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Receptor Modulation by FcγRI-Specific Fusion Proteins Is Dependent on Receptor Number and Modified by IgG[1]

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The high-affinity IgG receptor, FcγRI (CD64), is constitutively expressed exclusively on professional APCs. Human FcγRI binds monomeric IgG with high affinity and is, therefore, saturated in vivo. The binding of IgG to FcγRI causes receptor recycling, while Abs that cross-link FcγRI cause rapid down-modulation of surface FcγRI. Because studies performed in the absence of ligand may not be representative of FcγRI modulation in vivo, we investigated the ability of FcγRI-cross-linking Abs and non-cross-linking derivatives to modulate FcγRI in the presence and absence of ligand. In the absence of ligand mAb H22 and wH22xeGFP, an enhanced green fluorescent protein (eGFP)-labeled fusion protein of H22, cross-linked and rapidly down-modulated surface FcγRI on the human myeloid cell line, U937, and its high FcγRI-expressing subclone, 10.6. This effect was dependent on the concentration of fusion protein and the level of FcγRI expression and correlated with internalization of both wH22xeGFP and FcγRI, itself, as assessed by confocal microscopy. A single-chain Fv version, sFv22xeGFP, which does not cross-link FcγRI, was unable to modulate FcγRI in the absence of IgG. However, if ligand was present, treatment with either monovalent or cross-linking fusion protein led to intracellular receptor accumulation. These findings suggest at least two alternate mechanisms of internalization that are influenced by ligand and demonstrate the physiologic potential of FcγRI to transport a large antigenic load into APCs for processing. These studies may lead to the development of better FcγRI-targeted vaccines, as well as therapies to down-modulate FcR involved in autoimmune diseases. ***The Journal of Immunology*****, 2001, 167: 6303–6311.**

The high-affinity receptor for IgG, FcγRI (or CD64), is constitutively expressed exclusively on professional APCs (monocytes, macrophages, and dendritic cells). Human FcγRI binds monomeric human IgG1 and IgG3, as well as mouse IgG2a and IgG3, with high affinity (10^8–10^9 M^{-1}) and is consequently saturated in vivo (reviewed in Refs. 1–6). Several mAbs have been developed which bind FcγRI outside the ligand-binding site and therefore are able to bind in the presence of IgG (7). When cross-linked, these mAbs have been shown to trigger FcγRI functions such as phagocytosis, Ab-dependent cellular cytotoxicity, and superoxide generation (1, 8–11). Targeting Ags specifically to FcγRI via one of these mAbs has been shown to enhance Ag presentation, as evidenced by T cell proliferation (12–14) as well as cytotoxicity in vitro (15) and vaccine-like effects in vivo (16, 17). Thus, FcγRI-targeted Ags are currently being developed as vaccines (16, 18–21). However, the mechanism by which FcγRI-targeted Ags are processed and presented is unknown.

Abs that cross-link FcγRI have been shown to down-modulate surface expression of FcγRI and, therefore, have been used in the treatment of immune thrombocytopenia purpura, an autoimmune disease characterized by the FcR-mediated destruction of platelets. Treatment of immune thrombocytopenia purpura patients with either mAb 197 (22) or mAb H22 (23) led to down-modulation of surface FcγRI on peripheral blood monocytes and correlated with elevated platelet count.

In vitro studies on human monocytes have shown that when FcγRI is cross-linked, receptors are internalized (rather than shed) within hours of cross-linking. Furthermore, the down-modulation of surface FcγRI by mAb H22 was shown to be concentration-dependent, such that 0.1 μg/ml of H22 was optimal and lower or higher concentrations were less effective (24). It has been demonstrated that FcγRI recycles into and out of the cell when ligand is bound, and that the addition of an anti-FcγRI Ab which binds outside the ligand-binding site causes receptors to accumulate inside the cell and not recycle to the cell surface (25). Because prior studies performed in the absence of natural ligand (human IgG1 or mouse IgG2a) may not be representative of FcγRI modulation in vivo, we chose to investigate further the capacity of H22 and FcγRI-targeted fusion proteins to modulate FcγRI in the presence and absence of ligand.

In this work we describe FcγRI down-modulation (hereafter referred to as "modulation") on the human myeloid cell line U937 and its high FcγRI-expressing subclone 10.6, using H22 and wH22xeGFP, a fusion protein of H22 and enhanced green fluorescent protein (eGFP).[3] Both modulators (H22 and wH22xeGFP) can bind to FcγRI trivalently, because they contain an intact human IgG1 Fc region in addition to two Fab regions specific for FcγRI. We also examined sFv22xeGFP, an eGFP molecule genetically fused to the single-chain version of H22, which binds FcγRI

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[3] Abbreviations used in this paper: eGFP, enhanced green fluorescent protein; MFF, methanol-free formaldehyde; NHS, normal human serum; MFI, mean fluorescence intensity.

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monovalently. The goal of these studies was to examine the efficiency of FcγRI internalization and modulation in response to the two new eGFP fusion proteins and the parent H22. We also explored the contribution of the relative level of receptor expression and the presence of ligand on modulation. Our findings suggest that trivalent anti-FcγRI constructs modulate FcγRI in the absence of IgG in a concentration-dependent manner similar to the modulation of monocyte FcγRI reported by Wallace et al. (24), although the kinetics of modulation are more rapid on 10.6 cells. However, the presence of IgG leads to greater modulation at higher concentrations of modulator and permits the internalization of non-cross-linked anti-FcγRI constructs. Two different models are proposed for receptor modulation in the presence and absence of ligand.

Materials and Methods

Cells

U937, a human myeloid cell line expressing FcγRI, was obtained from American Type Culture Collection, (Manassas, VA) (26). 10.6 cells, a high FcγRI-expressing subclone of the parent U937 line, were a kind gift of Dr. P. M. Guyre (Dartmouth Medical School, Lebanon, NH) (8). Cells were cultured in RPMI 1640 supplemented with 10% FBS, L-glutamine, 25 mM HEPES, and 50 μg/ml gentamicin (all from BioWhittaker, Walkersville, MD) (complete medium) in the presence or absence of 25 ng/ml IFN-γ (Genentech, South San Francisco, CA) at 37°C/5% CO_2.

Abs and reagents

Anti-FcγRI Abs used as modulators included H22 (7, 27); H22 F(ab′)$_2$; MDX-H210, a bispecific Ab comprised of the Fab′ of mAb H22 and anti-HER2/neu (28); and 197, a mAb which has been shown to cross-link and modulate FcγRI (10, 22, 29) (all kind gifts of Medarex, Bloomsbury, NJ). Also used were two FcγRI-targeted eGFP fusion proteins, sFv22xeGFP and wH22xeGFP, constructed from mAb H22 and eGFP (Ref. 30 and C. Guyre, M. Barreda, S. Swink, and M. Fanger, manuscript in preparation). sFv22xeGFP, which binds FcγRI monovalently, is a single-chain Fv fragment of mAb H22 fused to eGFP. wH22xeGFP, which binds FcγRI both by its Fab ends and its Fc end, thereby cross-linking FcγRI, is a fusion protein of the whole H22 mAb with eGFP fused to the CH3 domains of the H22 H chains (31). The amount of aggregates in the fusion proteins varied from 0–4%, and the percentage of aggregates did not correlate with modulation activity. Saturation curves were generated for each individual batch of fusion protein that was made, and the 1/2 maximum binding for individual batches was determined. In all cases, 5 nM was <1/2 maximum for wH22xeGFP, and 15 nM was < 1/2 maximum for sFv22xeGFP. The 1/2 maximum binding for H22 was ~4 nM, and 5 nM was subsaturating. Some modulation assays were performed in the presence or absence of human gammaglobulin (Sigma-Aldrich, St. Louis, MO) at a final concentration of 3 mg/ml in medium containing 0.5 mg/ml BSA. FcγRI was detected using a murine mAb, 32.2 (Medarex), which binds FcγRI outside the ligand-binding domain at an epitope distinct from the H22 binding site (7, 32), followed by a R-PE-conjugated F(ab′)$_2$ goat anti-mouse IgG (H and L chains, minimal cross-reaction to human, bovine, horse, rabbit, and swine; Jackson ImmunoResearch Laboratories, West Grove, PA). For confocal microscopy studies, detection Ab was Cy3-conjugated F(ab′)$_2$ goat anti-mouse IgG plus IgM (Jackson ImmunoResearch Laboratories). P3, an irrelevant murine IgG1, was used as an isotype control.

Modulation assay

Cells were treated with varying amounts of modulator ± 3 mg/ml IgG in complete medium from 30 min to 24 h either at 37°C or on ice. In some studies, normal human serum (NHS; Gemini Bio-Products, Calabasas, CA) or mouse serum (Sigma-Aldrich) was used in place of FBS. Following treatment, cells were washed twice with ice-cold PBA (PBS containing 1% BSA (Sigma-Aldrich) and 0.05% sodium azide; Fisher Scientific, Fairlawn, NJ) and stained on ice for surface FcγRI in a 96-well round-bottom polypropylene microtiter plate (Costar, Corning, NY). Briefly, cells were resuspended in 20 μg/ml mAb 32.2 and incubated on ice for 1 h. Cells were then washed three times with ice-cold PBA, resuspended in a 1/40 dilution of R-PE-conjugated goat anti-mouse IgG, and incubated on ice for 45 min. Cells were washed twice with ice-cold PBA and resuspended in 1% methanol-free formaldehyde (MFF; Polysciences, Warrington, PA) in PBS. Fixed cells were stored at 4°C for at least 16 h before flow cytometric analysis was performed.

Flow cytometric analysis

Fixed cells were analyzed by flow cytometry using a FACScan (BD Biosciences, San Jose, CA). For each experiment compensation was set using unstained ("autofluorescent") or isotype control, green-only, and red-only samples to eliminate fluorochrome crossover between FL-1 and FL-2. Ten thousand events were collected for each sample in a given experiment. FL-2 data represent the red channel (585/42 nm filter), demonstrating the relative amounts of FcγRI on the surface of cells following treatment. Modulation was calculated from FL-2 data using the following equation, where MFI represents the geometric mean fluorescence intensity:

$$\left(1 - \frac{\text{mAb 32 MFI of cells treated with modulator}}{\text{mAb 32 MFI of untreated cells}}\right) \times 100\%$$

MFI of isotype control mAb P3, which reflects the background fluorescence due to nonspecific binding of Abs and cell autofluorescence, was first subtracted. In experiments using serum or IgG, ligand was present in both "untreated" and "plus modulator" conditions. Based on this calculation, decreased MFI (i.e., decreased surface FcγRI expression) is reflected in increased percent modulation.

Confocal microscopy

Cells were subjected to receptor modulation as described above and then fixed in ice-cold 1% MFF (Polysciences) for a minimum of 1 h. Fixed cells were then permeabilized in PBAS (PBA containing 0.5% saponin; Sigma-Aldrich) and stained as described above using PBAS as wash buffer and Ab diluent. Following staining, cells were fixed in 1% MFF and stored at 4°C for a minimum of 16 h. Cells were then pelleted and ~3.5 μl of cells were placed on a glass slide (Gold Seal Products, Portsmouth, NH). Approximately 3.5 μl of Prolong Antifade reagent (Molecular Probes, Eugene, OR) was added, followed by an 18-mm glass coverslip (Costar) which was then sealed with nail varnish. Imaging was performed exactly as described elsewhere (31) on a Bio-Rad MRC-1024 Confocal Scanning Laser Microscope system (Bio-Rad, Hercules, CA) using a krypton/argon laser and LaserSharp version 3.2 software (Bio-Rad).

Molar conversions

To compare data displayed in micrograms per milliliter to molar concentrations, molar equivalents for each fusion protein and Ab used were calculated using the estimated m.w., as determined by Gene Inspector software (Textco, West Lebanon, NH). The molar equivalents of 1 μg/ml of fusion protein are 17.7 nM sFv22xeGFP, 4.96 nM wH22xeGFP, and 6.77 nM H22. The m.w. of mAb 197 was estimated to be equal to H22, because both are IgG.

Results

Multivalent binding to FcγRI is required for receptor modulation in the absence of ligand

Modulation assays were performed by treating cells with a potential modulator at 37°C and then staining cells on ice for residual surface FcγRI using mAb 32.2 followed by R-PE-conjugated goat anti-mouse IgG. Studies using directly labeled R-PE-32.2 demonstrated results similar to this two-step approach (data not shown). MFI of treated cells was compared with that of untreated cells to determine the loss of cell surface receptors due to the modulator. Fig. 1 illustrates the capacity of fusion proteins sFv22xeGFP and wH22xeGFP to modulate surface FcγRI. Although previous studies showed that H22 does not block the binding of 32.2, we tested whether the inclusion of eGFP in our fusion proteins inhibited the binding of 32.2. Surface expression of FcγRI on cells treated with sFv22xeGFP or wH22xeGFP on ice was not different from cells treated with PBS, indicating that neither fusion protein blocked the binding of the detection Ab, 32.2 (Fig. 1*A*). Cells treated for 2 h with PBS or monovalent sFv22xeGFP were unable to modulate FcγRI at either temperature, while the multivalent wH22xeGFP reduced surface expression ~50% at 37°C (Fig. 1*A*). Time course studies confirmed the inability of sFv22xeGFP to modulate FcγRI (Fig. 1*B*). However, modulation of surface FcγRI by wH22xeGFP was ~40% within 10 min and appeared to be maximal by 30 min (Fig. 1*B*).

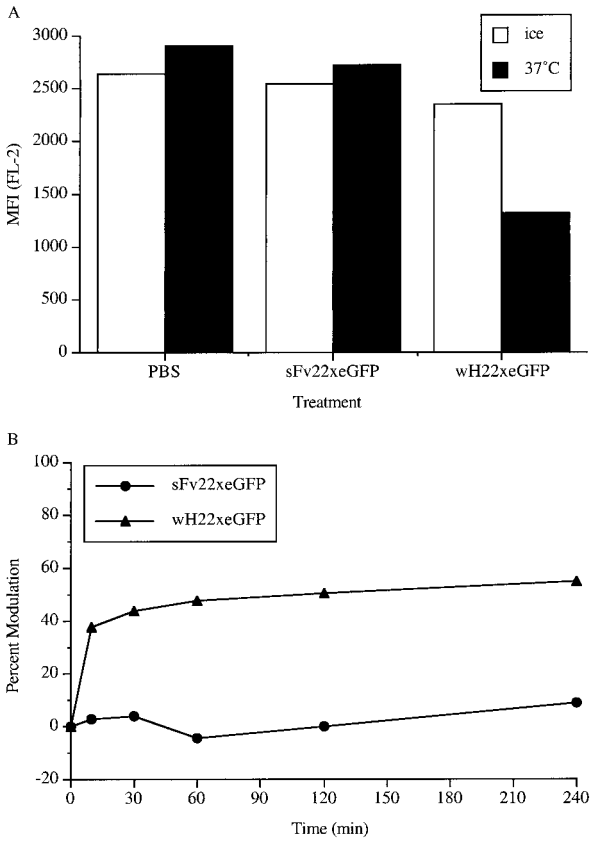

FIGURE 1. FcγRI modulation by saturating concentrations of FcγRI-targeted proteins in the absence of ligand. Saturating concentrations (2 μg/ml) of modulator were added to IFN-γ-treated 10.6 cells either on ice (*A*, open bars) or at 37°C (*A*, filled bars). Cells were then washed and stained on ice at various times with mAb 32.2 followed by R-PE-conjugated goat anti-mouse IgG. MFI (FL-2) data represent surface expression of FcγRI. Percent modulation = (1 − mAb 32 MFI of cells treated with modulator / mAb 32 MFI of untreated cells) × 100, as described in *Materials and Methods*. Values were calculated from single samples and are representative of at least three experiments. Two micrograms per milliliter are equal to 35.4 nM sFv22xeGFP and 9.92 nM wH22xeGFP. *A*, Expression of FcγRI at 2 h. Neither PBS nor sFv22xeGFP treatment led to decreased surface expression of FcγRI, while wH22xeGFP treatment at 37°C reduced expression by ~50%. *B*, Modulation time course. Treatment with sFv22xeGFP (●) did not lead to FcγRI modulation. However, wH22xeGFP (▲) modulated 38% of surface FcγRI within 10 min and maximally within 1–2 h.

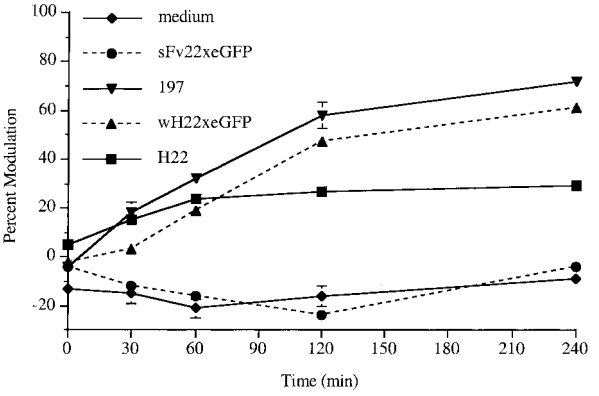

FIGURE 2. FcγRI modulation by subsaturating concentrations of FcγRI-targeted proteins in the absence of ligand. Subsaturating concentrations of modulator previously determined to modulate FcγRI were added to IFN-γ-treated 10.6 cells for various amounts of time either on ice or at 37°C. Following treatment, cells were washed and stained on ice with mAb 32.2, followed by R-PE-conjugated goat anti-mouse IgG. For cells treated with mAb 197, only secondary Ab was used to detect surface-bound 197. Percent modulation was determined using the following formula: [1 − (37°C MFI − MFI isotype control) / (ice MFI − MFI isotype control)] × 100. Treatment with neither medium (◆) nor 15 nM sFv22xeGFP (●) led to receptor modulation. However, treatment with 5 nM of mAb 197 (▼), wH22xeGFP (▲), and H22 (■) led to 72, 61, and 29% modulation, respectively, by 4 h. Mean values of duplicate samples ± SD are plotted.

Our initial studies showed that subsaturating concentrations of anti-FcγRI mAbs and fusion proteins were superior to saturating concentrations for maximally modulating FcγRI in 4- and 16-h assays (data not shown). Therefore, to directly compare the modulation kinetics of wH22xeGFP and other Abs, cells were treated with subsaturating concentrations of each. Because the binding of one modulator (mAb 197) to FcγRI blocks the binding site of the detection Ab, mAb 32.2, the standard modulation assay had to be modified. FcγRI was measured indirectly for samples treated with 197 using PE-conjugated goat anti-mouse IgG, which detects the surface-bound 197 itself. For samples treated with the other fusion proteins and Abs, FcγRI was measured using mAb 32.2, as in the standard modulation assay. For each construct, modulation was calculated based on cells treated with the given construct at 37°C to cells treated with that same construct on ice. Fig. 2, like Fig. 1, demonstrates that treatment with medium alone or sFv22xeGFP did not lead to FcγRI modulation. However, cells treated with equimolar amounts of wH22xeGFP, H22, or 197 led to varying degrees of modulation, with 197 modulating most rapidly, followed by wH22xeGFP. This variation among modulators at equimolar concentrations may be due to differences in binding kinetics and/or how well each can cross-link FcγRI. Of note, subsaturating concentrations of wH22xeGFP led to slower FcγRI modulation than did saturating concentrations (Fig. 1).

Receptor modulation correlates with the internalization of FcγRI

To better understand the nature of receptor modulation, confocal microscopy was performed to determine whether the reduction in surface FcγRI was due to receptor internalization. IFN-γ-treated U937 cells were incubated with sFv22xeGFP, wH22xeGFP, or H22 as modulators and then fixed and examined for intracellular FcγRI using mAb 32.2. In 4-h modulation assays, FcγRI appeared only on the cell surface if no modulator was used (data not shown) or if the monovalent sFv22xeGFP was used as modulator (Fig. 3, *top panel*). This lack of internalized receptor correlated with the lack of FcγRI modulation in flow cytometric assays. By contrast, when wH22xeGFP was used as modulator, intracellular FcγRI was observed at all concentrations that led to receptor modulation in flow assays (0.1–10 μg/ml) (Fig. 3, *middle panel*; compare with Fig. 4*A*). Similarly, when H22 was used as modulator, internalized FcγRI was more prominent at the low concentrations (0.1–1 μg/ml), which showed maximal receptor modulation in flow assays (Fig. 3, *bottom panel*; compare with Fig. 4). Similar patterns of internalization were observed in 1-h assays (data not shown). Interestingly, FcγRI staining often appeared polarized to one side of the cell at high concentrations of modulator. Although we do not know the basis for the polarized staining, it was often observed when modulator was present and may be due to the assembly of FcγRI into membrane rafts.

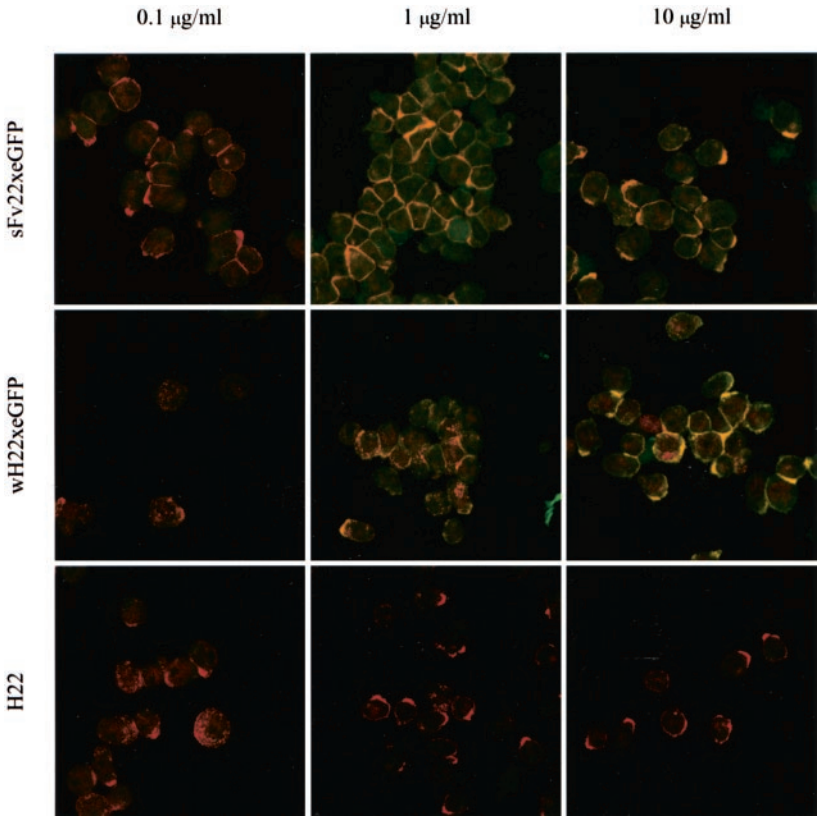

FIGURE 3. Treatment with wH22xeGFP and H22, but not sFv22xeGFP, leads to receptor internalization in the absence of ligand. U937 cells were cultured in the presence of IFN-γ for 3 days and then treated at 37°C for 4 h with modulator. Cells were then fixed and stained for intracellular FcγRI with mAb 32.2, followed by Cy3-conjugated goat anti-mouse IgG plus IgM. Confocal micrographs represent a section through the center of the plane of cells. Green stain represents eGFP fluorescence, red stain represents 32.2 staining, and yellow/orange staining is a result of green plus red staining. One microgram per milliliter is equal to 17.7 nM sFv22xeGFP, 4.96 nM wH22xeGFP, and 6.77 nM H22.

Ligand markedly influences modulation by H22 constructs

Because human serum contains high levels of IgG, the ligand-binding domain of FcγRI is usually occupied in vivo. Therefore, it seemed important to determine whether ligand affected the ability of H22 constructs to modulate FcγRI. Fig. 4 demonstrates that ligand altered modulation by H22 and wH22xeGFP. In the presence of saturating concentrations of human gammaglobulin, both H22 and wH22xeGFP were able to modulate FcγRI at high concentrations, including those which, in the absence of ligand, led to little or no modulation (e.g., 1 and 10 μg/ml H22) or lower levels of modulation (e.g., 10 μg/ml wH22xeGFP) (Fig. 4*A*). We next compared modulation in the presence of serum known to contain IgG not capable of binding FcγRI (FBS) (33) to that which contains human FcγRI-binding isotypes (human and mouse serum). FcγRI modulation by 0.1 μg/ml H22 in the absence of ligand was significant, but 10-fold lower or higher concentrations did not cause modulation. However, if ligand was present (in the form of NHS (Fig. 4*B*) or mouse serum (Fig. 4*C*)), modulation occurred in the presence of higher concentrations of H22 (1 and 10 μg/ml). These data support the role of ligand in altering the ability of H22 to modulate FcγRI.

Because ligand seemed to modify the ability of Abs that cross-link FcγRI to modulate FcγRI, we investigated the effect of ligand on modulation by anti-FcγRI Ab variants that do not cross-link. In the absence of ligand, H22 was able to modulate FcγRI as expected, but neither MDX-H210 nor H22 $F(ab')_2$, two FcγRI-specific molecules that lack an Fc region, caused modulation (Fig. 5*A*). This indicates that the trivalent H22, but not its monovalent or divalent derivatives could modulate FcγRI. However, when ligand was present in the form of NHS, all three Abs were able to modulate FcγRI (Fig. 5*B*). These data suggest that, in addition to altering the modulation of FcγRI by Abs that cross-link the receptor, ligand affected the ability of non-cross-linking anti-FcγRI Abs to modulate FcγRI.

Ligand permits intracellular accumulation of anti-FcγRI constructs and FcγRI

Flow cytometric analyses revealed that modulation of FcγRI by high concentrations of H22 and wH22xeGFP could be attained if ligand was present (Fig. 4). To determine whether this modulation correlated with the internalization of FcγRI, confocal microscopy studies were performed. Cells were treated with 10 μg/ml modulator in the absence (Fig. 3, *right panels*) or presence (Fig. 6) of IgG. In the absence of IgG, wH22xeGFP and FcγRI appeared both on the surface and intracellularly (Fig. 3, *middle right panel*). However, when IgG was present, both fusion protein and receptor appeared predominantly inside the cell (Fig. 6, *middle panel*). For cells treated with H22 in the absence of ligand, FcγRI appeared mainly on the cell surface (Fig. 3, *bottom right panel*), while internalized receptor was evident when IgG was present (Fig. 6, *right panel*). These data correlated with modulation data showing that high levels of wH22xeGFP were capable of modulating FcγRI but the addition of ligand enhanced the modulation, and that H22 was unable to modulate FcγRI at this concentration unless ligand was present (Fig. 4).

We further demonstrated that the monovalently targeted sFv22xeGFP was capable of internalization, along with FcγRI, in the presence (Fig. 6, *left panel*), but not absence (Fig. 3, *top right panel*), of IgG, indicating the ability of monovalent FcγRI-targeted proteins to be internalized with the receptor in the presence of ligand. This is in accordance with data showing that a monovalently targeted anti-FcγRI Ab, MDX-H210, was able to modulate FcγRI in the presence of ligand (Fig. 5*B*).

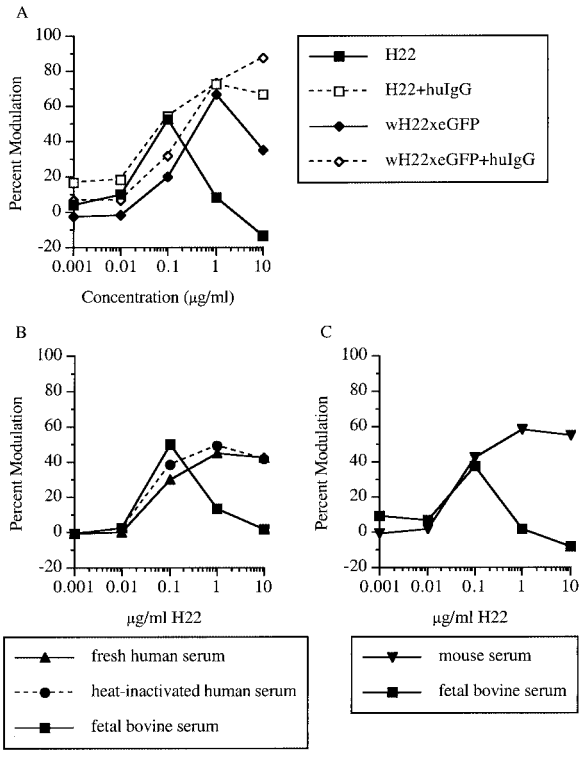

FIGURE 4. Ligand modifies modulation of FcγRI by H22 and wH22xeGFP. U937 cells were cultured in the presence of 25 ng/ml IFN-γ for 3 days and then used in a modulation assay. Cells were incubated with modulator in IgG or serum-containing medium for 16 h at 37°C/5% CO_2, and FcγRI levels were determined by mAb 32.2, followed by R-PE-conjugated goat anti-mouse IgG. Mean modulation of duplicate samples is based on control cells incubated in the absence of modulator, as described in *Materials and Methods*. One microgram per milliliter is equal to 4.96 nM wH22xeGFP and 6.77 nM H22. *A*, The presence of human gammaglobulin (open symbols) permitted modulation by high concentrations of H22 (□) and wH22xeGFP (◇), while modulation was low or absent in the absence of IgG (filled symbols). *B*, The presence of fresh human serum (▲) and heat-inactivated human serum (●), but not FBS (■), permitted modulation of FcγRI at higher concentrations of H22. *C*, The presence of mouse serum (▼), but not FBS (■), permitted modulation of FcγRI at higher concentrations of H22.

The ability of anti-FcγRI Abs to modulate is dependent on receptor number

To determine whether the concentration dependency of H22 and wH22xeGFP to modulate FcγRI changed with FcγRI levels, two different U937 cell lines were studied: U937 cells obtained from American Type Culture Collection (U937 cells) and its high FcγRI-expressing subclone (10.6 cells). Both cell lines were tested with and without prior culture in the presence of IFN-γ, a cytokine known to up-regulate FcγRI. IFN-γ has been shown to influence the processing of Ag (34, 35) but was reported to not alter the ability of immune complexes to be internalized through FcR (36). Table I illustrates the relative receptor expression of each of these cell lines cultured for 3 days in the presence and absence of IFN-γ. As expected, treatment with IFN-γ up-regulated FcγRI expression ~4- to 5-fold, and FcγRI expression for each cell line was significantly different (Table I). In 16-h modulation assays using these cells, treatment with sFv22xeGFP did not lead to FcγRI modulation (Fig. 7). However, when H22 or wH22xeGFP was used, modulation occurred, and the concentration that led to maximal modulation increased with receptor number (Fig. 7). Furthermore, in some cases wH22xeGFP was able to modulate FcγRI over a wider

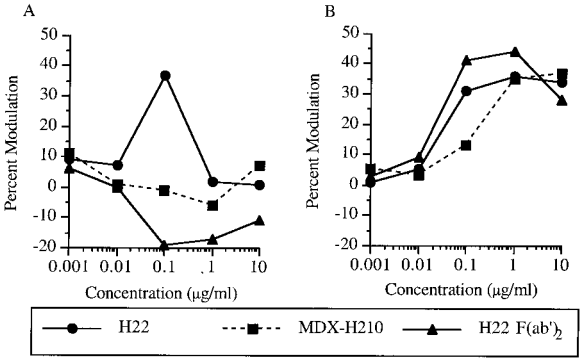

FIGURE 5. Effect of ligand on FcγRI modulation by H22 Abs with different valencies. H22 (●), MDX-H210 (■), and H22 F(ab′)$_2$ (▲) modulated FcγRI in the presence of NHS (*B*) but only H22 (0.1 μg/ml) modulated in the presence of FBS (*A*). IFN-γ-treated U937 cells were incubated with H22 constructs in serum-containing medium for 16 h at 37°C/5% CO_2. FcγRI levels were determined by R-PE-conjugated 32.2 staining. Percent modulation is based on control cells incubated without Ab. Mean values of duplicate samples are plotted. One microgram per milliliter is equal to 6.77 nM H22, ~10 nM MDX-H210, and ~10 nM H22 F(ab′)$_2$.

range of concentrations than H22 (e.g., 3 and 10 nM wH22xeGFP vs 3 nM only H22, Fig. 7*C*). In addition, wH22xeGFP led to higher levels of modulation than H22 in most cases (Fig. 7, *A*, *B*, and *D*). Similar trends in receptor modulation were observed in 4-h modulation assays (data not shown).

Overall, these data suggest that higher receptor numbers required a higher concentration of modulator, thereby maintaining the optimal modulator:receptor molar ratio for maximal FcγRI modulation. To further illustrate this point, the FcγRI expression data for each modulator were plotted. As shown in Fig. 8, the concentration of H22 or wH22xeGFP that led to maximal modulation (seen as decreased MFI) shifted to the right as receptor number increased, whereas sFv22xeGFP did not lead to decreased FcγRI expression at any concentration. The addition of ligand, generally in the form of saturating concentrations of IgG, consistently led to an increase in levels of FcγRI. Interestingly, high concentrations (≥30 nM) of H22, an IgG1 mAb that cross-links and modulates FcγRI at lower concentrations, resulted in increased receptor expression, even approaching that of cells treated with a saturating concentration of IgG (Fig. 8). This supports the hypothesis that high levels of a proven cross-linker can bind FcγRI in a functionally monovalent fashion and, like the binding of ligand, lead to increased levels of surface FcγRI.

Discussion

Modulation of FcγRI using anti-FcγRI Abs has been described for cultured human monocytes (24, 37) and peritoneal exudate macrophages from human FcγRI-transgenic mice (17), as well as in vivo in patients with immune thrombocytopenia purpura (22, 23). In the present study, our aim was to further investigate the nature of FcγRI modulation by a humanized mAb, H22, as well as H22 fusion proteins, using the human myeloid cell lines U937 and its high FcγRI-expressing subclone, 10.6, as a model. Using wH22xeGFP, an H22-eGFP fusion protein which cross-links FcγRI, we found receptor modulation to be rapid, with the majority of the modulation occurring within the first 10 min of the assay and a steady-state reached by 30 min. This is similar to what has been found in studies by others using U937 cells, which showed internalization to occur within 5 min after cross-linking receptor-bound IgG (25). In studies by Wallace et al. (24), FcγRI modulation on normal human monocytes was evident at 2 h and maximal

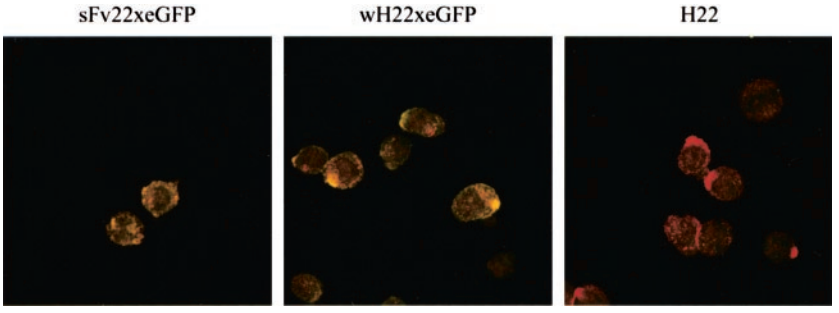

FIGURE 6. FcγRI internalization in the presence of ligand. The presence of human IgG permitted intracellular accumulation of FcγRI by monovalent sFv22xeGFP and high concentrations of wH22xeGFP and H22. U937 cells were cultured in the presence of IFN-γ for 3 days and then treated at 37°C for 4 h with 10 μg/ml modulator in the presence of 3 mg/ml human IgG. Cells were then fixed and stained for intracellular FcγRI with mAb 32.2, followed by Cy3-conjugated goat anti-mouse IgG plus IgM. Green stain represents eGFP fluorescence, red stain represents 32.2 staining, and yellow/orange staining is a result of green plus red staining. One microgram per milliliter is equal to 17.7 nM sFv22xeGFP, 4.96 nM wH22xeGFP, and 6.77 nM H22.

at 8 h when H22 was used as modulator. The differences in kinetics observed in that study vs ours could be due to the nature of the cell populations studied, as well as the fact that freshly isolated monocytes are likely to have residual bound IgG, which can alter modulation kinetics. Modulation of FcγRI in our studies was not accompanied by modulation of two other surface molecules, FcγRII or class I (Ref. 31 and data not shown), which do not bind anti-FcγRI constructs. This is in accordance with modulation studies by Wallace et al. (24), which demonstrated the specificity of H22 for FcγRI. Furthermore, FcγRI modulation in our studies was not blocked by Abs to irrelevant cell surface receptors, FcγRII or CD11b (data not shown), suggesting that those two molecules did not take part in anti-FcγRI-mediated modulation of FcγRI.

Reduction of the surface expression of FcγRI may be due to internalization or to other events, such as shedding. We looked at internalization using confocal microscopy and found that internalization of both wH22xeGFP and receptor correlated with the receptor modulation that was observed in 4-h flow assays. Similar results were obtained in 1-h assays (data not shown). This suggests that the receptor is not rapidly recycling to the surface after the targeted fusion protein is delivered into the cell. Shedding of FcγRI is an unlikely cause of receptor modulation, as cells treated for 30 min with wH22xeGFP on ice, which showed no modulation, had similar levels of green fluorescence as cells treated at 37°C, which had ~50% reduction in surface FcγRI (data not shown). Because the wH22xeGFP fluorescence can be detected even if it has been internalized, similar levels of green fluorescence between control and modulated samples indicate that little, if any, modulator has been lost from the cell. This finding is not consistent with receptor shedding.

Because high concentrations of IgG are present in vivo, we performed modulation assays in the presence of ligand to get a sense of how these modulators will behave under normal physiologic conditions. In the presence of IgG, H22 and wH22xeGFP both modulated FcγRI at high concentrations of modulator, concentrations at which modulation was lower or did not occur in the absence of ligand. Confocal microscopy experiments confirmed the internalization of FcγRI under conditions where modulation was observed. Because high concentrations of modulator may favor monovalent binding to FcγRI, cross-linking and subsequent internalization will not occur. The addition of ligand has been shown by others to be required for receptor recycling (25). Therefore, we speculate that the addition of ligand in our system allows for the trafficking of FcγRI into the cell, and the bound modulator prevents recycling of the receptor back to the surface, leading to a net decrease in surface FcγRI expression. Alternatively, ligand binding may enhance the ability of the modulator to bind to the receptor, thereby increasing the efficiency of FcγRI internalization and

Table I. *Relative FcγRI expression on cell lines*[a]

Cell Line	Mean MFI	SD
U937	202	8.8
10.6	436	69.4
U937 + IFN-γ	948	35.6
10.6 + IFN-γ	1715	61.7

[a] FcγRI expression was determined by mAb 32.2, followed by R-PE-conjugated goat anti-mouse IgG. Mean MFI values and SD for quadruplicate samples are shown. ANOVA and Tukey-Kramer multiple comparisons post test p values were all highly significant ($p < 0.0001$).

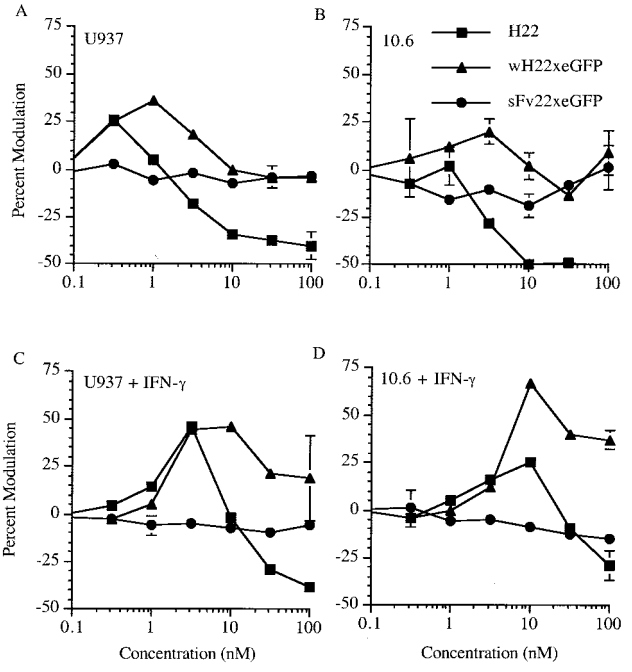

FIGURE 7. Modulation of FcγRI by H22 and wH22xeGFP is concentration dependent and varies with FcγRI expression. Cells were cultured in the presence or absence of 25 ng/ml IFN-γ for 3 days and then used in a modulation assay. Cells were incubated with modulator for 16 h at 37°C, and FcγRI levels were determined by mAb 32.2 followed by R-PE-conjugated goat anti-mouse IgG. Mean modulation ± SD of duplicate samples is based on control cells incubated in the absence of modulator, as described in *Materials and Methods*. Graphs were generated from one experiment representative of at least three similar studies. *A*, U937 cells. *B*, 10.6 cells. *C*, IFN-γ-treated U937 cells. *D*, IFN-γ-treated 10.6 cells.

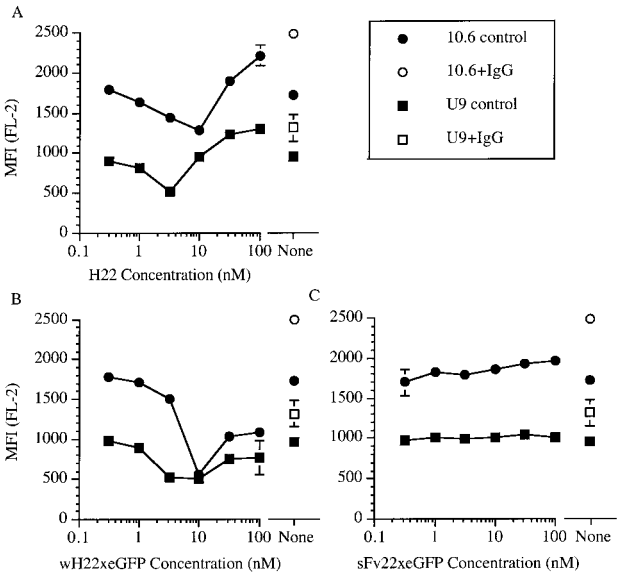

FIGURE 8. Comparison of FcγRI levels following H22, wH22xeGFP, and sFv22xeGFP treatment on cells expressing different levels of FcγRI. IFN-γ-treated 10.6 cells (circles) or IFN-γ-treated U937 cells (squares) were incubated with modulator for 16 h at 37°C/5% CO_2, and FcγRI levels were determined by mAb 32.2 followed by R-PE-conjugated goat anti-mouse IgG. Scatter symbols represent control unmodulated samples in the absence (filled symbols) or presence (open symbols) of 3 mg/ml IgG. Mean values of duplicate samples ± SD are plotted. Data are representative of at least three similar experiments. *A*, FcγRI expression following treatment with H22. *B*, FcγRI expression following treatment with wH22xeGFP. *C*, FcγRI expression following treatment with sFv22xeGFP.

modulation. Ligand was also shown to affect the ability of monovalently and divalently targeted fusion proteins to modulate FcγRI. In the absence of ligand, neither MDX-H210, a bispecific Ab which binds FcγRI via a Fab′ of H22, nor H22 F(ab′)$_2$, was able to modulate FcγRI. However, when ligand was present, FcγRI modulation was observed. Moreover, confocal analysis revealed that although FcγRI distribution for control cells treated with IgG was similar to control cells in the absence of ligand, suggesting there was no internalization of receptor due to immune aggregates that might be present in the IgG (data not shown), monovalently targeted sFv22xeGFP was internalized in the presence, but not absence, of IgG. Therefore, it is possible that even non-cross-linking Abs can sufficiently interrupt ligand-induced receptor recycling, such that receptors are internalized, but not re-expressed, on the cell surface.

The influence of ligand on FcγRI function has been explored in many ways. Studies by Pfefferkorn et al. (38, 39) have shown that signaling events triggered by cross-linking FcγRI can be altered by ligand. In both U937 cells and human monocytes, FcγRI cross-linking triggered significantly greater respiratory burst (O_2^-) in the presence of ligand. This effect was demonstrated with multiple ligands shown to bind human FcγRI, including human, mouse, rabbit, and rat isotypes, and did not occur with isotypes which fail to bind FcγRI (38). In addition, when FcγRI was cross-linked in the presence of human IgG1, increased phosphorylation of FcγRI-associated γ-chain was observed (39). In other studies by Harrison et al. (25), the Fab of anti-FcγRI mAb 32.2 was able to internalize via receptor recycling in U937 cells in the presence, but not absence, of IgG. However, in their study primaquine was required to visualize internalized FcγRI, as the Fab and the receptor recycled back out to the surface in the absence of this drug. The authors proposed that bound ligand permits FcγRI to recycle by causing dissociation of FcγRI from a cytoskeletal actin-binding protein which, in the absence of IgG, prevents the recycling of FcγRI to the surface (25, 40). Our observation that sFv22xeGFP can be internalized in the presence of ligand is consistent with this model, although we were able to visualize the internalized eGFP without the use of primaquine.

We observed that in the absence of IgG, H22 modulated FcγRI only at certain optimal concentrations, but not at 10-fold higher or lower concentrations. These patterns were observed in both 4- and 16-h assays, although modulation percentages were generally slightly higher in overnight assays. We considered the following model: For a given cell line with a certain number of FcγRI molecules, if few H22 molecules are present per cell (leading to a relatively low molar ratio of H22:FcγRI), extensive trivalent cross-linking (and therefore FcγRI modulation) does not occur. Alternatively, cross-linking may occur by the few H22 molecules present, but to a low enough level that the overall cell surface expression does not change significantly. In contrast, if an excess of H22 molecules is present (leading to a relatively high molar ratio of H22:FcγRI), monovalent binding is favored and receptor cross-linking does not occur. However, there exists an optimal concentration of H22 which binds trivalently, causing cross-linking and receptor modulation. If our hypothesis is correct, the concentration of modulator required to maximally modulate FcγRI should increase when a cell line with higher levels of FcγRI is used. Therefore, we compared two different U937 cell lines, U937 cells and the 10.6 high FcγRI-expressing subclone, cultured in the presence or absence of IFN-γ, a cytokine known to up-regulate FcγRI expression. We reasoned that if very few receptors exist on the surface of a cell, the probability of forming a trivalent complex will be low because the receptors are spatially too distant for efficient cross-linking within the ≤16-h treatment times used in these assays. With greater receptor numbers, either as a function of a high-FcγRI-expressing clone or IFN-γ treatment, a higher concentration of modulator would be required to maintain the optimal modulator:receptor molar ratio for maximal FcγRI modulation. Indeed, in the absence of ligand, we found that a higher concentration of modulator (H22 or wH22xeGFP) was required to optimally reduce surface FcγRI levels on cells expressing a higher receptor number. Incidentally, we found that the concentration of H22 required to maximally reduce surface expression of FcγRI was similar to that described by Wallace et al. (24) for human monocytes, which express similar levels of surface FcγRI (reviewed in Ref. 1). Although we used IFN-γ treatment to up-regulate surface FcγRI on U937 cells, it is possible that this cytokine has other effects that may influence modulation, such as altering the FcγRI α- and γ-chain association (41). The effects of anti-FcγRI Abs and fusion proteins on receptor modulation described for these cells may therefore be more characteristic of an IFN-γ-stimulated monocyte.

We observed that treatment with wH22xeGFP led to greater modulation than H22 in most cases and could modulate at concentrations up to 10-fold higher than H22. The optimal concentration for modulation was slightly higher (~half-log) for wH22xeGFP than for H22. This difference may be attributable to a lower affinity of wH22xeGFP for FcγRI, which has been observed in other studies (data not shown). Interestingly, high concentrations of H22 frequently led to "negative modulation." We believe this increased level of surface FcγRI is due to the functionally monovalent binding of H22, which mimics the natural ligand in its ability to increase receptor expression. This phenomenon may be due to the ability of ligand to prevent receptor shedding or enhance the binding of detection Abs. For instance, in the absence of ligand, some FcγRI molecules may localize in packed microdomains on the cell surface, which are less susceptible to the

detection Ab, whereas IgG could cause dispersion of the receptors, making them more readily detectable.

An important finding in the current studies is that, despite apparently lower affinity, wH22xeGFP generally led to faster and greater levels of FcγRI modulation than H22, suggesting that the addition of an Ag to H22 can alter the binding and cross-linking characteristics of the mAb. It is therefore important to assess each FcγRI-targeted Ag for its ability to modulate and internalize, because the binding and cross-linking properties of targeted Ags may play a role in the strength of an immune response. For example, Ags which cause more complete modulation would be expected to result in a larger antigenic load and increased Ag presentation per mmol of Ag. Our studies suggest that wH22xeGFP-mediated FcγRI modulation may be similar to that of 197, a mouse IgG2a mAb against FcγRI shown to be more effective in modulating FcγRI than H22 in the absence of ligand (27). One possible explanation for the observed differences between H22- and wH22xeGFP-mediated modulation and internalization is differences in the accessibility of the H22-binding site of FcγRI to each Ab, once the Fc portion is bound. For example, at a given concentration, each H22 molecule may favor binding to two FcγRI molecules: the Fc end and one Fab end to one receptor and the other Fab end to a second receptor. However, the addition of eGFP moieties to the C-terminal ends of the H chains may contribute enough steric hindrance that binding of wH22xeGFP to the ligand-binding domain and the H22-binding domain of the same FcR is limited or impossible. Therefore, wH22xeGFP may favor the binding of three FcR: one by the Fc end and two by the Fab ends. The latter conformation would favor more extensive and rapid cross-linking of FcγRI. Indeed, using R-PE-labeled polyclonal anti-human IgG as detection, we have observed higher binding with H22 than with equimolar concentrations of wH22xeGFP (data not shown), supporting such a model.

Two mechanisms of FcγRI internalization have been proposed by Harrison et al. (25): 1) binding of monovalent ligand causes rapid internalization and recycling of FcγRI back to the cell surface via an endosomal pathway; and 2) binding of an FcγRI cross-linker in the presence of ligand causes rapid internalization and retention of the receptor inside the cell, most likely diverting it to a lysosomal pathway. Our receptor modulation and internalization data with monovalent sFv22xeGFP and with multivalent H22 and wH22xeGFP are consistent with these respective models. Furthermore, we have shown that exogenous IgG was not required for the internalization and modulation that results from cross-linking FcγRI using H22 and wH22xeGFP, most likely because these constructs themselves will bind inside as well as outside the ligand-binding domain of FcγRI.

In summary, we have demonstrated that an anti-FcγRI Ab (H22) and an FcγRI-targeted fusion protein (wH22xeGFP) capable of cross-linking FcγRI internalized rapidly, along with the receptor, leading to modulation of surface FcγRI. The specific nature of internalization and modulation was dependent on the level of FcγRI expression on the cell and the concentration of modulator, and was altered by the presence of ligand. A monovalent version of the targeted fusion protein, sFv22xeGFP, was unable to modulate surface FcγRI but could internalize if ligand was present. These data, taken together with those of others, suggest that FcγRI-targeted fusion proteins that cross-link FcγRI are internalized and enter an intracellular pathway different from those which do not cross-link FcγRI. This may reflect the in vivo Ag sampling that occurs by FcR cross-linking and FcR-mediated endocytosis, respectively. Our studies suggest that under physiologic conditions, FcγRI has the capacity to mediate the uptake of a large antigenic load, carried into the cell by 40–80% of surface FcγRI. Therefore, a diverse array of Ags can be sampled, processed, and presented, potentially leading to an effective immune stimulation. The therapeutic implications of this work are twofold: 1) the optimization of FcR modulation may lead to better therapeutic strategies for FcγR-mediated autoimmune diseases such as immune thrombocytopenia purpura; and 2) the successful delivery of Ag into the appropriate processing pathways of APCs via FcγRI targeting may lead to more effective Ag presentation and subsequent immune response to foreign invaders or cancer. We have recently demonstrated the intracellular colocalization of FcγRI-targeted Ag with MHC class I (31) and believe that the pathway for internalization of FcγRI-targeted eGFP will be the same as for those FcγRI-targeted fusion proteins being developed as vaccines for cancer, AIDS, and other diseases (14, 16, 17, 19–21). Moreover, it has been shown that FcγRI-targeted Ag can be presented in the context of class I (15) as well as class II (13, 14). These fusion proteins may serve as useful tools in helping to elucidate how FcγRI-targeted Ags get processed inside the cell such that presentation occurs via both class I and class II MHC.

Acknowledgments

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