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Identification of FcαRI as an Inhibitory Receptor that Controls Inflammation: Dual Role of FcRγ ITAM

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Summary

Serum IgA is considered a discrete housekeeper of the immune system with multiple anti-inflammatory functions, whereas IgA-immune complexes mediate inflammatory responses. Here, we identify FcαRI as a molecular device that determines the nature of IgA responses. In the absence of sustained aggregation, receptor targeting by serum IgA or anti-FcαRI Fab inhibits activating responses of heterologous FcγR or FcεRI. The inhibitory mechanism involves recruitment of tyrosine phosphatase SHP-1 to FcαRI and impairment of Syk, LAT, and ERK phosphorylation induced by FcεRI engagement. SHP-1 recruitment is dependent on ERK. Conversely, sustained aggregation of FcαRI by multimeric ligands stimulates cell activation by recruiting high amounts of Syk and aborting SHP-1 binding. Both types of signals require the FcRγ-ITAM motif. Anti-FcαRI Fab treatment suppresses manifestations of allergic asthma in FcαRI transgenic mice. These findings redefine FcαRI as a bifunctional inhibitory/activating receptor of the immune system that mediates both anti- and proinflammatory functions of IgA.

Introduction

The maintenance of immune system homeostasis by immunoglobulins (Ig) is essential for protecting the host against inflammation and autoimmunity (Ravetch and Bolland, 2001). Human IgA is the most heterogeneous Ig, as it occurs in multiple molecular forms and two subclasses (IgA1 and IgA2), which are differentially distributed between the systemic and mucosal immune system. Serum IgA, the second most abundant isotype, are mainly monomers derived from bone marrow plasma cells (Kerr, 1990), whereas secretory IgA (SIgA) are synthesized as dimers by local plasma cells before being transported to mucosal surfaces through epithelial cells by the polymeric Ig receptor (Phalipon and Corthesy, 2003). Pathogens with mucosal tropism induce a local SIgA immune response that is protective. Data with serum IgA have revealed an intriguing paradox: they support an activating response when aggregated by antigens (van Egmond et al., 2001) yet have also been considered as an anti-inflammatory isotype (Kerr, 1990). IgA is not usually involved in humoral immune responses and does not activate complement. In the absence of antigen, IgA downregulates IgG-mediated phagocytosis, chemotaxis, bactericidal activity, oxidative burst activity, and cytokine release (Van Epps and Williams, 1976; Van Epps et al., 1978; Van Epps and Brown, 1981; Wilton, 1978; Wolf et al., 1994; Nikolova and Russell, 1995; Wolf et al., 1996). Supporting evidence for the regulatory role of IgA comes from patients with selective IgA deficiency, who show increased susceptibility to autoimmune and allergic disorders (Schaffer et al., 1991). The molecular basis underlying IgA inhibitory functions is unknown.

Other Ig isotypes have been reported to have inhibitory functions by interacting with Fc receptors containing immunoreceptor tyrosine-based inhibitory motifs (ITIM) (Long, 1999). They act by coaggregation with activating receptors: the crosstalk between the two receptors generates a negative signal. This signal is mediated either by SH2 domain-containing phosphatase (SHP-1) that consumes protein tyrosine kinase substrates or SH2 domain-containing inositol phosphatase (SHIP) consuming phosphatidylinositol 3,4,5-trisphosphate, the product of phosphatidylinositol-3-kinase. Both phosphatases were found to associate through their SH2 domain with given ITIM-bearing receptors upon ITIM tyrosine phosphorylation by the coaggregated activating receptor (Coggeshall et al., 2002). These phosphatases are then ideally located to target their substrates, which aborts the activating signal.

FcαRI (CD89) is the only IgA Fc receptor expressed on blood myeloid cells, including monocyte/macrophages, dendritic cells, Kupffer cells, neutrophils, and eosinophils (Monteiro and Van De Winkel, 2003). It can bind IgA1 and IgA2 with low affinity ($K_a \sim 10^6$ M^{-1}). While monomeric IgA binding to FcαRI is transient, polymeric IgA and IgA immune complexes bind with a greater avidity (Wines et al., 2001). FcαRI is considered as a unique member of the FcR family. Its two Ig-like domains are oriented at right angles; two FcαRI molecules can bind one IgA molecule within the EC1 domain, at a site completely different from the other FcRs (Herr et al., 2003). The FcαRI gene is not located in the FcR gene cluster but on chromosome 19, inside the leukocyte receptor cluster (LRC). FcαRI is distantly related to other FcRs, being more homologous to LRC-encoded activating and inhibitory receptors (Davis et al., 2002). Another peculiarity of FcαRI is that it can be expressed with or without physical association to FcRγ (Launay et al., 1999). This subunit contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic tail (Kinet, 1999). The γ-less FcαRI internalizes and recycles IgA to the cell surface (Launay et al., 1999), while FcRγ-associated FcαRI mediate activating responses (Morton et al., 1995;

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Launay et al., 1999; Shen et al., 2001). Therefore, FcαRI has been described as a solely activating receptor following aggregation by IgA immune complexes (Monteiro and Van De Winkel, 2003).

As anti-inflammatory effects of serum IgA require binding of the Fcα portion to the cell (Van Epps and Williams, 1976; Wilton, 1978), we tested whether FcαRI could mediate IgA-induced inhibition. We found that targeting FcαRI in the absence of sustained aggregation markedly inhibits IgG-dependent phagocytosis and IgE-induced exocytosis, whereas sustained FcαRI aggregation promotes cell activation. Both activation and inhibition require the FcRγ ITAM. The FcαRI inhibitory pathway of heterologous receptors involves ERK-dependent recruitment of SHP-1, leading to impairment of signaling induced by heterologous FcεRI. By contrast, in FcαRI-activating signals, SHP-1 recruitment is prevented, while Syk recruitment is enhanced. FcαRI inhibitory function was demonstrated in vivo in an asthma model using human FcαRI transgenic mice. This study redefines FcαRI as a bifunctional activating/inhibitory receptor that controls inflammatory responses.

Results

Identification of FcαRI as an Inhibitory Receptor

Serum IgA can dampen IgG-mediated phagocytosis (Wilton, 1978; Nikolova and Russell, 1995). To determine the role of FcαRI in this process, we examined whether Fab fragments of an anti-FcαRI mAb (clone A77) could inhibit IgG-mediated phagocytosis of *E. coli* by blood monocytes. Fab usage permitted FcαRI targeting independently of IgG receptors. Preincubation of monocytes with A77 Fab inhibited IgG-mediated phagocytosis (>80%) compared to irrelevant Fab fragments (Figure 1). These results identify FcαRI as a molecular device involved in the previously described IgA-induced decrease in IgG-mediated phagocytosis.

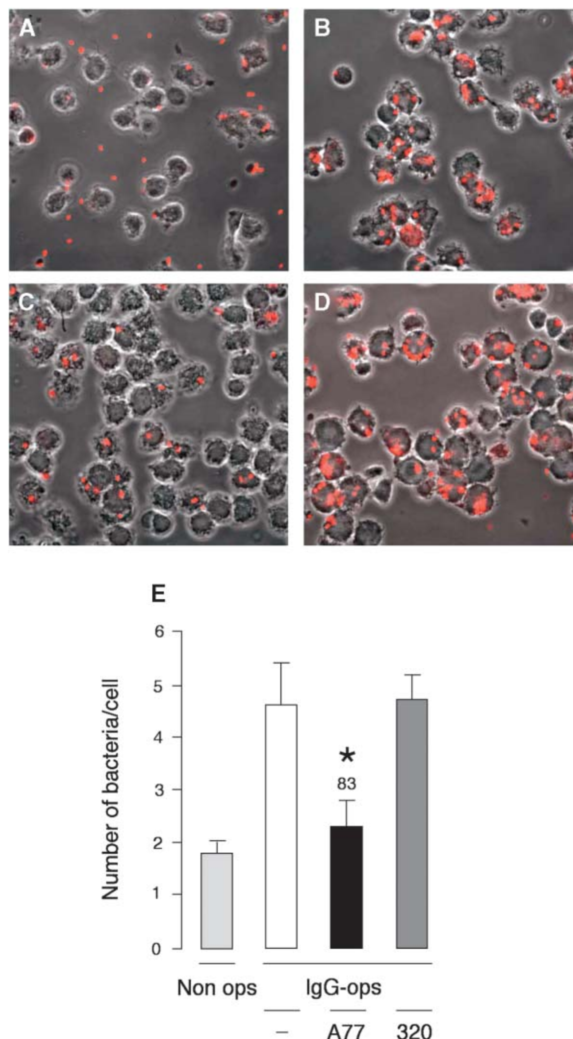

Figure 1. FcαRI Inhibits IgG-Mediated Phagocytosis

Human monocytes were preincubated with 10 μg/ml anti-FcαRI Fab (A77) (C), irrelevant Fab (320) (D), or buffer (A and B). Phagocytosis was measured with Texas-red-conjugated *E. coli* opsonized (B–D) or not (A) with rabbit anti-*E. coli* IgG, using confocal microscopy. Overlaid transmission and fluorescence images (mid sections) are shown (n = 6). Mean number (±SD) of ingested bacteria per cell from different healthy donors was determined by counting at least three fields (E). Mean percentage inhibition by Fab (*$p < 0.02$, Student's unpaired t test) is indicated above the bar.

Type of Interaction with the Ligand Determines the Balance between Inhibitory and Activating Functions of FcαRI

To characterize the requirements for inhibitory or activating function, stable FcαRI transfectants were established in the rat mast cell line RBL-2H3 expressing high-affinity IgE receptor (FcεRI). Antigen stimulation of IgE-sensitized transfectants induced a strong degranulation response (Figure 2A). Preincubation with anti-FcαRI Fab markedly inhibited this reponse (74%) as compared to an irrelevant Fab and to nontransfected cells (Figure 2A). This was observed in two other transfectants (not shown). The effect was dose dependent (see Supplemental Figure S1A at http://www.immunity.com/cgi/content/full/22/1/31/DC1/) and observed with several anti-FcαRI mAbs (A77, A59, A62) recognizing epitopes within the EC2 domain (Morton et al., 1999) but not with the anti-FcαRI mAb A3 (Supplemental Figure S1B). Of note, anti-FcαRI Fab did neither modify IgE binding nor FcαRI expression in the presence or absence of IgE or of IgE plus antigen (Supplemental Figures S1C–S1E). Anti-FcαRI Fab purified by gel filtration had a similar inhibitory action, ruling out a role of aggregates (not shown). The ligand valence in the inhibitory response was also examined. Monovalent anti-FcαRI Fab had a stronger inhibitory effect than divalent $F(ab')_2$ fragments (Supplemental Figure S1F), whereas multivalent FcαRI crosslinking induced cell activation (Figure 2B). No degranulation was observed with anti-FcαRI Fab or $F(ab')_2$ alone (not shown).

The anti-inflammatory effect of IgA, the physiological ligand of FcαRI, was next investigated. Incubation of transfectants with serum IgA but not with IgG significantly inhibited IgE-dependent degranulation (43%) (Figure 2C). As IgA may exert its biological activity at inflammatory sites where numerous mediators (including proteases) accumulate, we examined whether cell treatment with trypsin modulated IgA-mediated inhibi-

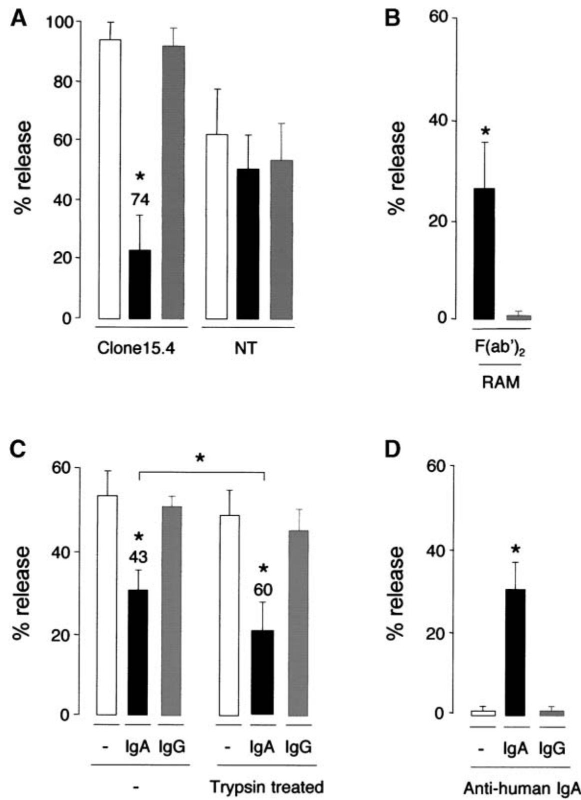

Figure 2. Characterization of FcαRI Inhibitory/Activating Functions

(A) FcαRI inhibits IgE-mediated exocytosis. FcαRI transfectants (clone 15.4) and nontransfected (NT) RBL cells were sensitized with IgE (open bars) or IgE plus 10 μg/ml A77 anti-FcαRI Fab (black bars) or irrelevant Fab (320) (gray bars). Degranulation was triggered with DNP-HSA (0.1 μg/ml), and net β-hexosaminidase release was determined (*$p < 0.02$, $n = 5$).

(B) FcαRI crosslinking induces degranulation. Cells were sensitized with 10 μg/ml of A77 $F(ab')_2$ fragments (black bars) or 320 (gray bars), stimulated with rabbit anti-mouse $F(ab')_2$ (RAM at 40 μg/ml), and assayed as in (A) (*$p < 0.02$, $n = 4$).

(C) Human serum IgA induces inhibition modulated by proteolytic treatment. FcαRI transfectants were pretreated or not with 1 mg/ml trypsin and sensitized overnight with IgE alone (open bars) or with IgE plus either serum IgA (black bars) or IgG (gray bars) at 0.2 mg/ml. Degranulation was triggered with DNP-HSA (0.03 μg/ml) and assayed as in (A) (*$p < 0.01$, $n = 6$).

(D) IgA complexes mediate cell activation. Cells were sensitized with 0.2 mg/ml IgA (black bars), IgG (gray bars), or medium (open bars) before stimulation with goat anti-human IgA $F(ab')_2$ (50 μg/ml) and assayed as in (A) (*$p < 0.02$, Student's unpaired t test, $n = 4$). Mean percentage inhibition of degranulation is indicated above the bar.

tory function. This treatment did not affect FcαRI expression (Monteiro et al., 1990) or IgE-mediated degranulation, yet the inhibitory effect of serum IgA was significantly enhanced (~40%) (Figure 2C). A similar enhancement was observed with purified myeloma IgA (not shown). Two different batches of serum IgA inhibited degranulation in a dose-dependent manner with a maximal inhibition at 0.5 mg/ml (Supplemental Figure S1G). Both IgA1 and IgA2 induced significant inhibition (30%–40%), as compared to human IgG (<5%) (Supplemental Figure S1H). The different IgA preparations did not induce degranulation on their own and showed no inhibition in nontransfected cells (not shown). In contrast, sustained aggregation of FcαRI by IgA complexes resulted in degranulation (Figure 2D). These data establish that IgA interactions with FcαRI in the absence of antigen are profoundly inhibitory, while aggregation by IgA complexes leads to cell activation.

FcαRI Targeting Prevents IgE-Mediated Asthma

We next examined the effect of monomeric targeting of FcαRI on inflammatory responses in vivo using an IgE-mediated asthma model. As mice do not express FcαRI (Monteiro and Van De Winkel, 2003), the inhibitory function was tested in transgenic (Tg) mice expressing human FcαRI on myeloid cells (Launay et al., 2000). After repeated intranasal challenge with IgE immune complexes in the presence of an irrelevant Fab, $FcαRI^+$ Tg mice developed bronchial hyperreactivity to inhaled methacholine, as compared to PBS-challenged counterparts (Figures 3A and 3B). This was abrogated in A77 anti-FcαRI Fab-treated Tg mice, whereas no effect was seen in $FcαRI^-$ littermates (Figures 3A and 3B). Pulmonary histology of antigen-challenged Tg mice treated with the irrelevant Fab showed diffuse alveolar capillary congestion, with a perivascular and peribronchial inflammatory infiltrate consisting mainly of granulocytes and mononuclear cells (Figures 3F–3H). These features were absent from lungs of PBS-challenged mice (Figures 3C–3E). Anti-FcαRI Fab administration prevented antigen-induced airway congestion and infiltration by inflammatory cells (Figures 3I–3K). No effects were observed in lungs of $FcαRI^-$ littermates treated with anti-FcαRI Fab (not shown).

FcRγ ITAM Involvement in FcαRI-Inhibitory Signaling

To determine which type of FcαRI, associated or nonassociated with FcRγ, accounts for the inhibitory response, we examined transfectants expressing a R209L transmembrane FcαRI mutant (Figures 4A and 4B) that cannot associate with the FcRγ chain (Launay et al., 1999). $FcαRI_{R209L}$ elicited neither inhibitory nor activating responses (Figures 4C and 4D). Therefore, γ-less FcαRI does not directly account for these functions. To assess the involvement of FcRγ, a chimeric receptor was constructed by fusing the extracellular and R209L transmembrane domains of FcαRI to the intracytoplasmic tail of human FcRγ (Figure 4A). Binding of anti-FcαRI Fab to $FcαRI_{R209L}/γ$ chimeric receptor transfectants restored the inhibitory effect to an extent similar to that observed in wild-type transfectants (91% versus 74%) (Figure 4C). Aggregation of chimeric receptors induced degranulation, demonstrating that, like wild-type FcαRI, they were able to mediate both activation and inhibition (Figure 4D). As FcRγ does not contain a known inhibitory motif, the role of FcRγ ITAM in the inhibitory effect was examined. Single or double Y-to-F ITAM point mutations of the chimeric receptor were made and transfected into RBL cells (Figures 4A and 4B). All mutants were no longer able to mediate inhibitory and activating responses (Figures 4C and 4D). Of note, all transfectants expressed significant levels of FcαRI at the cell surface (Figure 4B) and exhibited over 50% degranulation mediated by FcεRI (Figure 4C).

We next examined whether FcRγ could mediate both

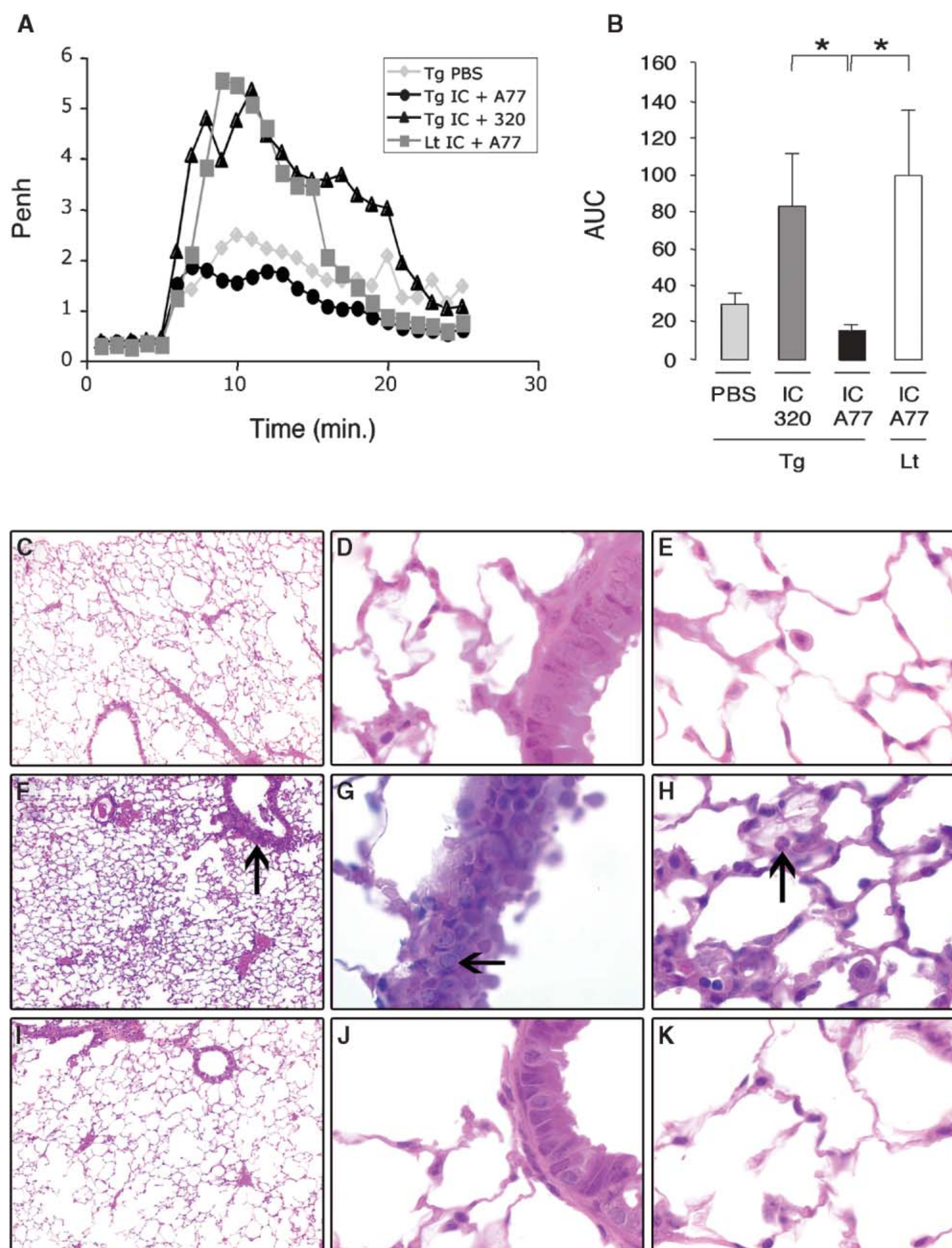

Figure 3. FcαRI Targeting Prevents Asthma Manifestations

FcαRI-transgenic Balb/c mice (Tg) and littermate controls (Lt) were immunized twice with TNP-OVA and repeatedly challenged intranasally with PBS or with IgE-TNP-OVA immune complexes (IC) in the presence of anti-FcαRI (A77) or irrelevant 320 Fab. Bronchial reactivity to inhaled methacholine was analyzed by whole-body plethysmography.

(A) Changes in airway resistance are expressed as Penh (mean of three mice per group).

(B) Cumulative areas under the curve (AUC) of corresponding Penh values are means ± SD of three independent experiments involving at least eight mice per group (*$p < 0.05$, Student's unpaired t test).

(C–K) Morphological analysis (H&E staining) of lung tissue sections from FcαRI Tg mice. Control PBS-challenged mice showing normal morphology are shown in (C)–(E). Antigen-challenged mice treated with irrelevant Fab, showing peribronchial (F) and epithelial (G) inflammatory infiltrates and alveolar capillary congestion (H) (see arrows). Antigen-challenged anti-FcαRI Fab-treated mice showing substantially less inflammation and congestion are shown in (I)–(K). Magnification: 10×, (C), (F), (I); 100× (D), (E), (G), (H), (J), (K).

activating and inhibitory functions in the context of another related FcR. As monomeric IgE has been reported to trigger FcεRI signaling (Asai et al., 2001; Kalesnikoff et al., 2001), we assessed whether monomeric IgE could reversely inhibit FcαRI-mediated activation in RBL transfectants. Two mouse monomeric IgE failed to inhibit FcαRI-induced exocytosis (Supplemental Figure S2A). Similarly, human monomeric IgE, which does not bind to rat FcεRI, was unable to inhibit mouse IgE-mediated degranulation in previously described FcεRI humanized RBL cells (Marchand et al., 2003) (Supplemental Figure S2B). We also established transfectants expressing a chimeric receptor composed of the extracellular and transmembrane domains of FcγRIIb fused

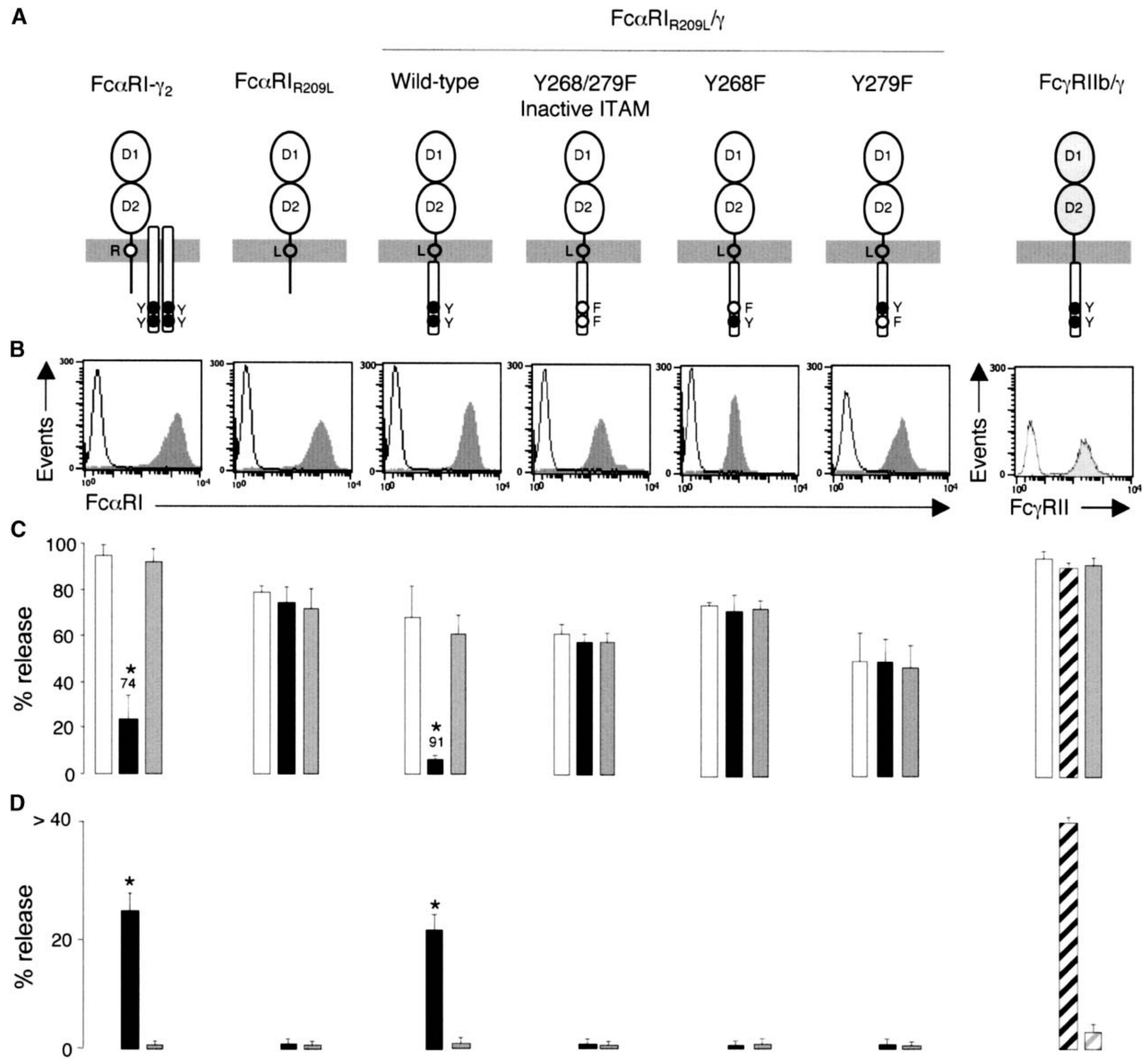

Figure 4. FcRγ ITAM Mediates Both Inhibition and Activation in the Context of FcαRI

(A) FcαRI and FcγRIIb constructs are schematically presented.

(B) FcR construct surface expression by transfectants using A77 anti-FcαRI, 2.4G2 anti-FcγRII/III, or irrelevant 320 mAb (gray versus open histograms).

(C) FcR-mediated inhibition of IgE-dependent degranulation. Transfected clones were IgE sensitized alone (open bars) or with 10 μg/ml of anti-FcαRI A77 Fab (black bars), anti-FcγR 2.4G2 Fab (hatched bars), or irrelevant 320 Fab (gray bars). Degranulation was assayed as in Figure 2A (*$p < 0.02$, $n = 3$). Numbers above the bars indicate the percentage inhibition as compared to IgE-induced degranulation (open bars).

(D) FcR-mediated degranulation. Cells were sensitized with 10 μg/ml of A77 (black bars), biotinytaled 2.4G2 (hatched black bars), 320 (gray bars), or biotinytaled 320 (hatched gray bars) F(ab′)$_2$ and stimulated with RAM F(ab′)$_2$ (40 μg/ml) or with SA-alkaline phosphatase (50 μg/ml). Net β-hexosaminidase release was determined (*$p < 0.02$, Student's unpaired t test, $n = 4$).

to the intracytoplasmic tail of mouse FcRγ (FcγRIIb/γ), as described (Bonnerot et al., 1992). Although FcγRIIb/γ was capable to mediate an activating signal upon multimeric engagement with anti-FcγR mAb 2.4G2, Fab fragments of this mAb were unable to inhibit FcεRI-induced degranulation (Figures 4C and 4D). These data underline the importance of the FcαRI context for FcRγ to mediate the inhibitory function.

SHP-1 Recruitment and Cell Desensitization by FcαRI

Multivalent crosslinking of FcαRI permits recruitment and activation of tyrosine kinases and adaptors (Gulle et al., 1998; Launay et al., 1998; Park et al., 1999). To identify initial events involved in FcαRI-inhibitory signaling, we determined the phosphorylation state of the FcαRI-associated FcRγ after multivalent or monovalent ligand interaction. Targeting of the FcαRI$_{R209L}$/γ chimeric receptor by A77 Fab triggered a weaker phosphorylation of the FcRγ than after FcαRI-multimeric aggregation (Figure 5A, left panel). Noninhibitory anti-FcαRI A3 Fab failed to induce phosphorylation (Supplemental Figure S3). A weak phosphorylation of chimeric receptor was also observed after FcεRI activation and treatment with A77 Fab (Figure 5A, right panel). As phosphatases are

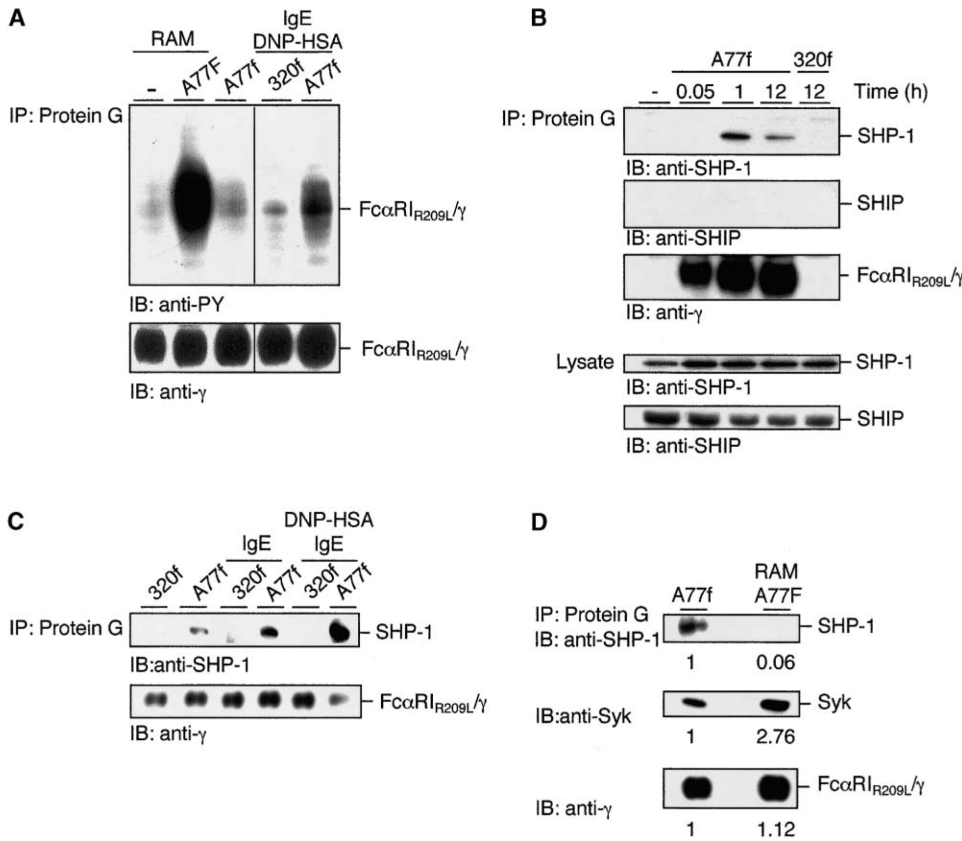

Figure 5. Monomeric FcαRI Targeting Induces Weak Phosphorylation of FcRγ and SHP-1 Recruitment

(A) Phosphorylation state of FcαRI/FcRγ under multimeric or monomeric targeting conditions. For FcαRI stimulatory experiments (left panel), chimeric FcαRI$_{R209L}$/γ RBL transfectants (clone 9.4) were incubated or not (−) with 10 μg/ml of anti-FcαRI A77 F(ab′)$_2$ (A77F) for 30 min at 4°C, washed, and stimulated with 40 μg/ml rabbit anti-mouse Ig F(ab′)$_2$ (RAM) for 3 min at 37°C. Cells were also incubated with 10 μg/ml A77 Fab (A77f) under the same conditions but omitting RAM. For FcαRI-inhibitory experiments of FcεRI signaling (right panel), cells were sensitized with IgE anti-DNP plus 10 μg/ml A77 Fab (A77f) or 320 Fab (320f) overnight at 37°C before stimulation with DNP-HSA (0.1 μg/ml) for 3 min at 37°C. Cells were lysed in 0.3% Triton X-100 lysis buffer, and equal amounts of A77 Fab were added prior to immunoprecipitation with protein G, allowing chimera precipitation. Samples were separated by SDS-10% PAGE and transferred onto PVDF membranes before immunoblotting with 4G10-HRP mAb. To control for equal amounts of chimera, blots were stripped and reprobed with anti-FcRγ Ab plus goat anti-rabbit Ig-HRP.

(B) Monomeric targeting of FcαRI/FcRγ elicits SHP-1 recruitment to FcαRI. FcαRI$_{R209L}$/γ transfectants were incubated with either 10 μg/ml A77 or 320 Fab for indicated times. Cells were lysed in 1% digitonin lysis buffer before immunoprecipitation with protein G and immunoblotting with anti-SHP-1 Ab plus goat anti-mouse Ig-HRP or anti-SHIP Ab plus anti-rabbit Ig-HRP. To judge effective immunoprecipitation of the chimeric receptor, blots were stripped and reprobed with anti-FcRγ Ab plus goat anti-rabbit Ig-HRP. Whole-cell lysates were also analyzed for the presence of each phosphatase.

(C) SHP-1 recruitment to the FcαRI is modulated by FcεRI stimulation. Cells were incubated overnight at 37°C with or without IgE and in the presence of either A77 Fab or 320 Fab (10 μg/ml). Where indicated, cells were stimulated with DNP-HSA (0.1 μg/ml) for 3 min at 37°C. Cells were lysed in 1% digitonin lysis buffer, and 2 μg of A77 Fab was added on lysates of cells that had been stimulated with irrelevant 320 Fab before immunoprecipitation with protein G to enable receptor isolation. The presence of SHP-1 and FcαRI/γ was examined as in (B).

(D) Prevention of SHP-1 recruitment to FcαRI following multimeric engagement. FcαRI$_{R209L}$/γ transfectants were incubated with 10 μg/ml of anti-FcαRI A77 Fab (A77f) or with preformed complexes of F(ab′)$_2$ (A77F) (10 μg/ml) plus RAM F(ab′)$_2$ (40 μg/ml) for 1 hr at 37°C. The presence of SHP-1, Syk, and FcαRI/γ was examined by immunoblotting as in (B). Densitometric analysis of proteins was performed, and the fold increase in intensity of the recruitment of proteins from crosslinked receptors compared to monovalently targeted receptors (arbitrarily set as 1) is shown.

known as key mediators in inhibitory pathways, their recruitment to the FcαRI was then investigated. SHP-1 but not SHIP was recruited in a time-dependent manner to the FcαRI chimera (Figure 5B). No association of FcαRI with SHP-2 was observed (not shown). SHP-1 recruitment required the FcRγ ITAM, as inactive ITAM mutant chimera were unable to recruit SHP-1 to FcαRI (not shown). SHP-1 recruitment was enhanced by IgE and even more so by IgE plus antigen (Figure 5C). We also examined whether bifunctional inhibitory/activating properties of FcαRI/γ reflect a difference between the recruitment of phosphatases and kinases. Monovalent targeting induced recruitment of SHP-1 to the FcαRI$_{R209L}$/γ chimeric receptor, whereas multimeric engagement prevented this recruitment (Figure 5D). Conversely, a stronger recruitement (2- to 3-fold increase) of Syk kinase was observed after receptor crosslinking as compared to monovalent Fab interaction based on the total amount of receptor precipitated (Figure 5D).

As SHP-1 is known to dephosphorylate different proteins of the activation pathway (Coggeshall et al., 2002), we examined the phosphorylation state of crucial FcεRI signaling effectors. FcαRI targeting by A77 Fab prevented in a time-dependent manner FcεRI-induced

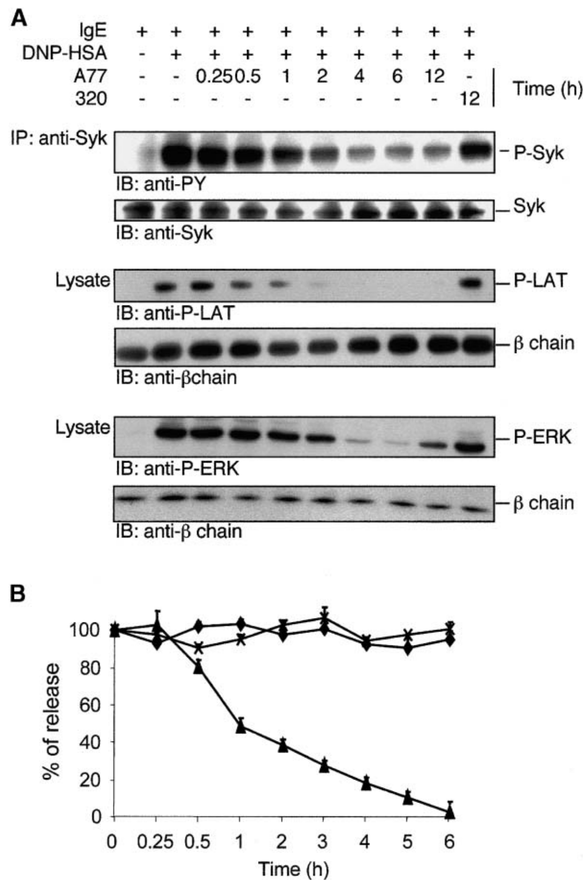

Figure 6. Monomeric FcαRI Targeting Induces Sustained Dephosphorylation of Syk, LAT, and ERK Activated by FcεRI

(A) $Fc\alpha RI_{R209L}/\gamma$ transfectants (clone 9.4) were sensitized overnight with IgE, washed, and incubated with either A77 or 320 Fab (10 μg/ml) for the time indicated. Cells were incubated with 0.1 μg/ml DNP-HSA for 3 min at 37°C. Cells were lysed in 0.3% Triton X-100 lysis buffer and immunoprecipitated with anti-Syk Ab plus protein A/G beads or directly used as a lysate. Samples were resolved by SDS-10% PAGE, transferred onto PVDF membranes, and immunoblotted with 4G10-HRP mAb or antibodies to Syk, p-LAT, or p-ERK plus goat anti-rabbit or anti-mouse Ig-HRP. To judge equal loading, blots were stripped and reprobed with anti-Syk or anti-β chain Ab plus goat anti-rabbit Ig-HRP or goat anti-mouse Ig-HRP, respectively.

(B) Kinetics of inhibition of IgE-mediated exocytosis after monomeric FcαRI targeting. RBL cells (crosses) or transfectants expressing chimeric $Fc\alpha RI_{R209L}/\gamma$ (triangles) or $Fc\alpha RI_{R209L}/\gamma$ with double Y-to-F point mutations within the ITAM motif (Y268/279F) (diamonds) were sensitized overnight with IgE anti-DNP and with 10 μg/ml A77 Fab. Degranulation was triggered with DNP-HSA (0.1 μg/ml), and net β-hexosaminidase release was determined (mean ± SD, n = 3).

phosphorylation of Syk, LAT, and ERK, whereas no inhibition was observed with irrelevant Fab (Figure 6A). The relatively long incubation time needed to observe maximal inhibition of kinase phosphorylation by the monovalent ligand paralleled the kinetic data measuring inhibition of IgE-dependent degranulation (Figure 6B).

ERK Controls SHP-1 Recruitment to the FcαRI

As ERK has been proposed to positively regulate Syk activity by FcεRI (Xu et al., 1999) and to inhibit the recruitment of SHP-1 to the TCR (Stefanova et al., 2003), we addressed the function of ERK in the FcαRI-mediated inhibitory cascade. Monovalent targeting of FcαRI resulted in weak phosphorylation of Syk and ERK, whereas multimeric targeting enhanced both types of responses (Figure 7A). As expected, pretreatment of cells with a MEK kinase inhibitor decreased Syk phosphorylation induced by crosslinking of FcαRI but did not substantially modify anti-FcαRI Fab-induced phosphorylation of $Fc\alpha RI_{R209L}/\gamma$ chimera (not shown) and Syk (Figure 7B). In contrast, MEK kinase inhibitor abolished SHP-1 recruitment to FcαRI after monovalent targeting (Figure 7C). The small amount of SHP-1 recruited after multimeric crosslinking of FcαRI was also reduced by MEK inhibitor (Figure 7C). Moreover, inactivation of ERK completely reversed FcαRI-inhibitory capacity on FcεRI-mediated exocytosis (Figure 7D), while FcαRI degranulation responses were only partially affected (30 ± 4%) (Figure 7E). Of note, FcεRI-induced degranulation was also partially inhibited (36 ± 7%) in the presence of an excess of MEK inhibitor (30 μM), as reported (Zhang et al., 1997).

Discussion

This study unveils important aspects of the physiologic role of IgA and FcαRI as negative regulators of the immune system. In the past, Ig interaction with the relevant FcR in the absence of antigen was considered a passive phenomenon with no biological effects. Here, we demonstrate that, in the absence of sustained aggregation, ligand binding to FcαRI inhibits cellular responses initiated by a heterologous receptor, while sustained clustering of FcαRI leads to cell activation. Although monomeric IgE was recently shown to mediate a spectrum of response in mast cells (Kitaura et al., 2003), our data indicate that it did not inhibit exocytosis induced by heterologous receptors. The dual function of FcαRI provides a molecular basis for the previously observed paradoxical roles of IgA as both an anti- and proinflammatory isotype (Russell et al., 1997). The low natural frequency of antigen-specific serum IgA antibodies (Kerr, 1990) would favor the physiologic inhibitory function of FcαRI and suggests that the main role of circulating IgA is to keep the immune system under tight steady-state control. Our findings also suggest that the site where IgA interacts with its receptor may be influential, as protease treatment of cells substantially enhanced the inhibitory effect of FcαRI. As inflammatory sites contain an array of proteases, their action may enhance the inhibitory potential of the IgA-FcαRI interaction, and this in turn would regulate local inflammatory reactions.

A consensus model of negative signaling in the immune system involves ITAM/ITIM coclustering. Here, the FcαRI inhibitory function takes place in the absence of receptor coaggregation and of an ITIM motif. Therefore, this function cannot be explained by the paired FcR model, where an ITIM-bearing FcR inhibits the pathway triggered by ITAM-containing FcR after their coaggregation (Long, 1999). Our structure-function studies identify the FcRγ ITAM as the module involved in the FcαRI inhibitory pathway. Both FcRγ ITAM tyrosines are required for inhibitory and activating functions. This demonstrates that under certain conditions ITAM, instead of promoting activation as initially described, can be inhibitory toward a heterologous receptor and can thus

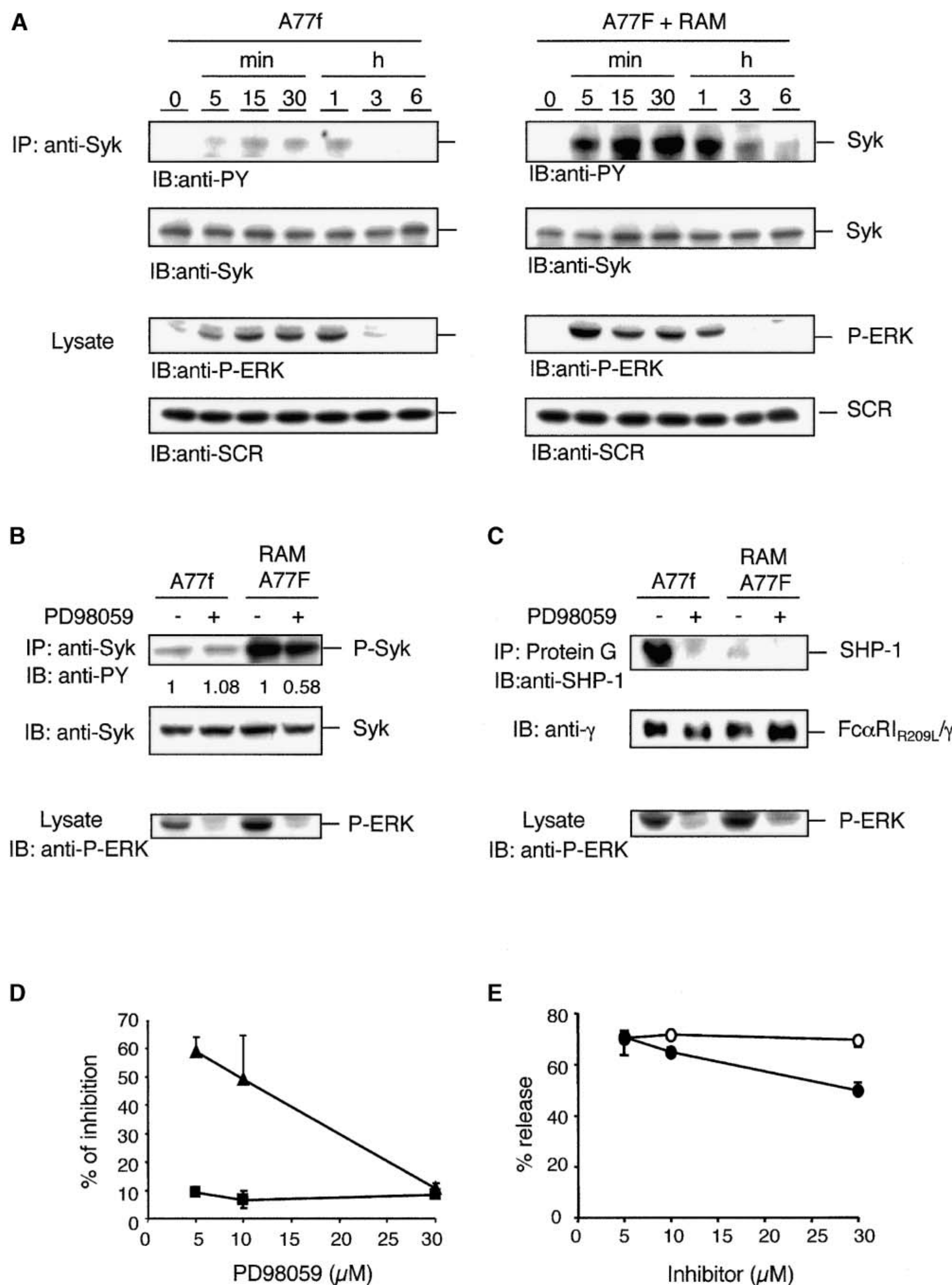

Figure 7. Involvement of ERK in SHP-1 Recruitment and FcαRI-Inhibitory Function

(A) Monomeric targeting of FcαRI induces weak phosphorylation of Syk and ERK. FcαRI$_{R209L}$/γ transfectants were incubated with 10 μg/ml of A77 Fab or preformed complexes of A77 F(ab′)$_2$ plus RAM F(ab′)$_2$ as in Figure 5D for indicated times. The phosphorylation of Syk and ERK was examined by immunoblotting as in Figure 6. To judge equal loading and/or effective immunoprecipitation, blots were stripped and reprobed with anti-Syk Ab or anti-scramblase (SCR) plus goat anti-rabbit or mouse Ig-HRP, respectively.

(B) MEK inhibitor (PD98059) decreases Syk phosphorylation after multimeric FcαRI crosslinking. FcαRI$_{R209L}$/γ transfectants were treated (+) or not (−) with 30 μM of PD98059 for 30 min and then incubated with 10 μg/ml of A77 Fab or preformed complexes of 10 μg/ml A77 F(ab′)$_2$ plus 40 μg/ml RAM F(ab′)$_2$ in the presence of MEK inhibitor for 1 hr at 37°C, lysed in Triton lysis buffer, and immunopreciptated by anti-Syk Ab plus protein G. To judge effective immunoprecipitation, blots were stripped and reprobed with anti-Syk Ab plus goat anti-rabbit Ig-HRP. Whole-cell lysates were also analyzed for effective inhibition of ERK by immunoblotting using anti-pERK.

(C) MEK inhibitor abolishes recruitment of SHP-1 after monomeric FcαRI targeting. FcαRI$_{R209L}$/γ transfectants pre-treated (+) or not (−) with 30 μM MEK inhibitor were incubated with A77 Fab or preformed complexes of A77 F(ab′)$_2$ plus RAM F(ab′)$_2$, as in (B), in the presence of MEK inhibitor for 1 hr. Cells were lysed in 1% digitonin lysis buffer, immunoprecipitated with protein G, and immunoblotted with anti-SHP-1 Ab plus goat anti-mouse Ig-HRP. To judge effective immunoprecipitation of the chimeric receptor, blots were stripped and reprobed with anti-FcRγ Ab plus goat anti-rabbit Ig-HRP. Whole-cell lysates were also analyzed for effective inhibition of ERK by immunoblotting using anti-pERK.

(D) MEK inhibitor reverses FcαRI-inhibitory function. FcαRI$_{R209L}$/γ transfectants were sensitized with IgE anti-DNP overnight and pretreated for 30 min with indicated concentrations of MEK inhibitor prior to incubation with 10 μg/ml of A77 Fab (triangles) or 320 Fab (squares) in the presence of MEK inhibitor for 1 hr. Degranulation was triggered with DNP-HSA (0.1 μg/ml), and net β-hexosaminidase release was determined.

be considered as a dual-function motif. Such duality was not observed in the context of FcϵRI or of FcγRIIb, which failed to be inhibitory when targeted monovalently, indicating the particular nature of the FcαRI-γ receptor complex. Interestingly, while multimeric binding induces strong FcRγ ITAM phosphorylation (and cell activation), monomeric targeting leads to weak phosphorylation of FcRγ (and inhibition of cell activation), suggesting that the inhibitory signal originates from incomplete phosphorylation of FcRγ. This weak phosphorylation is revelant with the FcαRI-IgA binding reaction, which has moderately fast on and off rates: IgA binding is transient, whereas IgA complexes stabilize FcαRI aggregates by decreasing the off rate (Wines et al., 2001; Herr et al., 2003). As in the absence of antigen IgA binding may involve two FcαRI (Herr et al., 2003), one can propose that transient IgA binding could lead to an inhibitory signal, while sustained aggregation by IgA complexes with greater avidity could trigger cellular activation. Although this is reminiscent of the kinetic proofreading model (Torigoe et al., 1998), our findings argue against such a mechanism. According to this model, low-affinity ligands of an activating receptor, such as FcϵRI, lead to strong receptor phosphorylation and trapping of critical effectors in unstable and thus ineffective signaling complexes. Here, by contrast, targeting of FcαRI with anti-FcαRI Fab leads to weaker phosphorylation of FcRγ and to weaker recruitment of Syk than observed after multimeric aggregation of FcαRI. These observations led us to further explore what appeared as a novel inhibitory mechanism.

Although FcαRI-mediated inhibition does not require coaggregation with the targeted receptor, our data provide evidence for involvement of the tyrosine phosphatase SHP-1. ITAM-dependent recruitment of SHP-1 and SHIP has been previously demonstrated following activation of FcϵRI or FcγRIIa (Kimura et al., 1997; Nakamura et al., 2002; Ganesan et al., 2003) and is thought to act as a negative feedback loop regulating activation signals induced by their own FcR. In the case of FcαRI, SHP-1 acts toward a heterologous receptor. Monomeric targeting of FcαRI promotes strong recruitment of SHP-1 as compared to multimeric crosslinking. This recruitment was delayed (1 hr) but long lasting (12 hr), which differs from previous results showing rapid recruitment of SHP-1 by TCR (Dittel et al., 1999) under inhibitory conditions. It correlates with a decreased pattern of phosphorylation of Syk, LAT, and ERK, which are crucial effectors of the FcϵRI activation pathway, explaining the strong inhibition of cell activation. Interestingly, simultaneous engagement of FcαRI (monovalent targeting) and of the heterologous FcϵRI (multivalent targeting) enhanced SHP-1 recruitment to FcαRI, leading to attenuation of FcϵRI signaling. This suggests that a novel type of crosstalk between FcαRI and activating receptors in the absence of coaggregation increases local concentration of SHP-1 at the membrane, eliciting a desensitization mechanism. This may represent physiologic situations where different FcR can be independently engaged in a given cell.

Recent observations described a mechanism by which the TCR, an ITAM-bearing receptor, can alternatively mediate inhibition or activation (Stefanova et al., 2003). While weakly binding ligands predominantly trigger a negative signal by recruitment of SHP-1, inducing receptor desensitization, strongly binding ligands efficiently induce an ERK-dependent activating feedback loop that competes with SHP-1 recruitment. In contrast, our data point to a requirement of ERK for the recruitment of SHP-1 to FcαRI. Since in our experiments monovalent targeting of FcαRI preceeds multimeric engagement of targeted FcϵRI, we propose that FcαRI weak stimulating conditions initiate a low-intensity signaling pathway (low phosphorylation of FcRγ, Syk, and ERK), leading to progressive recruitment of SHP-1. This process may promote accumulation of SHP-1 at the plasma membrane, reaching a threshold level that would in time allow the dephosphorylation of signaling proteins activated by the heterologous receptor, as is the case of FcϵRI. In contrast, under multimeric crosslinking of FcαRI, a high-intensity signaling pathway is triggered (high phosphorylation of FcRγ, Syk, and ERK), where Syk is strongly recruited to FcαRI, favoring an activation signal in which SHP-1 recruitment is aborted. Thus, ERK critically participates both in an activating pathway that originates from Syk recruitment and in an inhibitory pathway that involves SHP-1 recruitment.

The powerful inhibitory signal triggered by FcαRI in vitro led us to investigate its therapeutic potential in a murine IgE-mediated model of asthma. Anti-FcαRI Fab treatment prevented allergic hyperresponsiveness and the accompanying airway inflammation in FcαRI^{+}-Tg mice. One of the hallmarks of asthma, leukocyte infiltration, was markedly inhibited by this treatment. Although FcαRI has not been shown to be expressed on mast cells in humans, it is expressed on several cell types involved in the inflammatory allergic network, including macrophages, dendritic cells, neutrophils, and eosinophils (Monteiro and Van De Winkel, 2003). Allergic patients have also an upregulation of FcαRI expression on eosinophils (Monteiro et al., 1993), emphasizing its potential role as a negative regulator. Therefore, strategies specifically targeting this receptor, particularly in patients refractory to conventional therapy, might be useful to prevent allergic disorders.

Collectively, the present findings redefine FcαRI as a novel type of molecular switch of the immune system, directing signals toward either an activating or an inhibitory function. Fine tuning of this mechanism depends on several parameters, including the presence of anti-

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Data are presented as percent inhibition of FcϵRI-induced β-hexasaminidase release by A77 or 320 Fab (mean $\pm$ SD, n = 3). No reverse inhibition was observed with vehicle (DMSO).

(E) MEK inhibitor partially affects FcαRI-mediated degranulation. FcαRI$_{R209L}$/γ transfectants were pretreated for 30 min with indicated concentrations of MEK inhibitor (black circles) or DMSO (open circles) prior to stimulation with preformed complexes of 10 μg/ml of biotinylated A77 F(ab′)$_2$ plus SA-alkaline phosphatase (SA-AP; 50 μg/ml) in the presence of MEK inhibitor for 45 min, and net β-hexosaminidase release was determined (mean $\pm$ SD, n = 3).

gen, the proteolytic environment, and the relative proportions of the two types of FcαRI (with or without FcRγ). These unique features of FcαRI-IgA interaction, together with the observation that FcαRI is closely related to inhibitory/activating receptors in the LRC locus (Davis et al., 2002), lead us to propose that selective pressure during evolution may have generated a single switch molecule, FcαRI, capable of mediating either activation or inhibition.

Experimental Procedures

Animals

Balb/c mice transgenic for the human FcαRI (line 83) were used (Launay et al., 2000). Mice were bred and maintained at the facilities of Bichat Medical School. All experiments were done in accordance with national guidelines.

Ig, Antibodies, and Reagents

The mouse monoclonal Abs A3, A59, A62, and A77, specific for FcαRI (Monteiro et al., 1992), were used as either $F(ab')_2$ or Fab fragments. Other mAbs used were as follows: My 43 anti-FcαRI (Shen et al., 1989), 2.4G2 anti-mouse FcγRII/III (Unkeless, 1979), 30.9 anti-FcεRI β chain (Pastorelli et al., 2001), rabbit anti-mouse IgG, and two irrelevant mAbs (clones TPO 6.12 and 320.1). $F(ab')_2$ were obtained by pepsin digestion as described (Silvain et al., 1995). Fab were prepared by pepsin digestion and reduction with 0.01 M cysteine and alkylation with 0.15 M iodoacetamide at pH 7.5. Purified serum IgA (ICN Biomedicals Inc, Aurora, OH) was used. Human serum IgG, IgA, and IgE myeloma proteins as well as mouse anti-DNP-specific IgE (clones DNP48, a gift from R. Siraganian, and H1-ε-26.82) were obtained as described (Liu et al., 1980; Monteiro et al., 1990; Roa et al., 1997; Marchand et al., 2003). MEK kinase inhibitor PD 98059 was purchased from Calbiochem, La Jolla, CA.

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 (Monteiro et al., 1990). RBL-2H3 cells were maintained as described (Roa et al., 1997). In some experiments, cells were treated with 1 mg/ml trypsin-TCPK (Sigma, St Louis, MO) in DMEM for 30 min at 37°C.

cDNA Constructs, Expression Vectors, and Transfection

Wild-type human FcαRI and the R209L mutant were transfected into RBL cells as described (Launay et al., 1999). The $FcαRI_{R209L}/γ$ chimera was generated as follows. The extracellular and transmembrane domains of the R209L mutant were amplified by PCR (F_{wt}: GGG**CTCGAG**ATGGACCCCAAACAGACCACC and $R_{γ\text{-}α}$: CTTTC GCACTTGGATCTTCAGATTTTCAACCAGTATGGCCAA) as well as the intracellular domain of human FcR γ-chain ($F_{α\text{-}γ}$: TTGGCCATAC TGGTTGAAAATCTGAAGATCCAAGTGCGAAAG, $R_{γ}$: GGG**GGATCC** TTACTGTGGTGGTTTCTCATG). PCR products were fused by overlapping extension PCR. Point mutations (Y268F, Y279F, and double Y268/279F) were introduced in the ITAM motif of the $FcαRI_{R209L}/γ$ chimera by using the same strategy. All constructs were cloned into pSRαNeo (Launay et al., 1999) between XhoI-BamHI restriction sites (bolded in primers), and products were sequenced. The FcγRIIb/γ chimera was provided by C. Bonnerot (Bonnerot et al., 1992). RBL cells were transfected with 15 μg of DNA by electroporation and selected for FcαRI expression and releasing capacity. For each construct, at least three independent transfectants were tested and found to give comparable results.

Flow Cytometry

Cells preincubated or not with 100 μg human IgG to block FcγRs were incubated with phycoerythrin-labeled anti-human FcαRI mAb (A59-PE) (BD PharMingen) or biotinylated anti-mouse FcγRII/III 2.4G2 mAb plus streptavidin (SA)-PE (Southern Biotech Associates). Irrelevant mAb were used as controls. In some experiments, cells were cultured for 1 hr or overnight at 37°C with test reagents (anti-FcαRI Fab, IgE, or IgE plus DNP-HSA) before staining on ice with IgE-FITC or My43 anti-FcαRI mAb plus anti-Igh6a (RS3) biotin (Schuppel et al., 1987) and SA-PE. Cells were analyzed using a FACScalibur (Becton Dickinson).

Phagocytosis Assay

Adherent PBMC were incubated with 10 μg/ml A77 Fab or irrelevant Fab for 30 min at 37°C, washed, and incubated at 37°C for 30 min with Texas-red-conjugated *E. coli* (50 bacteria/cell) (Molecular Probes, Eugene, OR) opsonized or not with polyclonal rabbit anti-*E. coli* IgG antibodies according to the manufacturer's instructions. Slides were mounted and examined with a confocal laser microscope (LSM 510 Carl Zeiss, Jena, Germany).

β-Hexosaminidase Assay

Exocytosis was determined by measuring release of β-hexosaminidase (Roa et al., 1997). Briefly, cells were sensitized with IgE anti-DNP (1:200) and test reagents for 1 hr or overnight at 37°C, washed, and stimulated with 0.1 or 0.03 μg/ml DNP-HSA (Sigma) or with rabbit anti-mouse IgG $F(ab')_2$ fragment (40 μg/ml) for 45 min at 37°C as indicated. In some experiments, cells were stimulated with 2.4G2 $F(ab')_2$ biotin or A77 anti-FcαRI $F(ab')_2$ biotin (10 μg/ml), and degranulation was triggered by addition of 0.05 mg/ml SA-alkaline phosphatase (Southern Biotech Associates).

IgE Immune-Complex-Mediated Allergic Asthma Model

FcαRI-Tg mice and littermates were immunized intraperitoneally with 10 μg TNP-OVA (Sigma) in 2 mg of aluminum hydroxide gel per 25 g of body weight on days 0 and 7. Starting on day 14, mice were challenged intranasally for 7 consecutive days with 2 μg TNP-OVA complexed with 20 μg anti-DNP IgE (Zuberi et al., 2000) in the presence of 5 μg A77 or irrelevant Fab (clone 320). On days 14 and 20, mice received 50 μg A77 or control Fab intraperitoneally. Twelve hours after the final intranasal challenge, unrestrained conscious mice were placed in a whole-body plethysmograph chamber (BUXCO Electronics, Sharon, CT), and 300 mM methacholine was delivered for 60 s. Airway resistance was calculated every minute for 20 min after methacholine exposure (Zuany-Amorim et al., 1998). Animals were anesthetized, lungs were inflated by tracheal injection of 1 ml of Optimum Cutter Temperature Compound (BDH, Poole, UK), fixed in 4% paraformaldehyde, dehydrated in graded alcohols, and embedded in paraffin. Comparative histopathologic evaluation of the degree of inflammation was performed on entire H&E-stained lung sections.

Tyrosine Phosphorylation Assay, Immunoprecipitation, and Immunoblotting

Cells were solubilized in lysis buffer: 50 mM HEPES (pH 7.4), 0.3% Triton X-100 (or 1% digitonin), 50 mM NaF, 50 mM NaCl, 1 mM Na_3VO_4, 30 mM $Na_4P_2O_7$, 50 U/ml aprotinin, 10 μg/ml leupeptin, and post-nuclear supernatants were prepared. FcαRI were immunoprecipitated for 2 hr at 4°C with A77 Fab fragment and protein G-Sepharose (Amersham-Pharmacia Biotech). Syk was immunoprecipitated using anti-Syk Ab (Launay et al., 1998). Proteins were resolved by SDS-PAGE, transferred onto PVDF membranes, and immunoblotted with 4G10 anti-PY mAb, anti-SHP-1 mAb (Upstate, Charlottesville, VA), polyclonal anti-SHIP rabbit Ab (provided by A. Veillette), rabbit anti-FcRγ Ab, anti-Syk Ab (Launay et al., 1998), anti-β chain, anti-scramblase (Pastorelli et al., 2001), anti-phospho LAT (Cell Signaling, Beverly, MA), or anti-phospho ERK mAbs (Upstate, NY) for 1 hr at room temperature, followed by goat anti-mouse Ig or goat anti-rabbit Ig (Jackson ImmunoResearch Laboratories, West Grove, PA) both coupled to HRP. Membranes were developed by ECL (Amersham-Pharmacia).

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