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Research Article

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 (Fc gamma RI; CD64), which is exclusively present on myeloid cells. To evaluate a comparable role of Fc gamma RI in antigen presentation in vivo, we generated human Fc gamma RI transgenic mice. Under control of its endogenous promoter, human Fc gamma RI was selectively expressed on murine myeloid cells at physiological expression levels. As in humans, expression was properly regulated by the cytokines IFN-gamma, 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 Fc gamma RI can induce enhanced antibody responses, mice were immunized with an anti-human Fc gamma RI 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 Fc gamma RI on myeloid cells is highly active in mediating enhanced antigen presentation in vivo, and show that anti-Fc gamma RI mAbs are promising vaccine adjuvants.

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 (FcγRI; CD64), which is exclusively present on myeloid cells. To evaluate a comparable role of FcγRI in antigen presentation in vivo, we generated human FcγRI transgenic mice. Under control of its endogenous promoter, human FcγRI 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 FcγRI can induce enhanced antibody responses, mice were immunized with an anti–human FcγRI 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 FcγRI on myeloid cells is highly active in mediating enhanced antigen presentation in vivo, and show that anti-FcγRI 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 (FcγR) in the immune defense against infectious agents. The class I FcγR (CD64) represents a unique member among the broadly distributed and heterogeneous FcγR family. FcγRI is the sole receptor that binds with high affinity both monomeric and immune-complexed IgG, and its expression is restricted to cells of the myeloid lineage. Mononuclear phagocytes constitutively express FcγRI, whereas low numbers of receptors are found on polymorphonuclear neutrophils (PMNs).[1] Human (h) FcγRI expression can be enhanced by exposure to IFN-γ, 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 FcγRI expression on human monocytes and PMN (3–7). FcγRI 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 FcγRI can also mediate enhanced antigen presentation through internalization of antigen complexed with IgG (9). Moreover, antigen conjugated to FcγRI-specific antibodies, thus targeted to this receptor, markedly enhanced T cell activation in vitro (9). Targeting of antigen to FcγRI 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 antibody-forming 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 FcγRI-mediated enhancement of antigen presentation by myeloid cells is physiologically relevant (9). As a consequence, targeting of antigen to FcγRI potentially constitutes an effective strategy for augmentation of antigen-specific antibody responses. Here, we describe experiments aimed at validating the concept of hFcγRI targeting in vivo by using a novel transgenic mouse model. We show that hFcγRI 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 FcγRI in immune responses in vivo and show that targeting of antigen to hFcγRI 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; hFcγR, 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 ^{32}P-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 (2×10^5 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 ^{32}P-labeled p135 cDNA probe.

Antibodies. Anti–human FcγRI mAbs 22, 32.2, and 197, and anti–human FcγRIII mAb 3G8, were provided by Medarex, Inc. (Annandale, NJ). Anti–mouse FcγRII/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 hFcγRI (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 IgG1κ antibody framework, and retains high affinity for human FcγRI. Antibody SB82 (generously provided by Scotgen Biopharmaceuticals Ltd.) is a comparable humanized IgG1κ 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 (2×10^5 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.

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 washed, resuspended in DMEM (GIBCO BRL, Grand Island, NY) supplemented with 4.5 g/liter glucose, 10% (vol/vol) FCS, and 50 μg/ml streptomycin, and cultured at a concentration of 5×10^5 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, Mijdrecht, The Netherlands) as targets coated with bispecific antibodies (BsAbs) [$F(ab')_2 \times F(ab')_2$] 22 × anti-OE or 3G8 × anti-OE (generous gifts of Dr. L. Shen, Dartmouth Medical School, Lebanon, NH) (8). Sensitized targets (1×10^7) were added to macrophage monolayers (2.5×10^5 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.

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 μg/ml), or with heat-aggregated human IgG (15 μg/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 μg per mouse) diluted in sterile saline. After 6 wk, mice were reimmunized subcutaneously with the same antigen (100 μg 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 μg/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 phosphatase-labeled 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 hFcγRI by myeloid cells of transgenic mice. Recent studies have defined several key regulatory motifs within the promoter region of the hFcγRIA 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 FcγRI (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 hFcγRIA promoter were, therefore, expected to target gene expression in myeloid cells of transgenic mice. A human genomic fragment encompassing the FcγRIA 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 hFcγRI 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 hFcγRI 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 hFcγRI (Fig. 2 *A*). 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). hFcγRI was, furthermore, expressed at intermediate levels on peripheral blood monocytes and at low levels on neutrophils (Fig. 2 *A*). Lymphocytes did not express detectable hFcγRI (Fig. 2 *A*). No cross-reactive staining of human CD64 mAbs was seen on cells from nontransgenic mice tested in parallel (Fig. 2 *A*). 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 FcγRII/III mAb 2.4G2 or the macrophage marker Mac-1 (CD11b/CD18) yielded interesting results. Expression levels of Mac-1 integrin was identical in transgenic and control mice. The level of mouse FcγRII/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 hFcγRI affects mouse FcγR surface expression.

Regulation of hFcγRI 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 hFcγRI expression. First, we treated mice with IFN-γ and analyzed transgene RNA expression. As seen in Fig. 1 *C* (*middle panel*), hFcγRI mRNA production was enhanced by IFN-γ. 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-γ–treated transgenic mice.

Another cytokine that triggers increased FcγRI 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 (~45% of total white blood cells; $n = 3$) compared with saline-treated animals (~12%). Moreover, expression of hFcγRI was strongly up-regulated on circulating neutrophils of G-CSF–

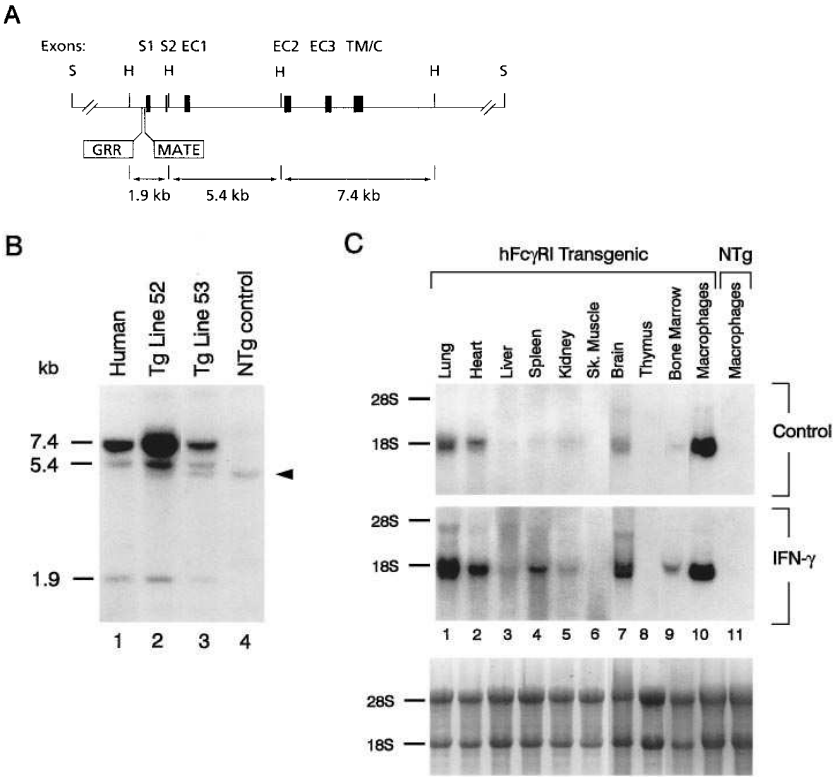

Figure 1. Transgenic mice expressing human FcγRI. (*A*) Structure of the transgene consisting of an 18-kb human genomic DNA fragment carrying the FcγRIA gene locus (11). Exons are represented by closed boxes. GRR and MATE indicate locations of regulatory motifs within the promoter region (see Results). *S1,2*, signal sequences; *EC1–3*, extracellular domains; *TM/C*, transmembrane and cytoplasmic region; *H*, HindIII; *S*, SalI. (*B*) Southern blot analysis of hFcγRI-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 hFcγRI 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 (2×10^5 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.

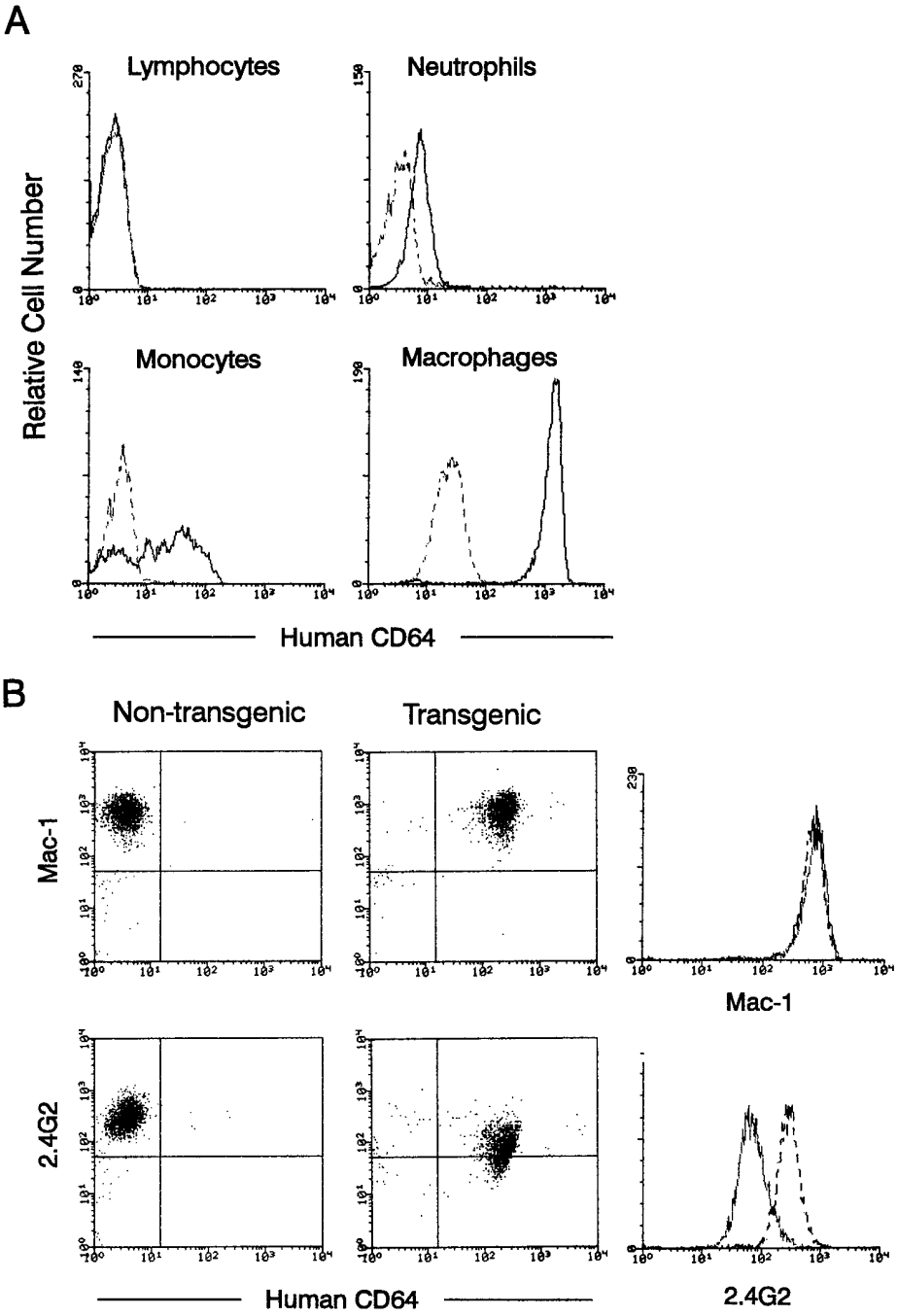

Figure 2. Flow cytometric analysis of hFcγRI cell surface expression. (*A*) Peripheral blood leukocytes and peritoneal macrophages of transgenic line 53 (*solid lines*) and nontransgenic mice (*dotted lines*), were stained with anti-hFcγRI mAb 22-FITC. Cells were stained with PE-labeled anti-CD45R/B220 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 FcγRII/III and Mac-1 on peritoneal macrophages of transgenic line 52 and control mice. Cells were stained with mAb 32.2-FITC (anti-hFcγRI) and either with 2.4G2-PE (anti–mouse FcγRII/III) or with Mac-1-PE. Macrophages expressing hFcγRI showed a reduced 2.4G2 staining (*solid line*) compared with controls (*dotted line*).

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, hFcγRI expression levels on transgenic neutrophils were back to baseline.

In addition to IFN-γ and G-CSF, several other cytokines are known to regulate FcγRI 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 hFcγRI 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 hFcγRI 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 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 hFcγRI (Fig. 3 *C*). When lesions on the skin were healed, blood staining profiles returned to normal (not shown).

Internalization and phagocytosis mediated by hFcγRI. To evaluate whether hFcγRI 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-

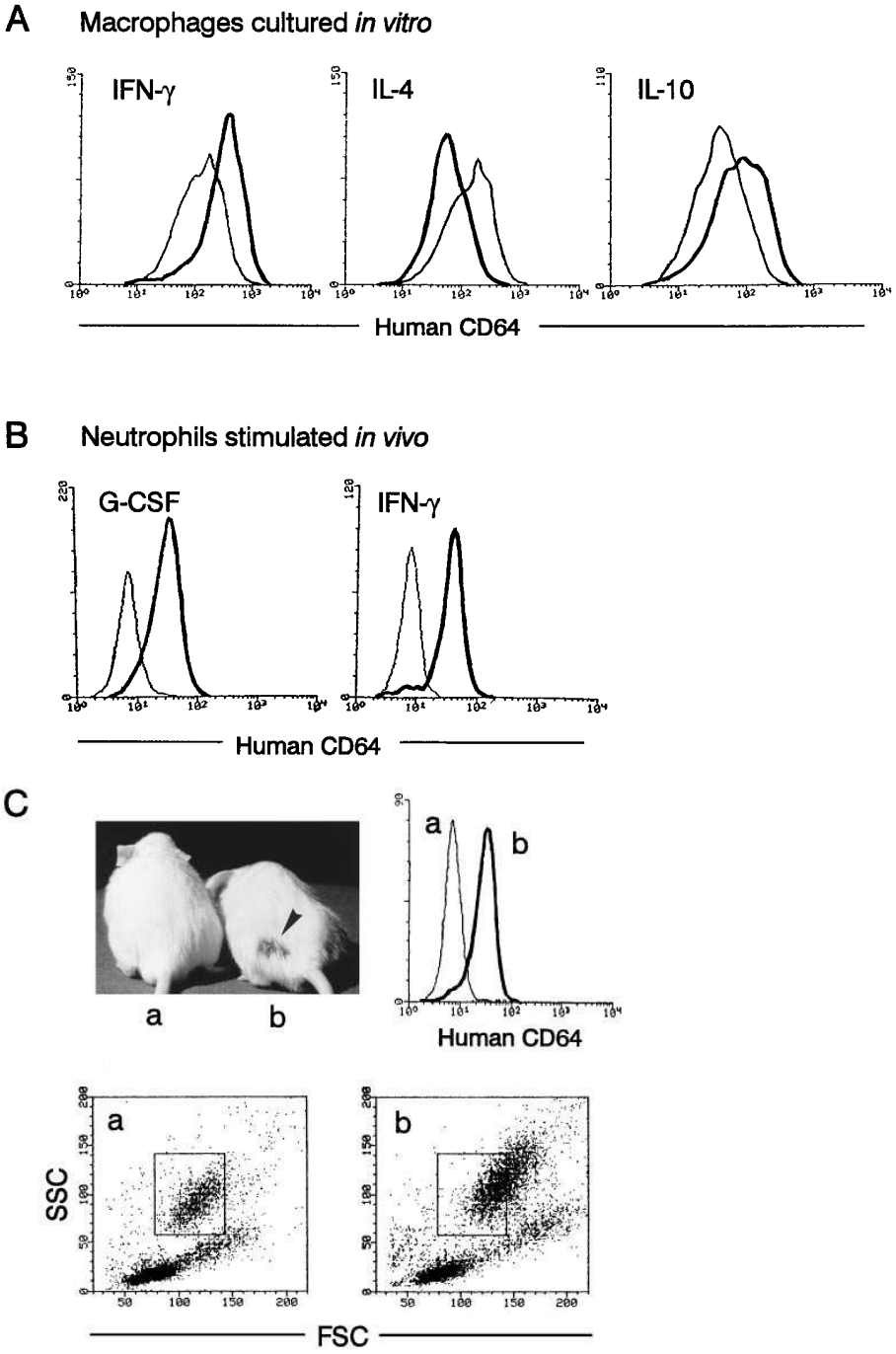

Figure 3. Regulation of hFcγRI expression in transgenic mice. (*A*) Effects of cytokines on hFcγRI cell surface expression on cultured macrophages. Thioglycollate-elicited peritoneal macrophages of line 52 transgenic mice were cultured with murine IL-4 (30 ng/ml), IL-10 (15 ng/ml), or IFN-γ (250 U/ml) for 22 h. Expression of hFcγRI 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*) hFcγRI 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 (2×10^5 U per mouse). Expression of hFcγRI 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 hFcγRI expression profiles of blood neutrophils from both animals are depicted.

tween transgenic and endogenous mouse FcγR, we used an alternative method to opsonize particles. BsAbs were used to direct the OE specifically to hFcγRI (8). The anti-hFcγRI × anti-OE BsAb comprises mAb 22, which is reactive with an epitope of hFcγRI outside the ligand binding site yet is capable of triggering hFcγRI-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-hFcγRIII [mAb 3G8] × anti-OE) (Fig. 4 *C*). Similarly, macrophages of nontransgenic littermates failed to bind anti-hFcγR–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

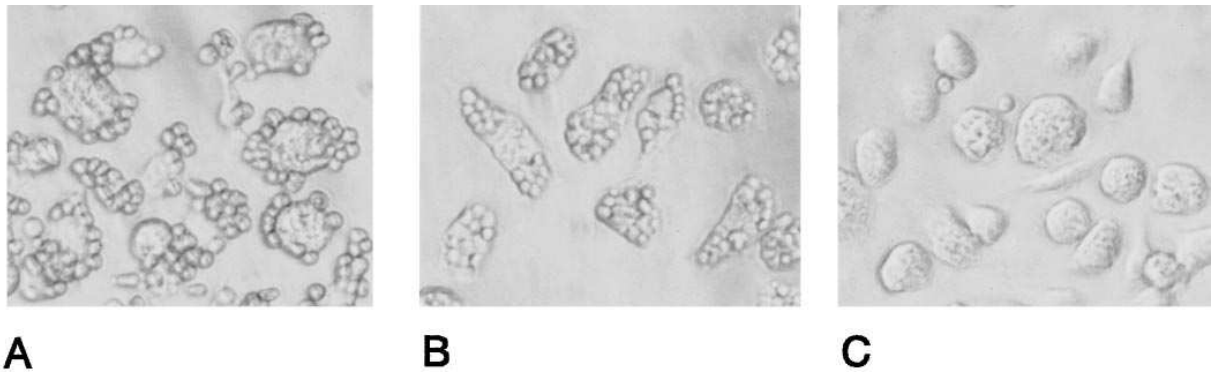

Figure 4. hFcγRI-mediated phagocytosis of OE by thioglycollate-elicited peritoneal macrophages of line 52 transgenic mice. Binding at 4°C (*A*) and phagocytosis at 37°C (*B*) of OE directed to hFcγRI via BsAb 22 × anti-OE. In control experiments using OE coated with BsAb 3G8 (anti-hFcγIII) × anti-OE, 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 hFcγRI (1, 2). These characteristics were expected to result in receptor cross-linking similar to mAb 197. Antibody H22 was found to bind with equal reactivity to hFcγRI 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 hFcγRI-targeted antigens in transgenic mice. Targeting antigen to FcγRI on human monocytes via mAb 22 results in enhanced T cell activation in vitro (9). This prompted us to investigate whether FcγRI targeting enhances specific humoral immune responses in transgenic mice. Antibody H22, which recognizes hFcγRI despite occupation of the latter with IgG, served as an hFcγRI-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 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 FcγRI. Most data, however, were generated in in vitro studies, and it appears pertinent to question whether these functions reflect a role for FcγRI in vivo. Attempts to evaluate the contribution of FcγRI to immune responses in

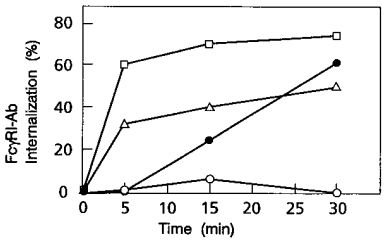

Figure 5. Internalization of hFcγRI cross-linking antibodies. Surface FcγRI molecules on thioglycollate-elicited peritoneal exudate cells were labeled at 4°C either with heat-aggregated human IgG (□), mAb 197 (mIgG2a) (△), murine mAb 22 (mIgG1) (○), or with humanized mAb H22 (hIgG1) (●). The amounts 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.

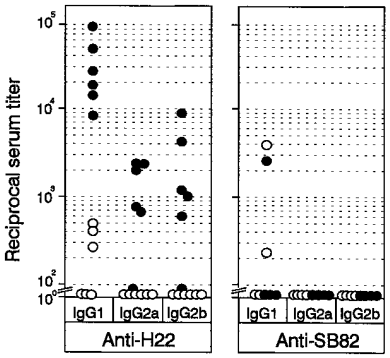

Figure 6. Antigen-specific IgG responses after immunization of mice with either anti-hFcγRI humanized mAb H22 (*left*) or with control humanized mAb SB82 (*right*) as antigen. 8–10-wk-old line 52 transgenic mice (●) and nontransgenic littermates (○) were immunized subcutaneously with 100 μg of antigen. 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 FcγRI (25). Obviously, experiments analyzing FcγRI functioning in humans are restricted by ethical considerations. Our strategy to overcome these difficulties was to generate human FcγRI-transgenic mice. This study demonstrates that an 18-kb human genomic DNA fragment contains sufficient regulatory information to target the expression of hFcγRIa 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 hFcγRI 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 hFcγRI 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 hFcγRI 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 hFcγRI expression in transgenic mice. PMN hFcγRI 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 hFcγRIA 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 FcγR 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 hFcγRI-transgenic mice (data not shown), suggesting that the human receptor does not significantly affect myeloid/lymphoid differentiation. The presence of hFcγRI, however, had an apparent effect on expression levels of endogenous mouse FcγR. Macrophages of transgenic mice expressed consistently lower levels of mouse FcγRII/III (Fig. 2 *B*). As FcγRIII is known to depend on the Fc receptor γ chain for surface expression (34), we speculate that competition by hFcγRI for mouse γ chains underlies the lower mouse FcγRII/III expression on phagocytes. This hypothesis is supported by an observation in FcεRI-deficient mice. In these animals, absence of FcεRI molecules resulted in an increased availability of FcR γ chain for FcγRIII and led to higher FcγRIII levels on mast cells (35). Studies in γ chain–deficient mice showed that mouse FcγRI was functionally absent on macrophages, indicating that the γ chain is crucial for functional mouse FcγRI expression (25). Here, functionality of hFcγRI 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-hFcγRI BsAbs. Thus, our findings support the formation of a functional hFcγ receptor complex. Collectively, these data suggest that the presented hFcγRI-transgenic mouse represents a relevant model for evaluation of FcγRI functioning in vivo.

Immunization of mice with an anti-hFcγRI antibody that contains antigenic determinants, and monitoring sera for presence of antigen-specific antibodies, clearly showed that hFcγRI 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 FcγRI could have important consequences in humans. For instance, BsAbs with one specificity for FcγRI and another for a tumor-associated 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 FcγRI 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 FcγRI 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 FcγRI 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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