Divergent Intracellular Sorting of FcγRIIA and FcγRIIB

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The human low affinity FcγRII family includes both the activating receptor FcγRIIA and the inhibitory receptor FcγRIIB. These receptors have opposing signaling functions but are both capable of internalizing IgG-containing immune complexes through clathrin-mediated endocytosis. We demonstrate that upon engagement by multivalent aggregated human IgG, FcγRIIA expressed in ts20 Chinese hamster fibroblasts is delivered along with its ligand to lysosomal compartments for degradation, while FcγRIIB dissociates from the ligand and is routed separately into the recycling pathway. FcγRIIA sorting to lysosomes requires receptor multimerization, but does not require either Src family kinase activity or ubiquitylation of receptor lysine residues. The sorting of FcγRIIB2 away from a degradative fate is not due to its lower affinity for IgG and occurs even upon persistent receptor aggregation. Upon co-engagement of FcγRIIA and FcγRIIB2, the receptors are sorted independently to distinct final fates after dissociation of co-clustering ligand. These results reveal fundamental differences in the trafficking behavior of different Fcγ receptors.

Fcγ receptors (FcγR) are key players in immune responses. Widely expressed on cells of the hematopoietic system, these receptors mediate a multitude of biological responses that are triggered upon receptor engagement by multivalent IgG-containing immune complexes (1). These responses include production of inflammatory cytokines, antibody-dependent cellular cytotoxicity, and induction of dendritic cell maturation. FcγR also mediate the internalization of immune complexes. Soluble immune complexes are internalized by clathrin-mediated endocytosis whereas large (>0.5 μm) antibody-coated particles are internalized via phagocytosis. These uptake processes are important in host defense against infection; moreover, defects in immune complex clearance are associated with the development of autoimmunity (2).

FcγRs can be categorized into two functional groups. The activating FcγR have an immunoreceptor tyrosine-based activation motif (ITAM) within the cytoplasmic domain of the receptor itself or an associated Fcγ signaling subunit. Phosphorylation of tyrosine residues in the ITAM by Src family kinases after receptor aggregation initiates signaling cascades that trigger downstream effector responses (3). In contrast, inhibitory FcγR contain an immunoreceptor tyrosine-based inhibitory motif (ITIM), which recruits phosphatases that antagonize ITAM-mediated signaling. Therefore, the responsiveness of effector cells to immune complexes is determined by the balance between activating and inhibitory receptors (1).

A feature of the human immune system that distinguishes it from that of mouse is the presence of both activating and inhibitory members of the low affinity FcγRII subfamily. FcγRIIA is an activating FcγR unique to humans and other primates, and is the most widely expressed FcγR on human leukocytes (4). In addition, FcγRIIA is unusual among activating FcγR in having both ligand binding and ITAM signaling domains contained in a single polypeptide chain. FcγRIIA exists as two codominantly expressed polymorphic variants, with either histidine or arginine at residue 131; the His-131 form has a higher affinity for several IgG subclasses (5). FcγRIIA can mediate phagocytosis of large IgG-opsonized particles, which depends on ITAM-mediated signaling and rearrangements of the actin cytoskeleton (6). FcγRIIA is also able to mediate clathrin-dependent endocytosis of soluble IgG-containing immune complexes (7, 8).

Human leukocytes also express the inhibitory receptor FcγRIIB. Whereas the extracellular domains of FcγRIIA and FcγRIIB have 92% amino acid identity, their intracellular domains are divergent, with FcγRIIB containing an ITIM. Two different isoforms of FcγRIIB can be generated through alternative splicing. FcγRIIB1 is expressed mainly in B cells, where it inhibits signaling from the B cell receptor when the two receptors are coengaged by antigen-antibody complexes (9). FcγRIIB2 is expressed mainly in myeloid cells. Human FcγRIIB2 lacks the ability to support phagocytosis of IgG-opsonised large particles; on the contrary, it negatively regulates signaling for phagocytosis and other ITAM-dependent responses when coengaged with activating FcγR (10). FcγRIIB2 is, however, able to mediate endocytosis of soluble immune complexes because of the presence of a di-leucine motif in its cytoplasmic domain (11–13).

As both of these receptors can mediate immune complex uptake, we sought to investigate how they traffic within the cell after such internalization. This is important for understanding both the overall regulation of inflammatory signaling as well as the processing of immune complexes internalized via FcγR. We demonstrate that, in addition to their opposing effects on cell activation, FcγRIIA and FcγRIIB2 exhibit divergent trafficking behavior after internalization, with FcγRIIA, but not FcγRIIB2, being targeted for degradation.
**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—Fetal bovine serum, α-minimal essential medium, and G418 were from Wisent (St. Bruno, Quebec, Canada). Mouse anti-Myc antibody 9E10 was from Covance (Berkeley, CA). Anti-FcγRIIA antibody IV.3 was purified from hybridoma supernatants and Fab fragments were prepared using a Pierce ImmunoPure Fab Kit (Thermo Fisher Scientific, Rockford, IL). Anti-FcγRII antibody AT10 was from Abcam. Anti-hamster LAMP1 antibody UH1 was from the Developmental Studies Hybridoma Bank (University of Iowa). Cy3-, Cy5-, and horseradish peroxidase-conjugated secondary antibodies were from Jackson Immunoresearch Laboratories. Alexa488-conjugated secondary antibodies, Alexa488-transferrin and Rhodamine-transferrin were from Molecular Probes. Paraformaldehyde was from Canemco (16%, EM grade). PP1 was from Biomol. GM-CSF and cycloheximide were from BioShop (Burlington, Ontario, Canada). Supersignal West Pico chemiluminescent substrate, Restore It stripping buffer, and phosphor-conjugated secondary antibodies were from Pierce. Alexa488-conjugated anti-mouse antibodies, anti-β actin antibody, and other chemicals were from Sigma-Aldrich.

**Cell Culture**—ts20 cells were grown at 34 °C and 5% CO2 in α-minimal essential medium +10% fetal bovine serum. For preparation of human monocyte-derived macrophages, peripheral blood mononuclear cells were obtained from blood of healthy donors by Ficoll-Paque Plus (GE Healthcare) density centrifugation, followed by isolation of monocytes by adherence to tissue culture plastic. Cells were then cultured for 6 days in RPMI+10% fetal bovine serum with GM-CSF (1000 units/ml).

**DNA Constructs and Transfection**—cDNAs for wild-type or mutated forms of FcγRIIA (His131 variant) and FcγRIIB2 were cloned into pcDNA3.1/Myc-His or pcDNA3.1/His (Invitrogen) for expression with C-terminal Myc-His or His tags. Chimeric FcγRIIA/FcγRIIB2 Myc-His-tagged receptors were generated by PCR amplification of receptor sequences from GFP-tagged chimeric receptors (14) and cloning into pcDNA3.1/Myc-His. Transfections were performed with FuGENE 6 (Roche Applied Science) or Lipofectamine 2000 (Invitrogen) according to the manufacturers’ instructions. Stable cell lines expressing FcγRIIA, FcγRIIB2 and mutant receptors were selected with G418 (0.5 mg/ml). For treatment of cells with inhibitors, cells were pretreated for 30 min by addition of the inhibitor directly to the culture medium at 30 μM (PP1) or 300 nM (bafilomycin).

**Development of Rabbit Polyclonal Antibodies Specific to the Intracellular Domains of FcγRIIA or FcγRIIB2**—The intracellular domains of human FcγRIIA (residues 251–316) or FcγRIIB2 (residues 255–290) were individually cloned into the pGEX-3X vector to express the domains as fusion proteins to glutathione S-transferase (GST). The GST-tagged proteins were expressed in BL21 bacteria, purified on GST-agarose and used for immunization of New Zealand White rabbits (Division of Comparative Medicine, University of Toronto). Anti-GST antibodies were removed from antisera using GST-agarose.

**Endocytosis Assay**—Human IgG (10 mg/ml) was aggregated at 62 °C for 20 min followed by centrifugation at 16,000 × g for 10 min to precipitate insoluble IgG aggregates; supernatants containing soluble aggregates were used at 1:100 dilution to induce endocytosis. In experiments where endocytosis was triggered with anti-receptor antibodies, IV.3 (anti-FcγRIIA) or AT10 (pan anti-FcγRII) were added to transfected ts20 cells at 0.5 μg/ml for 20 min at 4 °C. After washing, cells were incubated for 20 min at 4 °C with 1 μg/ml Cy5-donkey anti-mouse antibody to cross-link the receptors, followed by warming to 34 °C to trigger endocytosis. In some experiments where subsequent immunofluorescence was performed using mouse antibodies, isotype-specific secondary antibodies were employed. For transferrin loading, rhodamine- or Alexa488-conjugated transferrin was added at 50 μg/ml for the last 10 min of incubation. For dextran loading, Alexa647-conjugated dextran was added at 50 μg/ml for 1 h followed by a chase for 1 h at 37 °C.

**Microscopy and Immunofluorescence**—Cells were washed and fixed with 4% paraformaldehyde for 30 min, then permeabilized with 0.1% Triton X-100 at room temperature for 20 min. To detect FcγR, cells were blocked with 5% BSA in phosphate-buffered saline (PBS) and incubated with anti-Myc antibody 9E10 or anti-FcγRIIB2 antibody at 1:1000 dilution in blocking buffer for one hour. For LAMP1 immunofluorescence, cells were permeabilized with methanol at −20 °C for 20 min followed by blocking with 5% BSA and treatment with UH1 antibody at 1:1 dilution. Samples were then treated with fluorophore-conjugated secondary antibodies at 0.8 μg/ml in PBS for 30 min before mounting cells with DAKO mounting medium for microscopy analysis. AgIgG was detected with fluorophore-conjugated anti-human secondary antibody.

Cells were analyzed using a Zeiss Axiovert 200 m microscope with 40× or 100× objectives or a Zeiss LSM 510 confocal scanning microscope with a 63× objective. Alexa488, Cy3, and Cy5 signals were detected using standard filter sets.

**Flow Cytometry**—ts20 cells expressing Myc-His-tagged FcγRIIA or FcγRIIB2 were detached from culture dishes and dispersed in PBS. Following fixation with 2% paraformaldehyde and permeabilization with 0.1% Triton X-100, cells were blocked with 5% BSA and stained with anti-Myc antibody 9E10 at 1:1000 dilution in blocking buffer for 30 min at room temperature. After washing, cells were stained with Alexa488-conjugated anti-mouse antibody (0.8 μg/ml for 15 min) and analyzed by flow cytometry using a FACSCalibur (Becton Dickinson). The background fluorescence observed with 9E10-stained untransfected ts20 cells was subtracted from the mean fluorescence intensity (MFI) values. For detection of aggregated IgG, cells were stained with 1.5 μg/ml Cy5-human secondary antibody, and background fluorescence observed with untransfected ts20 cells was subtracted.

**Immunoprecipitation and Western Blotting**—ts20 or THP-1 cells were lysed in lysis buffer (1% Triton X-100, 0.1% SDS, 0.5% deoxycholate, 1 mM NaF, 0.1% protease inhibitor mixture in PBS). Lysates were incubated on ice for 20 min, insoluble material was removed by centrifugation at 16,000 × g for 10 min at 4 °C, and lysates were frozen for future analysis. For immunoprecipitations, 1 μg of IV.3 antibody and 25 μl of protein G beads were mixed with cell lysates followed by overnight incubation at 4 °C. After washing beads twice with lysis buffer and two times with PBS-T (PBS with 0.05% Tween 20), beads were resuspended in Laemmli’s sample buffer. Samples were analyzed by SDS-PAGE, transferred to nitrocellulose membrane...
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(Bio-Rad), blocked with 5% BSA in PBS-T for 1 h at room temperature and probed with primary antibody at 1:1000 dilution in blocking buffer overnight. Blots were washed in PBS-T three times, incubated with anti-mouse or anti-rabbit horseradish peroxidase for 30 min, washed again, and developed with Supersignal West Pico chemiluminescent substrate (Pierce). A Genius2 Bioimager (Syngene) was used to visualize the blots.

RESULTS

Aggregated IgG Internalized by Either FcγRIIA or FcγRIIB2 Is Degraded in Lysosomes—To better understand how these two Fcγ receptors traffic, FcγRIIA or FcγRIIB2 carrying Myc-His tags were stably transfected into ts20 cells, a Chinese hamster fibroblast cell line. This heterologous expression system allows us to study the trafficking behavior of a single class of FcγR either in its wild type or mutated forms (8, 14, 15). Soluble complexes of heat aggregated IgG (agIgG), a mimic of soluble multivalent immune complexes, were used to trigger endocytosis of either FcγRIIA or FcγRIIB2. Both receptors mediated uptake of agIgG (Fig. 1, B and E), and by 80 min the agIgG was delivered to late endosomal/lysosomal compartments, as indicated by its co-localization with LAMP1 (Fig. 1, A–F). No significant uptake of agIgG was observed in untransfected cells (data not shown). Consistent with the observed delivery to lysosomes, agIgG internalized via either receptor underwent degradation (Fig. 1G).

FcγRIIA, but Not FcγRIIB2, Undergoes Degradation in Lysosomes after Internalization—Whereas agIgG ligand internalized by either FcγRIIA or FcγRIIB2 underwent similar degradation, a second important question is the fate of the receptors themselves. To address this, immunoblotting was performed to determine the amount of receptors remaining in cells after stimulation with agIgG. For FcγRIIA, receptor levels declined after induction of endocytosis, such that by 5 h after addition of agIgG very little FcγRIIA remained (Fig. 2A). In contrast, no drop in FcγRIIB2 levels was observed over the same time frame (Fig. 2A). The total amounts of FcγRIIA and FcγRIIB2 were also quantified by flow cytometry using intracellular staining with anti-myc antibody, which similarly showed loss of FcγRIIA but not FcγRIIB2 (Fig. 2, B and C and supplemental Fig. S1A). No drop in FcγRIIB2 level was observed after agIgG stimulation even in the presence of cycloheximide, indicating that the persistence of FcγRIIB2 following endocytosis is not due to new protein synthesis (data not shown). The loss of FcγRIIA after endocytosis was also analyzed in THP-1 cells, a human monocytic cell line that expresses endogenous FcγRIIA. As in the ts20 transfectants, FcγRIIA largely disappeared within 3 h of treatment with agIgG (Fig. 2D). This loss was inhibited by treatment with bafilomycin, indicating that degradation occurs in the endolysosomal system.

To characterize the subcellular localization of FcγRIIA and FcγRIIB2 after endocytosis, the tagged receptors were detected by immunofluorescence. Under unstimulated conditions, both FcγRIIA and FcγRIIB2 were observed to reside on the cell surface and in diffuse perinuclear vesicles (Fig. 3, A and J). This intracellular compartment is a recycling endosome pool, as judged by its co-localization with loaded transferrin (Fig. 3, A–C, J–L). After 80 min of agIgG treatment, FcγRIIA was largely co-localized with internalized agIgG ligand, which, as noted above (Fig. 1), has moved by this time into punctate perinuclear lysosomes (Fig. 3, G–I). These puncta, while located in the central region of the cell like the transferrin-positive recycling endosomes, are adjacent to these endosomes rather than co-localized with them (Fig. 3, D–F). Thus, consistent with the observed receptor degradation, FcγRIIA is targeted to lysosomes along with its ligand. In contrast, the same time point after agIgG addition, FcγRIIB2 showed a localization similar to that seen in unstimulated cells, namely, it was found at the cell surface and in transferrin-positive endosomes (Fig. 3, M–O), and did not follow internalized agIgG to lysosomes (Fig. 3, P–R), consistent with the observed lack of FcγRIIB2 degradation. Thus, FcγRIIB2 is sorted to recycling endosomes while agIgG ligand internalized via this receptor is degraded in lysosomes.

Extracellular Domains of FcγRIIA and FcγRIIB2 Do Not Determine Their Differential Trafficking—Although the extracellular domains of FcγRIIA and FcγRIIB2 are highly similar,
mERIC receptors were generated in which the extracellular domain of FcγRIIA was fused to the transmembrane and cytoplasmic domains of FcγRIIB2 (“IIA-IIIB2”) or vice versa (“IIIB2-IIA”). Western blotting was performed at different time points after induction of aglG endocytosis in cells expressing these receptors. As shown in Fig. 4, the chimeric receptor with FcγRIIA extracellular domain and FcγRIIB2 intracellular domain (IIA-IIIB2) showed no obvious drop in receptor level after aglG internalization, while conversely the chimeric receptor with FcγRIIB2 extracellular domain and FcγRIIA intracellular domain (IIB2-IIA) was degraded. Thus, the differential sorting of these receptors following endocytosis is presumably due to their divergent cytoplasmic domains.

Sorting of FcγRIIA and FcγRIIB2 Upon Persistent Cross-linking with Antibody Complexes—Whereas a difference in affinity for IgG apparently does not explain the difference between FcγRIIA and FcγRIIB2 sorting, the question nonetheless remains whether release from clustering ligand is necessary for sorting of the low affinity FcγRIIB2 away from a degradative fate, since persistence of ligand binding is thought to be an important factor determining lysosomal sorting of receptors (17). To address this question, we attempted to induce a more persistent clustering of FcγRIIB2 by engaging it with anti-FcγRII antibody AT10 followed by cross-linking receptors with secondary antibody, rather than through the low affinity binding of multivalent aglG. When FcγRIIB2-expressing cells were incubated with AT10 alone (with no secondary antibody), the AT10 was delivered to transferrin-positive endosomes, suggesting that either there is constitutive cycling of FcγRIIB2 between cell surface and recycling endosomes in the unclustered state or that dimerization of receptors by bivalent antibody is sufficient to trigger such cycling (Fig. 5, A–D). Upon clustering receptors with AT10 and secondary antibody, in contrast to the release of ligand that occurs with aglG, AT10-antibody complexes remained co-localized with the receptors, presumably due to their binding through a higher affinity interaction. Moreover, both FcγRIIB2 and the AT10 complexes were directed to recycling endosomes (Fig. 5, E–H). Consistent with this localization, neither FcγRIIB2 nor AT10 antibody complexes were degraded, in contrast to the case for FcγRIIA similarly engaged with AT10 and secondary antibody (supplemental Fig. S2). Thus, even under conditions of persistent oligomerization, FcγRIIB2 avoids a degradative fate.

Of note, when FcγRIIA-expressing cells were incubated with Fab fragments of the anti-FcγRIIA antibody IV.3 and chased at 34 °C, the Fab was internalized to transferrin-positive endosomes, suggesting that constitutive cycling of FcγRIIA can occur in the absence of clustering (Fig. 5, J–L). In contrast, when whole IV.3 antibodies were used, FcγRIIA was sorted away from transferrin-positive recycling endosomes (Fig. 5, M–P) and showed localization in LAMP1-positive lysosomes (Fig. 5, Q–S). Similar results were obtained using whole AT10 (data not shown). Thus, dimerization of FcγRIIA by bivalent antibodies (or possibly trimerization through additional engagement of the Fc region) appears to be sufficient to drive this sorting event. With whole IV.3 followed by cross-linking with secondary antibody, robust delivery of FcγRIIA to lysosomes was seen (Fig. 5, T–V).
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FIGURE 4. Extracellular domains of FcyRIIA and FcyRIIB2 do not determine their differential trafficking. ts20 cells were stably transfected with Myc-His-tagged wild type FcyRIIA (IIA), FcyRIIB2 (IIB2), or chimeric receptors comprised of the extracellular domain of FcyRIIA fused to the transmembrane and cytoplasmic domains of FcyRIIB2 (IIA-IIB2) or vice versa (IIB2-IIA). agIgG was added to the cells and incubated at 34 °C for indicated times. Lysates were analyzed by Western blotting with anti-Myc antibodies. The blot was stripped and reprobed with β-actin antibody. Representative of three experiments.

To recapitulate these findings in primary human cells, monocytic-derived macrophages were used. FcyRIIA was engaged with IV.3 with or without secondary antibody cross-linking. Cells were preloaded with fluorescent dextran to label the lysosomal compartment. At early times after engagement, IV.3 or IV.3 complexes were internalized into peripheral early endosomes that were positive for EEA1 (data not shown) and negative for dextran (Fig. 6, A–C, G–I). By 60 min post-internalization, both IV.3 alone and IV.3-secondary antibody complexes moved into central lysosomes (Fig. 6, D–F, J–L).

FcγRIIA Sorting for Degradation Does Not Require Src Family Kinase Activity or Receptor Lysines—Our results with chimeric receptors indicate that it is most likely the divergent intracellular domains of FcyRIIA and FcyRIIB2 that dictate their divergent sorting. The intracellular domain of FcyRIIA contains an ITAM that is phosphorylated by Src family kinases to induce downstream signaling cascades (3). We have previously reported that FcyRIIA engagement in transfected ts20 cells leads to receptor phosphorylation, and that both phosphorylation of FcγRIIA and phagocytosis driven by this receptor are inhibited by the Src family kinase inhibitor PP1 (8). Here, we tested the effect of PP1 on FcγRIIA intracellular sorting. While degradation was blocked by bafilomycin treatment, PP1 did not prevent aglG-induced FcγRIIA degradation (Fig. 7, A and B), indicating that Src family kinase-mediated receptor tyrosine phosphorylation is not essential to direct FcγRIIA to lysosomes.

Ubiquitylation of surface receptors can function both to trigger endocytosis and as a sorting signal driving their delivery to lysosomes (18–20). FcγRIIA is ubiquitylated upon engagement by immune complexes and an active ubiquitylation machinery is required for receptor endocytosis (8, 15). This raises the possibility that receptor ubiquitylation may also account for its lysosomal sorting. The intracellular domain of FcγRIIA contains five lysine residues that can serve as potential ubiquitylation sites. We assessed the degradation of a mutated version of FcγRIIA in which all five lysine residues were mutated to arginine (IIA-5KR). ts20 cells expressing either wild-type FcγRIIA or IIA-5KR were treated with IV.3 and cross-linking secondary antibody to trigger endocytosis. Antibody complexes internalized by either wild-type FcγRIIA or IIA-5KR were both delivered to LAMP1-positive lysosomes (Fig. 8, A–F). Moreover, the lack of lysines does not prevent FcγRIIA degradation (Fig. 8G), indicating that direct ubiquitylation of lysines in FcγRIIA is not essential for its degradation, and suggesting that other sorting signals in the cytoplasmic domain of FcγRIIA are involved in this step of its trafficking.

FcγRIIA and FcγRIIB2 Trafficking upon Co-engagement with agIgG—Our heterologous transfection model has the advantage of allowing the analysis of the intracellular trafficking capabilities of FcγRIIA and FcγRIIB2 in isolation. However, in cells expressing both FcγRIIA and FcγRIIB2, both receptors would be expected to be simultaneously co-engaged by multivalent immune complexes. If FcγRIIA and FcγRIIB2 carry different sorting signals, what is the result of such receptor co-engagement? On the one hand, FcγRIIA might have a dominant effect, pulling FcγRIIB2 toward lysosomal degradation. Conversely, co-engaged FcγRIIB2 might reroute FcγRIIA away from such a fate. A third possibility is that dissociation of immune complexes from the receptors allows them to sort to their respective fates independently of each other. To address this question, ts20 cells stably expressing Myc-His-tagged FcγRIIA were transiently transfected with untagged FcγRIIB2. AglG was added to these cells to co-engage FcγRIIA and FcγRIIB2 and the subcellular localization of FcγRIIA, FcγRIIB2, and aglG were determined by immunofluorescence (Fig. 9, A–H). After 10 min of aglG stimulation, FcγRIIA and FcγRIIB2 both co-localized with aglG in dispersed peripheral endosomes (Fig. 9, A–D). However, by 90 min after aglG stimulation, FcγRIIA and aglG were colocalized in central puncta but FcγRIIB2 showed
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**FIGURE 6.** Antibody complexes internalized by FcγRIIA are sorted to lysosomal compartments in human macrophages. Monocyte-derived human macrophages were preloaded with Alexa488-conjugated dextran. Cells were then incubated at 4 °C with Alexa488-conjugated IV.3 (A–F) or unlabeled IV.3 followed by Alexa488-labeled anti-mouse secondary antibody (G–L). Cells were then chased at 37 °C for 10 min (A–C, G–I) or 60 min (D–F, J–L). Merged images (C, F, I, L). Representative of three experiments.

expressing a range of levels of FcγRIIA (Fig. 9). Thus, our results suggest that dissociation of FcγRII from immune complexes after internalization allows independent sorting of FcγRIIA and FcγRIIB2 receptors to distinct final fates.

**DISCUSSION**

In this study, we have directly compared the intracellular trafficking of FcγRIIA and FcγRIIB2. Our results demonstrate that in addition to their opposing signaling functions, they have very different trafficking behaviors. Degradation of the activating receptor after its internalization may serve as a mechanism for ensuring appropriate termination of inflammatory signaling after initial encounter with immune complexes. In contrast, persistence of FcγRIIB2 may help to maintain the ability of myeloid cells to continue clearing immune complexes.

Incubation of FcγRIIA-expressing cells with Fab fragments of anti-FcγRIIA antibody IV.3 led to labeling of a recycling endosomal pool. This suggests that FcγRIIA, like human FcγRI (21), has the capacity to cycle between the cell surface and endosomal compartments in the absence of receptor clustering. Notably, we found that engagement of FcγRIIA with bivalent whole antibodies was sufficient to induce sorting of the receptors to lysosomes. It would be interesting to investigate to what extent such a minimal degree of clustering is also able to stimulate inflammatory signaling from FcγRIIA. It may be that small immune complexes (e.g. dimeric IgG) could have an overall anti-inflammatory effect by triggering down-regulation of activating FcγR with relatively little concomitant proinflammatory signaling. This may contribute to the therapeutic effect seen with small immune complexes or immune complex-like

**FIGURE 5.** Sorting of FcγRIIA and FcγRIIB2 after engagement with anti-FcγR antibodies. ts20 cells expressing Myc-His-tagged FcγRIIB2 (A–H) or FcγRIIA (I–V) were incubated at 4 °C with AT10 (A–H), IV.3 Fab (I–L), or intact IV.3 (M–V). Cells were then washed and incubated at 4 °C with Cy5-anti-mouse secondary antibodies. Fixed cells were labeled with Alexa488-conjugated IV.3 (A–F) or unlabeled IV.3 with Cy5-labeled pre-bound secondary antibodies (G–L). Cy5-labeled pre-bound secondary antibodies were used for immunofluorescence in N–V (anti-Myc and anti-LAMP1 are both isotype-specific anti-mouse secondary antibodies). Uncross-linked AT10 (C) or IV.3 (O, R) were detected by immunofluorescence with anti-mouse secondary antibodies after fixation. Alexa488-transferrin (A, E, K, M). LAMP-1 (Q, T). Merge images (D, H, L, P, S, V). Isotype-specific anti-mouse secondary antibodies were used for immunofluorescence in N–V (anti-Myc and anti-LAMP1 are both IgG1 antibodies; IV.3 is an IgG2b). Representative of three experiments.

a distinct localization in a diffuse central endosomal pool, as was seen with individually expressed receptors (Fig. 9, E–H). As an alternative approach, the effect of co-engagement on receptor degradation was assessed by transiently overexpressing FcγRIIB2 in cells that stably express FcγRIIA, or vice versa, and measuring degradation of the stably expressed receptor by flow cytometry (Fig. 9, I and J). Expression of cotransfected GFP was used as a measure of transient receptor expression (Fig. 9, I and J and supplemental Fig. 5, B–D). FcγRIIA was still degraded in cells expressing a range of levels of FcγRIIB2 (Fig. 9J), and conversely there was no obvious degradation of FcγRIIB2 in cells
agents in the context of autoimmune diseases in which activating FcγR on effector cells play deleterious roles (22, 23).

Our experiments with FcγRIIB2 highlight the fact that the receptor can dissociate from multivalent agIgG complexes after internalization. On first thought, one might expect that the interaction of receptors with multivalent complexes would be highly stable. Indeed, immune complexes can bind strongly to the cell surface due to the simultaneous interaction of multiple Fc domains with multiple Fc receptors (i.e., avidity). However, once the receptor-ligand complexes have been delivered to endosomal compartments, dissociation of individual receptors from the internalized immune complexes will be determined solely by their affinity for Fc, because each receptor binds only one Fc portion. In this context the multivalent nature of the complex may primarily have the effect of increasing the local concentration of IgG. The low affinity of the FcγRII thus will serve to facilitate release of receptors from internalized immune complexes, allowing their spatial segregation from the complexes through endosomal fission events. Our observations suggest that following co-engagement of FcγRIIA and FcγRIIB2 these two receptors are sorted independently, implying that dissociation of receptors from agIgG can occur before committed sorting events. The low affinity of the FcγRII may therefore confer two distinct evolutionary advantages. As is often noted, the low affinity of FcγR allows cells to respond specifically to multivalent immune complexes, rather than being permanently occupied by monomeric IgG present in serum. A second advantage, however, may be that low affinity allows FcγR to access distinct intracellular compartments even after co-engagement by immune complexes.

As for the effect of affinity not on the fate of the receptors but rather on that of internalized immune complexes, our findings with anti-FcγRII antibody imply that release of immune complexes internalized via FcγRIIB from the receptor is required for the immune complexes to undergo lysosomal degradation. The low affinity of human FcγRIIB for IgG would facilitate this release. It is noteworthy that among the murine low affinity FcγR, the inhibitory receptor FcγRIIB2 binds to mouse IgG1 with higher affinity than the activating receptors FcγRIII and FcγRIV, and binds to IgG2a and IgG2b with a comparable affinity to FcγRII (24). In contrast, among the human low affinity FcγR, FcγRIIB has a substantially lower affinity than the activating receptors FcγRIIA and FcγRIIIB for all human IgG isotypes except IgG4 (for which affinities are similarly low for all receptors) (5). FcγR-mediated uptake of antigen in immune complexes by antigen presenting cells can lead to greatly increased efficiency of subsequent antigen presentation to T cells (25). However, the consequences of antigen uptake via FcγRIIB in particular are unclear; in some studies murine
FcγRIIA and FcγRIIB2 sorting upon co-engagement with agIgG. A–J, ts20 cells stably expressing FcγRIIA-Myc-His (IIA-MH) were transiently transfected with untagged FcγRIIB2. The cells were incubated with agIgG on ice for 20 min, then chased at 34 °C for 10 min (A–D) or 90 min (E–H). AgIgG, FcγRIIA, and FcγRIIB2 were detected by immunofluorescence using anti-human (A, E), anti-Myc (B, F), and anti-FcγRIIB2 (C, G) antibodies, respectively. Merged images (D, H). Representative of three experiments. I, ts20 cells stably expressing FcγRIIA-Myc-His were transiently co-transfected with untagged FcγRIIB2 and GFP. FcγRIIA-Myc-His levels before and after a 3-h incubation with agIgG were analyzed by intracellular flow cytometry with anti-Myc. Right panel shows staining of untransfected ts20 cells. J, ts20 cells stably expressing FcγRIIB2-Myc-His were transiently co-transfected with untagged FcγRIIA and GFP. FcγRIIB2-Myc-His levels before and after a 3-h incubation with agIgG were analyzed by intracellular flow cytometry with anti-Myc. Right panel shows staining of untransfected ts20 cells. The red lines are added to facilitate comparison of receptor levels before and after agIgG treatment. Representative of three experiments.

FcγRIIB has been shown to be capable of facilitating antigen presentation (26–29), while in others it has been seen to have an inhibitory effect (30, 31). One means by which FcγRIIB can impair antigen presentation is by suppressing ITAM-induced dendritic cell maturation (32). In addition, however, FcγRIIB in mouse dendritic cells can recycle bound immune complexes to the cell surface and prevent their delivery to degradative compartments that is required for antigen processing (30, 31). Recycling of a portion of internalized immune complexes to the cell surface was also observed with rat FcγRIIB in liver sinusoidal endothelial cells (33). Such routing of immune complexes away from a degradative fate will depend on the persistence of their binding to receptors. If immune complexes are released from FcγRIIB after internalization in human antigen-presenting cells, with delivery of the released immune complexes to lysosomes, the uptake via FcγRIIB would be expected to facilitate antigen processing. The pH sensitivity of receptor binding to immune complexes may also be an important factor determining their release in endosomal compartments, and it would be interesting to determine the extent to which this varies among different Fc receptors.

Studies of FcγRIIA in neutrophils have shown an extremely rapid degradation of the receptor occurring in less than a minute following its ligation; this degradation appears to involve the action of the proteasome (34). Our results with monocytic THP-1 cells indicate that, as in the transfected cell model, degradation of FcγRIIA occurs in lysosomes over the longer time frames typical for such degradation. Moreover, trafficking of anti-FcγRIIA antibodies to lysosomes was observed in monocyte-derived macrophages. These results suggest that while loss of FcγRIIA after its ligation is a common theme in both monocytic cells and neutrophils, the very rapid degradation of FcγRIIA seen in neutrophils may be a mechanistically unique feature of these cells. Another recent study concluded that FcγRI, but not FcγRIIA, can be delivered to lysosomes in monocyes within 10 min of receptor engagement (35). Our results indicate that FcγRIIA can also traffic to lysosomes, with the slower kinetics more typical of lysosomal sorting of surface receptors.

While the ITAM of the common Fcγ subunit that associates with FcγRI is important for triggering downstream signaling upon stimulation of cells via this receptor, the cytoplasmic tail of FcγRI itself also has functional effects, controlling ligand binding and endocytosis through interactions with periplakin (36). Our finding that Src family kinase activity is not essential for FcγRIIA degradation suggests that the cytoplasmic tail of
this receptor also interacts with as yet unidentified cytosolic proteins distinct from the ITAM signaling pathway. The lack of requirement for lysine residues also argues against a requirement for direct ubiquitylation of the receptor for its degradation, though we cannot exclude a role for non-conventional ubiquitylation of nonlysine residues (37), or ubiquitylation of a receptor-associated protein. It is also possible that sorting is influenced by the transmembrane domains of FcRIIA and FcγRIB2, which differ from each other in three amino acids and which were swapped along with the cytoplasmic domains in our chimeric receptors. The transmembrane domains of both receptors have been shown to affect their localization to membrane microdomains (38, 39).

Preferential degradation of the activating FcγRIIA coupled with persistence of its inhibitory counterpart implies that after immune complex stimulation, not only does the level of the activating receptor decline, but the ratio of activating to inhibitory receptor also decreases. This reduction should serve to accentuate the termination of signaling from activating receptors, while maintaining cellular ability to clear immune complexes. Thus, these fundamental differences in the sorting of FcγRIIA and FcγRIB2 add an additional level of modulation of signaling over the longer term beyond the initial down-regulation of activating signals by ITIM-mediated dephosphorylation.

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