Antigen Targeting to Myeloid-specific Human FcγRI/CD64 Triggers Enhanced Antibody Responses in Transgenic Mice

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Abstract

Besides their phagocytic effector functions, myeloid cells have an essential role as accessory cells in the induction of optimal humoral immune responses by presenting captured antigens and activating lymphocytes. Antigen presentation by human monocytes was recently found to be enhanced in vitro through the high-affinity Fc receptor for IgG (FcyRI; CD64), which is exclusively present on myeloid cells. To evaluate a comparable role of FcyRI in antigen presentation in vivo, we generated human FcyRI transgenic mice. Under control of its endogenous promoter, human FcyRI was selectively expressed on murine myeloid cells at physiological expression levels. As in humans, expression was properly regulated by the cytokines IFN- γ , G-CSF, IL-4, and IL-10, and was up-regulated during inflammation. The human receptor expressed by murine macrophages bound monomeric human IgG and mediated particle phagocytosis and IgG complex internalization. To evaluate whether specific targeting of antigens to FcyRI can induce enhanced antibody responses, mice were immunized with an antihuman FcyRI antibody containing antigenic determinants. Transgenic mice produced antigen-specific antibody responses with high IgG1 titers and substantial IgG2a and IgG2b responses. These data demonstrate that human FcyRI on myeloid cells is highly active in mediating enhanced antigen presentation in vivo, and show that anti-FcyRI mAbs are promising vaccine adjuvants. (J. Clin. Invest. 1996. 97:331-338.) Key words: antigen presentation • Fc receptor • IgG • phagocytosis • adjuvant

Introduction

There is now abundant evidence for a major role of receptors for the Fc domain of IgG (Fc γ R) in the immune defense against infectious agents. The class I Fc γ R (CD64) represents a unique member among the broadly distributed and heterogeneous Fc γ R family. Fc γ RI is the sole receptor that binds

J. Clin. Invest. © The American Society for Clinical Investigation, Inc. 0021-9738/96/01/331/08 \$2.00 Volume 97, Number 2, January 1996, 331–338 with high affinity both monomeric and immune-complexed IgG, and its expression is restricted to cells of the myeloid lineage. Mononuclear phagocytes constitutively express FcyRI, whereas low numbers of receptors are found on polymorphonuclear neutrophils (PMNs).¹ Human (h) FcyRI expression can be enhanced by exposure to IFN-y, where PMNs respond most dramatically (1, 2). Recently, a number of other cytokines that are produced during infection and inflammation (i.e., G-CSF, IL-4, IL-10, and IL-13) have been shown to regulate FcyRI expression on human monocytes and PMN (3-7). FcyRI on these cells serves as a potent trigger molecule that initiates a variety of biological effector functions, including immune complex internalization and phagocytosis of opsonized particles (1, 2, 8). Recent in vitro studies indicated that FcyRI can also mediate enhanced antigen presentation through internalization of antigen complexed with IgG (9). Moreover, antigen conjugated to FcyRI-specific antibodies, thus targeted to this receptor, markedly enhanced T cell activation in vitro (9). Targeting of antigen to FcyRI in vivo, however, has not been reported.

Mononuclear phagocytes are critical antigen presenting cells (APCs) during the initiation of specific humoral immune responses. APCs capture and internalize antigen and provide costimulatory signals for activation of T cells, which are then, in their turn, capable of stimulating production of antibodyforming cells. A major drawback in the use of antigens for vaccine purposes is the requirement for potent adjuvants to elicit effective antibody responses. Recent advances have indicated that vaccine adjuvants that have in general been established empirically appear to facilitate the APC-mediated events (10). In light of these observations, it has been proposed that FcyRI-mediated enhancement of antigen presentation by myeloid cells is physiologically relevant (9). As a consequence, targeting of antigen to FcyRI potentially constitutes an effective strategy for augmentation of antigen-specific antibody responses. Here, we describe experiments aimed at validating the concept of hFcyRI targeting in vivo by using a novel transgenic mouse model. We show that hFcyRI in this model closely resembles the situation in humans with respect to its myeloid cell-specific expression, regulation by cytokines, and functioning in internalization. Our findings demonstrate that the transgenic model is suitable for analyses of the physiological role of FcyRI in immune responses in vivo and show that targeting of antigen to hFcyRI triggers enhanced antibody responses in transgenic mice.

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^{1.} *Abbreviations used in this paper:* APC, antigen presenting cell; BsAb, bispecific antibody; GRR, IFN- γ response region; hFc γ R, human IgG Fc receptor; MATE, myeloid-activating transcription element; OE, ox erythrocyte; PMN, polymorphonuclear neutrophil.

Methods

Generation of transgenic mice. The DNA construct used to produce transgenic mice was prepared from human leukocyte genomic DNA λ -FIX clones containing the hFcγRIA gene (11). DNA was digested with SalI, and an 18-kb linear fragment encompassing the entire 9.4-kb coding region of the FcγRIA gene as well as 9 kb of 5' up- and 3' downstream flanking material (Fig. 1 *A*) was isolated. This fragment was purified by electroelution, dissolved in 10 mM Tris, pH 7.4, 0.1 mM EDTA, and microinjected (4 µg/ml) into pronuclei of fertilized oocytes from FVB/N (12) mice. Transgenic founders were mated with FVB/N mice, and hemizygous transgenic offspring were routinely identified by analyzing peripheral blood monocytes for hFcγRI expression using human CD64 mAbs and the flow cytometer (see below).

Southern and Northern blot analyses. Genomic DNA was prepared from tail biopsies as described (13). Serially diluted DNA samples were digested with HindIII, blotted to Qiabrane nylon plus filters (Qiagen, Hilden, Germany), and probed with a ³²P-labeled 1.1-kb hFcγRI cDNA, p135 (14). Copy numbers of the transgene were determined by quantitating intensity of bands using ImageQuant PhosphorImager software (Molecular Dynamics, Inc., Sunnyvale, CA), using HindIII-digested human genomic DNA as a reference.

Total RNA was extracted from various mouse tissue samples homogenized with a tissue grinder (Ultra Turrax; Janke & Kunkel, Staufen, Germany), by using the RNAzol B method (Biotecx, Friendswood, TX) (3, 4). Some mice received two doses of recombinant rat IFN- γ (2 × 10⁵ U/dose) (provided by Dr. P. van der Meide; TNO, Rijswijk, The Netherlands), administered intravenously in 200 µl sterile PBS 48 and 24 h before tissue collection and RNA isolation. Upon isolation, RNA was fractionated by electrophoresis in 1% agarose/formaldehyde gels, transferred to Qiabrane nylon filters, and hybridized with the ³²P-labeled p135 cDNA probe.

Antibodies. Anti-human FcyRI mAbs 22, 32.2, and 197, and anti-human FcyRIII mAb 3G8, were provided by Medarex, Inc. (Annandale, NJ). Anti-mouse FcyRII/III mAb 2.4G2 and anti-Gr-1 mAb RB6-8C5 were obtained from PharMingen (San Diego, CA). Anti-Mac-1 mAb (M1/70) was purchased from Boehringer Mannheim (Mannheim, Germany), and anti-F4/80 from Serotec Ltd. (Kidlington, Oxford, UK). Anti-CD45R/B220 (RA3-6B2), anti-CD3 (145-2C11), anti-CD4 (RM4-5), anti-CD8 (53-6.7), and anti-TCR-α/β (H57-597) were used as hybridoma supernatants. mAb H22 is a humanized mAb directed against hFcyRI (the construction of this mAb is extensively described in reference 14a). This antibody was genetically engineered by Scotgen Biopharmaceuticals Ltd. (Aberdeen, Scotland, UK) using the antigen-binding loop (CDR) transplantation technique (15) and was produced by Medarex, Inc. The humanized antibody consists of the six CDRs derived of mouse mAb 22 (16) grafted onto a human IgG1k antibody framework, and retains high affinity for human FcyRI. Antibody SB82 (generously provided by Scotgen Biopharmaceuticals Ltd.) is a comparable humanized IgG1k mAb but with different CDRs and no reactivity with human or mouse antigens.

Flow cytometry. Single-cell suspensions of $(0.5-2 \times 10^5)$ thymocytes, spleen cells, resting peritoneal macrophages, bone marrow cells, or peripheral whole blood samples (25 µl) were stained with FITC-, PE-, and/or biotin-conjugated mAbs in PBS containing 2.5% FCS and 0.05% sodium azide, and analyzed on a FACScan[®] (Becton Dickinson & Co., San Jose, CA). Streptavidin-PE (Becton Dickinson & Co.) was used to detect biotinylated mAbs. Whole blood samples were subjected to FACS[®] lysing solution (Becton Dickinson & Co.) treatment after staining to lyse red blood cells and fix white cells, before flow cytometric analysis. In several experiments, mice received subcutaneous injections of rat IFN- γ (2 × 10⁵ U per mouse) or human G-CSF (50 µg per mouse) (Neupogen[®]; generously provided by Amgen, Inc., Thousand Oaks, CA) in sterile saline 24 h before peripheral blood collection. Control mice were injected subcutaneously with saline alone.

NA washed, resuspended in DMEM (GIBCO BRL, Grand Island, NY) supplemented with 4.5 g/liter glucose, 10% (vol/vol) FCS, and 50 μ g/ ml streptomycin, and cultured at a concentration of 5 × 10⁵ cells/ml at 37°C. After 3 h, nonadherent cells were removed by washing of monolayers with medium. To investigate the effects of cytokines on hFcγRI expression, purified macrophages were cultured for 22 h at 37°C in the presence or absence of recombinant mouse IFN-γ (250 U/ml) (Amgen, Inc.), mouse IL-4 (30 ng/ml), or mouse IL-10 (15 ng/ml) both from R&D Systems (Abingdon, UK). *Phagocytosis and internalization assays.* Uptake of particles by macrophages was assayed by using ox erythrocytes (OE) (BioTrading. Mi/drecht. The Netherlands) as targets coated with bispecific an-

macrophages was assayed by using ox erythrocytes (OE) (Bio Irading, Mijdrecht, The Netherlands) as targets coated with bispecific antibodies (BsAbs) $[F(ab')_2 \times F(ab')_2] 22 \times$ anti-OE or $3G8 \times$ anti-OE (generous gifts of Dr. L. Shen, Dartmouth Medical School, Lebanon, NH) (8). Sensitized targets (1×10^7) were added to macrophage monolayers $(2.5 \times 10^5$ cells) cultured in 24-well plates as described above. Cells were incubated for 30 min either at 4°C to determine binding or at 37°C to assess phagocytic activity. Unbound erythrocytes were removed by washing with PBS, and cells were fixed in 0.25% glutaraldehyde. Cells that had been incubated at 37°C were rinsed with distilled water for 30 s before fixation to lyse extracellularly bound erythrocytes.

Cell culture. Exudate cells were harvested from the peritoneal cavity of mice 4 d after intraperitoneal injection with 1 ml of 5% thioglycollate (Difco Laboratories, Inc., Detroit, MI). Cells were

Receptor internalization was assayed on peritoneal exudate cells, incubated in RPMI 1640 (GIBCO BRL) containing 5% FCS in polypropylene tubes on ice. Cells were incubated with one of the following antibodies: 22, 197, H22, or SB82 (5 μ g/ml), or with heataggregated human IgG (15 μ g/ml) (CLB, Amsterdam, The Netherlands). After 45 min at 4°C, cells were washed and temperature was shifted to 37°C for various times. Cells were then washed in ice-cold medium, the amount of mouse or human IgG remaining at the surface of cells was detected by FITC-labeled goat F(ab')₂ fragments to mouse or human IgG (Cappel Laboratories, Durham, NC), and cells were analyzed by flow cytometry. The percentage of antibody complexes internalized by monocytes/macrophages was calculated as described previously (17).

Immunizations and ELISA. 8–12-wk-old mice were immunized subcutaneously in the base of the tail with either mAb H22 or SB82 (100 μ g per mouse) diluted in sterile saline. After 6 wk, mice were reimmunized subcutaneously with the same antigen (100 μ g per mouse). 10 d after the last immunization, sera were collected and subjected to ELISA to quantitate antigen-specific antibody responses. Immunoplates (Maxisorp, Nunc, Roskilde, Denmark) were coated with H22 or SB82 (5 μ g/ml) in PBS overnight at 4°C and were then blocked with 2.5% BSA. After wells were washed with PBS containing 0.05% Tween 20, serially diluted sera were incubated for 2 h at 37°C. Plates were washed, and a panel of alkaline phosphataselabeled mouse isotype-specific antibodies (Southern Biotechnology Associates, Inc., Birmingham, AL) was added and incubated for 1 h at 37°C. Enzyme activity was revealed with a phosphatase substrate system (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

Results

Expression of hFc γ RI by myeloid cells of transgenic mice. Recent studies have defined several key regulatory motifs within the promoter region of the hFc γ RIA gene, including an IFN- γ response region (GRR) (18) and a more downstream myeloid cell-activating transcription element (MATE) responsible for myeloid cell-restricted expression (19). A sequence homologous to this MATE motif is also present in the promoter region of mouse Fc γ RI (20). It has recently been shown that promoters of myeloid-specific genes containing similar motifs were capable of driving correct cell type–specific expression of

reporter genes in transgenic mice (21, 22). Regulatory elements within the hFcyRIA promoter were, therefore, expected to target gene expression in myeloid cells of transgenic mice. A human genomic fragment encompassing the FcyRIA gene (11) under control of its own intact promoter, including GRR and MATE motifs, served as a transgenic construct (Fig. 1 A). Two stable lines of transgenic mice, designated 52 and 53, were established. Copy numbers of the transgene in lines 52 and 53 were estimated at 6-7 and 2-3, respectively, by quantitative Southern blot analyses (Fig. 1 B). Tissue-specific transgene transcription was examined by Northern analysis of total cellular RNA prepared from a variety of mouse tissue samples. As shown in Fig. 1 C (upper panel) for an animal from line 53, high levels of hFcyRI mRNA were detected in peritoneal macrophages and lungs, and lower levels in heart and brain. Minor amounts of transgene RNA were found in bone marrow, kidney, and spleen, whereas thymus, skeletal muscle, and liver contained no detectable levels. Transgenic line 52 showed an identical tissue distribution of hFcyRI transcripts, albeit with higher steady state expression levels. We noticed that this pattern could reflect the presence of myeloid cells within these organs.

Flow cytometry confirmed a cell type-specific expression profile of transgenic proteins. Transgenic peritoneal macrophages expressed high levels of cell surface hFcyRI (Fig. 2A). The human receptor was recognized by a panel of human CD64 mAbs (22, 32.2, and 197) reacting with different epitopes (17), suggesting that the receptor was expressed in proper conformation. Transgenic macrophages bound human IgG and, when analyzed by flow cytometry, showed profiles similar to those presented in Fig. 2 A. Under identical conditions, nontransgenic macrophages did not bind human IgG (5 µg/ml IgG). hFcyRI was, furthermore, expressed at intermediate levels on peripheral blood monocytes and at low lev-

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els on neutrophils (Fig. 2A). Lymphocytes did not express detectable hFcyRI (Fig. 2 A). No cross-reactive staining of human CD64 mAbs was seen on cells from nontransgenic mice tested in parallel (Fig. 2A). Expression profiles were identical in both transgenic lines, even though line 52 expressed higher cell surface receptor levels. Counterstaining of resting peritoneal macrophages with the anti-mouse FcyRII/III mAb 2.4G2 or the macrophage marker Mac-1 (CD11b/CD18) vielded interesting results. Expression levels of Mac-1 integrin was identical in transgenic and control mice. The level of mouse FcyRII/III, however, was consistently lower in transgenic animals (Fig. 2 B). No differences were seen in 2.4G2 staining of B cells between transgenic and control littermates (data not shown). These data suggested that the presence of hFcyRI affects mouse FcyR surface expression.

Regulation of hFcyRI expression by cytokines. To determine whether the regulatory regions of the transgene (e.g., GRR) act properly in the heterologous mouse system, we tested effects of cytokines with well-known influences on hFcyRI expression. First, we treated mice with IFN-y and analyzed transgene RNA expression. As seen in Fig. 1 C (middle panel), hFcyRI mRNA production was enhanced by IFN-y. In accordance with these data, cell surface expression of the transgenic receptor was dramatically increased on peripheral blood neutrophils (Fig. 3 B) and, less dramatically, on peritoneal macrophages (not shown) of IFN-y-treated transgenic mice.

Another cytokine that triggers increased FcyRI expression on human neutrophils during therapy in vivo is G-CSF(3, 4). Upon injection of this cytokine in mice, we observed, as expected, an increase in peripheral blood neutrophil counts (\sim 45% of total white blood cells; n = 3) compared with salinetreated animals (\sim 12%). Moreover, expression of hFcyRI was strongly up-regulated on circulating neutrophils of G-CSF-

> Figure 1. Transgenic mice expressing human $Fc\gamma RI.$ (A) Structure of the transgene consisting of an 18-kb human genomic DNA fragment carrying the FcyRIA gene locus (11). Exons are represented by closed boxes. GRR and MATE indicate locations of regulatory motifs within the promoter region (see Results). S1,2, signal sequences; EC1-3, extracellular domains; TM/C, transmembrane and cytoplasmic region; H, HindIII; S, SalI. (B) Southern blot analysis of hFcyRI-transgenic mice. Equal amounts (8 µg) of genomic DNA from human white blood cells (lane 1), transgenic lines 52 (lane 2) and 53 (lane 3), and nontransgenic (NTg) mice (lane 4) were digested with HindIII and hybridized with hFcyRI cDNA probe p135. The arrowhead on the right indicates the position of a mouse endogenous DNA fragment cross-hybridizing with p135. Sizes of DNA fragments are indicated. (C) Analysis of transgene RNA expression in tissues isolated from transgenic line 53 (upper panel, lanes 1-10), IFN- γ -treated transgenic line 53 (middle panel, lanes 1-10), and nontransgenic littermates (lanes 11). Treated mice were injected with recombinant rat IFN- γ (2 × 10⁵ U) 48 and 24 h before RNA isolation. Ethidium bromide staining patterns (lower panel) served as control for equivalent loading of lanes. Positions of the 28S and 18S ribosomal bands are indicated on the left.



treated transgenic mice (Fig. 3 *B*). In agreement with the findings obtained in humans (4), the observed G-CSF-triggered expression was transient. 4 d after G-CSF injection, $hFc\gamma RI$ expression levels on transgenic neutrophils were back to baseline.

In addition to IFN- γ and G-CSF, several other cytokines are known to regulate Fc γ RI expression on human monocytes in vitro (5, 6). Thioglycollate-elicited macrophages were cultured in the presence of IL-4, IL-10, or IFN- γ for 22 h. Expression of hFc γ RI was found to be up-regulated by both IL-10 and IFN- γ , whereas incubation with IL-4 led to decreased cell surface expression (Fig. 3 *A*), similar to that observed on cultured human monocytes. Together, these data indicate that the hFc γ RI transgene contains control elements relevant for specific cytokine regulation. This was further supported by an in vivo observation. It was noted that aggressive behavior in male

Figure 2. Flow cytometric analysis of hFcyRI cell surface expression. (A) Peripheral blood leukocytes and peritoneal macrophages of transgenic line 53 (solid lines) and nontransgenic mice (dotted lines), were stained with anti-hFcyRI mAb 22-FITC. Cells were stained with PE-labeled anti-CD45R/B220 and anti-TCR- α/β to define lymphocytes, and with anti-Gr-1 or anti-F4/80 to define neutrophils and monocytes, respectively. Macrophage expression was determined based on Mac-1 staining. (B) Expression of mouse FcyRII/III and Mac-1 on peritoneal macrophages of transgenic line 52 and control mice. Cells were stained with mAb 32.2-FITC (anti-hFcyRI) and either with 2.4G2-PE (anti-mouse FcyRII/III) or with Mac-1-PE. Macrophages expressing hFcyRI showed a reduced 2.4G2 staining (solid line) compared with controls (dotted line).

mice incidentally resulted in wound development (Fig. 3 *C*). Upon flow cytometric analysis of peripheral blood leukocytes from affected mice, we observed a considerable increase in the proportion of circulating neutrophils with concomitant changes in light-scatter profiles (Fig. 3 *C*; *lower panels*, gated cells), consistent with inflammatory responses in these mice. Interestingly, inflammatory neutrophils of transgenic mice expressed increased levels of hFc γ RI (Fig. 3 *C*). When lesions on the skin were healed, blood staining profiles returned to normal (not shown).

Internalization and phagocytosis mediated by $hFc\gamma RI$. To evaluate whether $hFc\gamma RI$ was functional in transgenic animals, we assessed the ability of the transgenic receptor to mediate phagocytosis of OE by peritoneal macrophages. Considering that regular IgG-opsonized particles do not discriminate be-



Figure 3. Regulation of hFcyRI expression in transgenic mice. (A) Effects of cytokines on hFcyRI cell surface expression on cultured macrophages. Thioglycollate-elicited peritoneal macrophages of line 52 transgenic mice were cultured with murine IL-4 (30 ng/ ml), IL-10 (15 ng/ml), or IFN-γ (250 U/ ml) for 22 h. Expression of hFcyRI was analyzed by flow cytometry using mAb 22-FITC (bold lines) and compared with cells cultured in medium alone (thin lines). One representative experiment out of three is shown. (B) $hFc\gamma RI$ expression on peripheral blood neutrophils upon in vivo treatment of line 53 transgenic mice with either G-CSF or IFN- γ . Mice were injected with a single dose of human G-CSF (50 µg per mouse) or rat IFN- γ (2 × 10⁵ U per mouse). Expression of hFcyRI on neutrophils was analyzed by mAb 22-FITC staining (bold lines) and compared with expression profiles of blood neutrophils isolated from saline-treated transgenic mice (thin lines). (C) Analysis of peripheral blood leukocytes of normal (mouse a) and inflamed (mouse b; inflammatory site marked by arrowhead) line 53 transgenic mice. Forward (FSC) versus side scatter (SSC) patterns and hFcyRI expression profiles of blood neutrophils from both animals are depicted.

tween transgenic and endogenous mouse $Fc\gamma R$, we used an alternative method to opsonize particles. BsAbs were used to direct the OE specifically to hFc γ RI (8). The anti-hFc γ RI × anti-OE BsAb comprises mAb 22, which is reactive with an epitope of hFc γ RI outside the ligand binding site yet is capable of triggering hFc γ RI-mediated functions (23). Macrophages from transgenic mice avidly bound OE coated with this BsAb (Fig. 4 *A*). Moreover, these bound particles were effectively internalized upon incubation of cells at 37°C (Fig. 4 *B*). Transgenic macrophages neither bound nor phagocytosed particles coated with a control BsAb (anti-hFc γ RIII [mAb 3G8] × anti-OE) (Fig. 4 *C*). Similarly, macrophages of nontransgenic littermates failed to bind anti-hFc γ R–directed particles (data not shown). In addition, we found transgenic macrophages to internalize nonparticulate aggregated human IgG, reaching a plateau after 15 min (Fig. 5). Under identical conditions, nontransgenic macrophages did not bind human IgG (data not shown), indicating that hFcγRI mediated the observed internalization in transgenic cells. Next, specific anti-CD64 mAbs were used to cross-link receptors and promote internalization. Antibody 197 (mouse IgG2a) is known to cross-link hFcγRI into a multireceptor complex by binding the receptor both by its Fab as well as via its Fc regions, thereby inducing receptor internalization (24). mAb 22 (mouse IgG1) recognizes hFcγRI solely by its Fab region and, thus, does not cross-link FcγRI nor stimulate internalization (24). In agreement with studies on human monocytic cells, transgenic macrophages rapidly internalized



Figure 4. hFc γ RI-mediated phagocytosis of OE by thioglycollate-elicited peritoneal macrophages of line 52 transgenic mice. Binding at 4°C (*A*) and phagocytosis at 37°C (*B*) of OE directed to hFc γ RI via BsAb 22 × anti-OE. In control experiments using OE coated with BsAb 3G8 (anti-hFc γ III) × anti-OE, neither binding nor phagocytosis was observed (*C*). Macrophages of nontransgenic mice were indiscriminable from the pattern shown in *C* regardless of the BsAb used.

> 40% of mAb 197. No significant internalization of mAb 22 was observed (Fig. 5). Finally, internalization of a humanized version of mAb 22 (H22) was tested. This antibody is a human IgG1, a subclass capable of interacting with hFcyRI (1, 2). These characteristics were expected to result in receptor crosslinking similar to mAb 197. Antibody H22 was found to bind with equal reactivity to hFcyRI as the parental mAb 22 (data not shown). In marked contrast to murine mAb 22, humanized mAb H22 was actively internalized by transgenic macrophages upon incubation at 37°C, albeit with different kinetics than mAb 197 (Fig. 5).

Antibody responses to hFcyRI-targeted antigens in transgenic mice. Targeting antigen to FcyRI on human monocytes via mAb 22 results in enhanced T cell activation in vitro (9). This prompted us to investigate whether FcyRI targeting enhances specific humoral immune responses in transgenic mice. Antibody H22, which recognizes hFcyRI despite occupation of the latter with IgG, served as an hFcyRI-targeted antigen. Transgenic mice and nontransgenic littermates were immunized with H22 without further adjuvant, and sera were analyzed by ELISA for anti-H22 antibody responses. All six transgenic mice produced high levels of H22-specific IgG1, and all but one generated significant levels of specific IgG2a and IgG2b antibodies to H22 (Fig. 6). In contrast, none of the six nontransgenic control mice generated detectable levels of either IgG2a or IgG2b antibodies to H22. Three of the six control mice did not produce detectable H22-specific IgG1, and



Figure 5. Internalization of hFc γ RI crosslinking antibodies. Surface Fc γ RI molecules on thioglycollate-elicited peritoneal exudate cells were labeled at 4°C either with heat-aggregated human IgG (\Box), mAb 197 (mIgG2a)

 (\triangle) , murine mAb 22 (mIgG1) (\bigcirc), or with humanized mAb H22 (hIgG1) (\bullet). The amounts of human/murine IgG remaining at the surface of labeled cells was determined by flow cytometry after incubation for different times at 37°C. The percentage of internalized receptor-antibody complexes was calculated exactly as described in reference 17. One representative experiment out of three is shown. the other three controls showed titers ~ 100 -fold lower than those observed in transgenic mice (Fig. 6).

To verify that the observed antibody responses resulted from antigen targeting to hFcγRI and not merely from the presence of the additional Fcγ receptor itself, we immunized mice with a control humanized mAb, SB82. This mAb is of the same isotype as H22 but is devoid of reactivity with mouse/human antigens and, thus, served as a nontargeted antigen. Control mAb SB82 did not induce detectable anti-SB82 IgG2a or IgG2b antibodies in either transgenic or nontransgenic animals (Fig. 6). Three of eight mice immunized with SB82 produced IgG1 antibody responses, even though no gross differences were observed between transgenic and control mice (Fig. 6). Thus, targeting of antigen to hFcγRI expressed on myeloid cells triggers enhanced antigen-specific antibody responses in vivo.

Discussion

Considerable progress has been made in our understanding of the function of $Fc\gamma RI$. Most data, however, were generated in in vitro studies, and it appears pertinent to question whether these functions reflect a role for $Fc\gamma RI$ in vivo. Attempts to evaluate the contribution of $Fc\gamma RI$ to immune responses in



Figure 6. Antigen-specific IgG responses after immunization of mice with either anti-hFc γ RI humanized mAb H22 (*left*) or with control humanized mAb SB82 (*right*) as antigen. 8–10wk-old line 52 transgenic mice (\bullet) and nontransgenic littermates (\bigcirc) were immunized subcutaneously with 100 µg of antigen. 6 wk

later, mice were reimmunized with the same antigen. Antigen-specific IgG1, IgG2a, and IgG2b responses were quantified by ELISA, using serum samples taken 10 d after the second injection. Antibody titers were determined by twofold serial dilutions of sera and are presented for individual mice.

vivo have been hampered by overlapping ligand-binding characteristics of different FcR (2) and lack of mAb reagents for murine FcyRI (25). Obviously, experiments analyzing FcyRI functioning in humans are restricted by ethical considerations. Our strategy to overcome these difficulties was to generate human FcyRI-transgenic mice. This study demonstrates that an 18-kb human genomic DNA fragment contains sufficient regulatory information to target the expression of hFcyRIa exclusively to myeloid cells in transgenic animals. In addition, the relative surface expression levels on heterologous mouse cells were similar to those observed in the human system (1, 2). Expression of hFcyRI was high on mononuclear phagocytes, relatively low on neutrophils, and not detectable on lymphocytes. Macrophages demonstrated higher expression levels than monocytes (Fig. 2 A). The MATE that has been shown to be conserved in promoters of several myeloid-specific genes (19, 26, 27) and that is present in the hFcyRI transgene might, thus, act as a dominant element conferring cell-specific expression. Furthermore, we demonstrated that the regulatory elements in the transgene were sufficient to control responsiveness to several key cytokines, an observation not reported in other transgenic studies investigating elements controlling myeloid gene expression (21, 22). The archetypic macrophage activator IFN- γ increased hFcyRI expression both on RNA and protein levels (Figs. 1 C; 3 A, B). The promoter element responsible for IFN- γ responsiveness (GRR) has been well characterized (18) and is also present in the transgene used. Consistent with recent reports showing that this GRR is a target for multiple cytokines (28, 29), we found G-CSF, IL-4, and IL-10 to regulate properly hFcyRI expression in transgenic mice. PMN hFcyRI was also up-regulated in mice with inflammatory lesions (Fig. 3 C), exactly as is observed in patients with bacterial infections (30). These results confirm that the human receptor is under strict control of endogenous mouse cytokines. Furthermore, we suggest that the presented genetic construct carrying the hFcyRIA gene can be used to direct myeloid-specific gene expression under control of regulatory cytokines.

Previous studies showed IgG Fc receptors to be present on human myeloid progenitors (CD64) (31) and on mouse T cell precursors (CD16/CD32) (32). It has even been suggested that FcyR on the latter cells interact with alternative ligands influencing lymphoid cell development (33). No overt abnormalities in myeloid and lymphoid cell numbers and subsets were observed in hFcyRI-transgenic mice (data not shown), suggesting that the human receptor does not significantly affect myeloid/lymphoid differentiation. The presence of hFcyRI, however, had an apparent effect on expression levels of endogenous mouse FcyR. Macrophages of transgenic mice expressed consistently lower levels of mouse FcyRII/III (Fig. 2 B). As Fc γ RIII is known to depend on the Fc receptor γ chain for surface expression (34), we speculate that competition by hFcyRI for mouse γ chains underlies the lower mouse FcyRII/ III expression on phagocytes. This hypothesis is supported by an observation in FceRI-deficient mice. In these animals, absence of FceRI molecules resulted in an increased availability of FcR γ chain for Fc γ RIII and led to higher Fc γ RIII levels on mast cells (35). Studies in γ chain-deficient mice showed that mouse FcyRI was functionally absent on macrophages, indicating that the γ chain is crucial for functional mouse FcyRI expression (25). Here, functionality of hFcyRI in transgenic mice was examined, and it was found that the receptor (a)bound monomeric human IgG; (b) was internalized upon antibody-induced receptor cross-linking; and (c) mediated efficient phagocytosis of OE coated with anti-hFc γ RI BsAbs. Thus, our findings support the formation of a functional hFc γ receptor complex. Collectively, these data suggest that the presented hFc γ RI-transgenic mouse represents a relevant model for evaluation of Fc γ RI functioning in vivo.

Immunization of mice with an anti-hFcyRI antibody that contains antigenic determinants, and monitoring sera for presence of antigen-specific antibodies, clearly showed that hFcyRI is capable of triggering enhanced antibody responses in transgenic mice. Notably, in addition to the observed mouse IgG1 antibody responses, transgenic mice produced antigen-specific IgG2a and IgG2b (Fig. 6). Production of these subclasses is generally not induced when mice are immunized without exogenous (synthetic) adjuvants (36). Targeting antigens to FcyRI could have important consequences in humans. For instance, BsAbs with one specificity for FcyRI and another for a tumorassociated antigen are currently tested in several phase I studies in breast cancer patients. These BsAbs constitute an approach to lyse and/or phagocytose tumor targets by monocytes and PMN. Analyses of sera showed that some patients had produced tumor-reactive IgG antibody responses 30-60 d after BsAb immunotherapy (Guyre, P. M., unpublished results).

Because $Fc\gamma RI$ has high affinity for monomeric IgG, it has been postulated that this receptor is constitutively occupied in vivo with (serum) IgG of diverse specificities (37). This enables myeloid cells to recognize a wide variety of antigens. Because of its capability to internalize antigens into myeloid APC that have the potential to provide costimulation, we hypothesize that $Fc\gamma RI$ plays a significant role in antigen presentation and, more specifically, in the initiation of humoral immune responses. As a consequence, we speculate that mAbs that react with an epitope of $Fc\gamma RI$ distinct from the occupied ligand binding site may be optimal to enhance such responses and represent novel and safe vaccine adjuvant candidates. The model described in this paper is suitable to exploit this in more detail.

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References

1. Van de Winkel, J. G. J., and P. J. A. Capel. 1993. Human IgG Fc receptors heterogeneity: molecular aspects and clinical implications. *Immunol. To- day*. 14:215–221.

2. Van de Winkel, J. G. J., and C. L. Anderson. 1991. Biology of human immunoglobulin G Fc receptors. *J. Leukocyte Biol.* 49:511–524.

3. Valerius, T., R. Řepp, T. P. M. de Wit, S. Berthold, E. Platzer, J. R. Kalden, M. Gramatzki, and J. G. J. van de Winkel. 1993. Involvement of the high-affinity receptor for IgG ($Fc\gamma RI$; CD64) in enhanced tumor cell cytotoxicity of neutrophils during granulocyte colony-stimulating factor therapy. *Blood*. 82:931–939.

4. Kerst, J. M., J. G. J. van de Winkel, A. H. Evans, M. de Haas, I. C. M. Slaper-Cortenbach, T. P. M. de Wit, A. E. G. Kr. von dem Borne, E. van der Schoot, and R. M. J. van Oers. 1993. Granulocyte colony-stimulating factor induces hFcγRI (CD64 antigen)-positive neutrophils via an effect on myeloid precursor cells. *Blood.* 81:1457–1464.

5. Te Velde, A. A., R. J. F. Huijbens, J. E. de Vries, and C. G. Figdor. 1990. IL-4 decreases $Fc\gamma R$ membrane expression and $Fc\gamma R$ -mediated cytotoxic activity of human monocytes. *J. Immunol.* 144:3046–3051.

6. Te Velde, A. A., R. de Waal Malefijt, R. J. F. Huijbens, J. E. de Vries, and C. G. Figdor. 1992. IL-10 stimulates monocyte FcγR surface expression and cytotoxic activity: distinct regulation of ADCC by IFN-γ, IL-4, and IL-10. *J. Immunol.* 149:4048–4052.

7. De Waal Malefijt, R., C. G. Figdor, R. Huijbens, S. Mohan-Peterson, B. Bennet, J. Culpepper, W. Dang, G. Zurawski, and J. E. de Vries. 1993, Effects of IL-13 on phenotype, cytokine production, and cytotoxic function of human monocytes: comparison with IL-4 and modulation by IFN-γ or IL-10. *J. Immunol.* 151:6370–6381.

8. Anderson, C. L., L. Shen, D. M. Eicher, M. D. Wewers, and J. K. Gill. 1990. Phagocytosis mediated by three distinct Fcγ receptor classes on human leukocytes. *J. Exp. Med.* 171:1333–1346.

9. Gosselin, E. J., K. Wardwell, D. R. Gosselin, N. Alter, J. L. Fisher, and P. M. Guyre. 1992. Enhanced antigen presentation using human Fcγ receptor (monocyte/macrophage)-specific immunogens. *J. Immunol.* 149:3477–3481.

10. Warren, H. S., F. R. Vogel, and L. A. Chedid. 1986. Current status of immunological adjuvants. *Annu. Rev. Immunol.* 4:369–388.

11. Van de Winkel, J. G. J., L. K. Ernst, C. L. Anderson, and I. M. Chiu. 1991. Gene organization of the human high affinity receptor for IgG, FcγRI (CD64). J. Biol. Chem. 266:13449–13455.

12. Taketo, M., A. C. Schroeder, L. E. Mobraaten, K. B. Gunning, G. Hanten, R. R. Fox, T. H. Roderick, C. L. Stewart, F. Lilly, C. T. Hansen, and P. A. Overbeek. 1991. FVB/N: an inbred mouse strain preferable for transgenic analyses. *Proc. Natl. Acad. Sci. USA*. 88:2065–2069.

 Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

14. Allen, J. M., and B. Seed. 1989. Isolation and expression of functional high-affinity Fc receptor cDNAs. *Science (Wash. DC)*. 243:378–381.

14a. Graziano, R. F., P. R. Tempest, P. White, T. Keler, Y. Deo, H. Ghebremariam, K. Coleman, L. C. Pfefferkorn, M. W. Fanger, and P. M. Guyre. 1995. Construction and characterization of a humanized anti- γ -Ig receptor type I (Fc γ RI) monoclonal antibody. *J. Immunol.* 155:4996–5002.

15. Riechmann, L., M. Clark, H. Waldmann, and G. Winter. 1988. Reshaping human antibodies for therapy. *Nature (Lond.)*. 332:323–327.

16. Guyre, P. M., R. F. Graziano, B. A. Vance, P. M. Morganelli, and M. W. Fanger. 1989. Monoclonal antibodies that bind to distinct epitopes on FcγRI are able to trigger receptor function. *J. Immunol.* 143:1650–1655.

17. Van den Herik-Oudijk, I. E., N. A. C. Westerdaal, N. V. Henriquez, P. J. A. Capel, and J. G. J. van de Winkel. 1994. Functional analysis of human FcyRII (CD32) isoforms expressed in B lymphocytes. *J. Immunol.* 152:574–588.

18. Pearse, R. N., R. Feinman, and J. V. Ravetch. 1991. Characterization of the promoter of the human gene encoding the high affinity IgG receptor: transcriptional induction by γ -interferon is mediated through common DNA response elements. *Proc. Natl. Acad. Sci. USA*. 88:11305–11309.

19. Perez, C., J. Wietzerbin, and P. D. Benech. 1993. Two cis-DNA elements involved in myeloid-cell-specific expression and gamma interferon (IFN- γ) activation of the human high-affinity Fc γ receptor gene: a novel IFN regulatory mechanism. *Mol. Cell. Biol.* 13:2182–2192.

20. Osman, N., C. A. Kozak, I. F. C. McKenzie, and P. M. Hogarth. 1992.

Structure and mapping of the gene encoding mouse high affinity $Fc\gamma RI$ and chromosomal location of the human $Fc\gamma RI$ gene. J. Immunol. 148:1570–1575.

21. Skalnik, D. G., D. M. Dorfman, A. S. Perkins, N. A. Jenkins, N. G. Copeland, and S. H. Orkin. 1991. Targeting of transgene expression to monocyte/macrophages by the gp91-phox promoter and consequent histiocytic malignancies. *Proc. Natl. Acad. Sci. USA*. 88:8505–8509.

22. Grisolano, J. L., G. M. Sclar, and T. J. Ley. 1994. Early myeloid cell-specific expression of the human cathepsin G gene in transgenic mice. *Proc. Natl. Acad. Sci. USA*. 91:8989–8993.

23. Wallace, P. K., A. L. Howell, and M. W. Fanger. 1994. Role of Fcγ receptors in cancer and infectious disease. *J. Leukocyte Biol.* 55:816–826.

24. Pfefferkorn, L. C., and M. W. Fanger. 1989. Transient activation of the NADPH oxidase through FcγRI: oxidase deactivation precedes internalization of cross-linked receptors. *J. Immunol.* 143:2640–2649.

25. Takai, T., M. Li, D. Sylvestre, R. Clynes, and J. V. Ravetch. 1994. FcR γ chain deletion results in pleiotropic effector cell defects. *Cell*. 76:519–529.

26. Skalnik, D. G., E. C. Strauss, and S. H. Orkin. 1991. CCAAT displacement protein as a repressor of the myelomonocytic-specific gp91-phox gene promoter. J. Biol. Chem. 266:16736–16744.

27. Pahl, H. L., A. G. Rosmarin, and D. G. Tenen. 1992. Characterization of the myeloid-specific CD11b promoter. *Blood.* 79:865–870.

28. Sadowski, H. B., K. Shuai, J. E. Darnell, Jr., and M. Z. Gilman. 1993. A common nuclear signal transduction pathway activated by growth factor and cytokine receptors. *Science (Wash. DC)*. 261:1739–1744.

29. Larner, A. C., M. David, G. M. Feldman, K.-I. Igarashi, R. H. Hackett, D. S. A. Webb, S. M. Sweitzer, E. F. Petricoin, III, and D. S. Finbloom. 1993. Tyrosine phosphorylation of DNA binding proteins by multiple cytokines. *Science (Wash. DC)*. 261:1730–1733.

30. Simms, H. H., M. M. Frank, T. C. Quinn, S. Holland, and T. A. Gaither. 1989. Studies on phagocytosis in patients with acute bacterial infections. *J. Clin. Invest.* 83:252–260.

31. Olweus, J., F. Lund-Johansen, and L. W. M. M. Terstappen. 1995. CD64/Fc γ RI is a granulo-monocytic lineage marker on CD34⁺ hematopoietic progenitor cells. *Blood.* 85:2402–2413.

32. Rodewald, H. R., P. Moingeon, J. L. Lucich, C. Dosiou, P. Lopez, and E. L. Reinherz. 1992. A population of early fetal thymocytes expressing Fc- γ RII/III contains precursors of T lymphocytes and natural killer cells. *Cell*. 69:139–150.

33. Sandor, M., J. Galon, L. Takacs, Y. Tatsumi, A. L. Mueller, C. Sautes, and R. G. Lynch. 1994. An alternative Fcγ-receptor ligand: potential role in T-cell development. *Proc. Natl. Acad. Sci. USA*. 91:12857–12861.

34. Ra, C., M. H. Jouvin, U. Blank, and J. P. Kinet. 1989. A macrophage $Fc\gamma$ receptor and the mast cell receptor for IgE share an identical subunit. *Nature (Lond.).* 341:752–754.

35. Dombrowicz, D., V. Flamand, K. K. Brigman, B. H. Koller, and J. P. Kinet. 1993. Abolition of anaphylaxis by targeted disruption of the high affinity immunoglobulin E receptor α chain gene. *Cell*. 75:969–976.

36. Kenney, J. S., B. W. Hughes, M. P. Masada, and A. C. Allison. 1989. Influence of adjuvants on the quantity, affinity, isotype and epitope specificity of murine antibodies. *J. Immunol. Methods*. 121:157–166.

37. Tax, W. J. M, and J. G. J. van de Winkel. 1990. Fcγ receptor II: a standby receptor activated by proteolysis. *Immunol. Today.* 11:308–310.