mediated human phagocytic responses (20). They also support the notion that anti-FcγRI Fab may be a promising treatment for human renal inflammatory diseases. Interestingly, Fab A77 also inhibited autologous responses induced by pathologic IgA immune complex present in the serum of patients with IgA nephropathy. FcγRI activation on monocytes by IgA immune complex was recently recognized as an aggravating factor in a spontaneous experimental model of IgA nephropathy (10).

In conclusion, our findings show that monovalent targeting of myeloid-expressed FcγRI generates a general, ITAM-dependent inhibitory signal (defined as ITAM) affecting responses induced by multiple inflammatory receptors and ligands. Both ITAM-dependent (49) and ITAM-independent inflammatory responses can be negatively regulated by this receptor. The inhibitory mechanism involves activation of SHP-1 likely by neutralizing receptor-activated phosphorylation responses. FcγRI-mediated inhibition reduced inflammatory markers in both immune and nonimmune experimental models of renal disease. Likewise, ex vivo targeting of human blood monocytes led to a substantial inhibitory effect on heterologous LPS-stimulated responses and autologous responses induced by IgA immune complex. ITAM inhibition of myeloid cell-mediated inflammatory responses thus appears to be a promising potential treatment for renal inflammatory diseases, including IgA nephropathy.

Disclosure
The authors have no financial conflict of interest.

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