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Production and characterization of mice transgenic for the A and B isoforms of human FcγRIII

Abstract Fcγ receptor (FcγR) engagement is pivotal for many effector functions of macrophages, polymorphonuclear neutrophils (PMN), and natural killer (NK) cells. Mice transgenic for the A and B isoforms of human (h) FcγRIII on macrophages, PMN, and NK cells were constructed to permit the study of mechanisms and potential in vivo strategies to utilize the cytotoxic effector and antigen-presenting functions of cells expressing the hFcγR. The present report characterizes the phenotypic and functional expression of hFcγRIII in transgenic mice derived by crossing hFcγRIIIA and hFcγRIIIB transgenic mice. Interleukin-2 (IL-2) induces hFcγRIII expression by myeloid cells and their precursors, and these transgenic receptors promote in vitro cytotoxicity and anti-hFcγRIII antibody internalization. Splenocytes from untreated and IL-2-treated hFcγRIIIA, hFcγRIIIB, and hFcγRIIIA/B mice exhibited enhanced in vitro cytotoxicity toward HER-2/*neu*-overexpressing SK-OV-3 human ovarian carcinoma cells when incubated with the murine bispecific mAb 2B1, which has specificity for HER-2/*neu* and hFcγRIII. These results indicate that hFcγRIII transgenes are expressed on relevant murine cellular subsets, exhibit inducible up-regulation patterns similar to those seen in humans, and code for functional proteins. hFcγRIII transgenic mice exhibiting specific cellular subset expression will permit the examination of strategies designed to enhance hFcγRIII-dependent immunological effector functions and will provide a model system in which to evaluate preclinically potential candidate molecules that recognize hFcγRIII for the immunotherapy of cancer.

Key words Fc receptors · Transgenic · Cytotoxicity · NK cells · Macrophages

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Introduction

Fcγ receptors (FcγR) mediate phagocytosis, induce cytokine release and superoxide production, and trigger antibody-dependent cellular cytotoxicity (ADCC) [23, 42, 62]. Three classes of human FcγR (hFcγR) have been described: FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16). FcγRI is constitutively expressed by monocytes and its expression is induced on polymorphonuclear neutrophils (PMN) by interferon γ (IFNγ) and granulocyte-colony-stimulating factor. Crosslinking of FcγRI mediates phagocytosis, triggers superoxide production, and induces interleukin-6 (IL-6) and tumor necrosis factor α (TNFα) [10, 11, 23, 24]. FcγRII is widely expressed by PMN, monocytes/macrophages, B cells, some T cells, dendritic cells and platelets [23, 32, 36, 59, 61, 69]. FcγRII engagement results in a respiratory burst by PMN, phagocytosis, ADCC, and TNFα release by monocytes [23, 62]. There are two isoforms of FcγRIII: FcγRIIIA and FcγRIIIB. FcγRIIIA is present on macrophages and natural killer (NK) cells [12], has transmembrane and cytoplasmic domains [58], and functions to trigger activation and phagocytic and cytolytic processes [8]. FcγRIIIB is expressed by PMN and is anchored to the cell membrane via a phosphatidylinositol glycan linkage [48]. Although FcγRIIIB is not involved in signal transduction for cellular cytotoxic responses, FcγRII and FcγRIII may act in a cooperative manner to mediate neutrophil degranulation and other FcγRII-mediated functions [9, 23, 47].

FcγR have been attractive trigger molecules for the development of bispecific antibodies (bsAb) to redirect

the cytotoxic capabilities of effector cells to neoplasms [3, 21, 22, 55, 60, 62, 66]. bsAb targeting FcγRI and CD15, or FcγRI and the protooncogene protein c-erbB-2 (HER-2/*neu*) have been employed in phase I clinical trials [3, 23, 60]. bsAb recognizing FcγRIII have been used to target phagocytic cells and NK cells to tumor cells [21, 22, 46, 55, 66, 67] Chemically heteroconjugated mAb with specificities for FcγRIII and various tumor-associated antigens promote human NK cell tumor lysis in vitro and prevent the growth of neoplasms in nude mice [55]. The bsAb HRS-3/A9 recognizing FcγRIII and CD30, which is expressed on Hodgkin's lymphoma cells, induced the lysis of Hodgkin's cells by unstimulated peripheral blood lymphocytes (PBL) and enriched NK cell populations [21]. HRS-3/A9 treatment of C.B17/Icr-*scid* mice bearing Hodgkin's tumors caused complete tumor regression in all of ten mice. We previously have characterized the bsAb 2B1, with specificities for FcγRIII and HER-2/*neu* [22, 66, 67]. 2B1 promotes the in vitro lysis of HER-2/*neu*-overexpressing tumor cells [22, 66, 67] by human large granular lymphocytes (LGL)/NK cells and macrophages, cures up to 70% of C.B17/Icr-*scid* mice bearing early established HER-2/ *neu*-positive xenografts [66], and induces anti-HER-2/ *neu* immune responses and scattered objective clinical responses in humans treated in a phase I clinical trial [68].

Preclinical characterization of the in vivo antitumor activities of bsAb has been mainly confined to rodent models employing immunocompromised C.B17/Icr-*scid* or nude mice bearing human tumor xenografts [17, 21, 33, 39, 40, 43, 55, 66]. Although successful treatment of human tumor xenografts has been achieved with the use of bsAb in immunocompromised mice reconstituted with human PBL, host influences and constraints on the function and trafficking of human effector cells may hinder the study of many human effector cell functions in xenogeneic hosts. To circumvent some of these problems, syngeneic rodent models have been used to assess efficacy of bsAb [4–7, 35, 51, 52, 63–65, 66, 67].

Human T cells, B cells, and NK cells have been shown to engraft in C.B17/Icr-*scid* mice, and such mice have been used to study various human immune cell functions [50, 51, 56]. Although the engrafted human B cells and NK cells have been shown to be functional, there is controversy about the functional capabilities of engrafted T cells [50, 51, 54]. In addition, the development of graft versus host disease limits the utility of this model [50].

Another approach to developing relevant murine models is the creation of immunocompetent or immunodeficient mice that are transgenic for human effector cell receptors. A hFcγRI transgenic mouse has been developed [20] in which hFcγRI is strongly expressed by monocytes and macrophages, and is minimally expressed by PMN. The transgene product is functional in ADCC, phagocytosis, and antigen presentation assays. As in humans, the expression of FcγRI in this model is regulated by IFNγ, IL-10 and IL-4.

The production of hFcγRIIIA and hFcγRIIIB mice has been previously reported [30]. This report presents the characterization of the phenotypic and functional expression of hFcγRIII in transgenic mice derived by crossing hFcγRIIIA and hFcγRIIIB transgenic mice. The resulting strains can be used to study potential in vivo strategies aimed at optimizing the cytotoxic effector and antigen presentation functions associated with hFcγRIII.

Materials and methods

Mice

Transgenic mice expressing the hFcγRIIIA isoform on macrophages and NK cells, and the hFcγRIIIB isoform on PMN were kindly provided by Dr. Jeffrey Ravetch and were generated as described [30]. The genetic background of the hFcγRIIIA and hFcγRIIIB transgenic mice included the C57BL/6, CBA/CA and CD-1 strains [30]. Mice expressing both isoforms of hFcγRIII were generated by breeding the hFcγRIIIA and hFcγRIIIB mice at the Fox Chase Cancer Center Laboratory Animal Facility. The progeny of hFcγRIIIA × hFcγRIIIB matings were screened by flow cytometry for the expression of hFcγRIII by peritoneal macrophages and peripheral blood PMN. Only mice expressing both isoforms were considered to be hFcγRIIIA/B mice, and only mice expressing the transgene most abundantly were used in successive matings.

In addition, an F1 male of the hFcγRIIIA/B strain was crossed into the C57BL/6JNIcr strain (obtained from the FCCC LAF) to create immunocompetent progeny on a C57BL/6JNIcr background. Progeny were screened as described above for the two hFcγRIII isoforms and were determined to have the C57BL/6JNIcr haplotype (H-2^b) by hemagglutination assays. Also, hFcγRIIIA mice were back-crossed with C.B17/Icr-*scid* mice to obtain immunodeficient progeny that express the hFcγRIIIA isoform. All progeny were screened for the presence of hFcγRIIIA on their peritoneal macrophages by flow cytometry, and screened for the SCID phenotype by assaying sera for membrane Ig secretion by enzyme-linked immunosorbent assay (ELISA). The mice ranged in age from 4 weeks to 22 weeks old, were housed in microisolator cages, and were fed standard chow and water ad libitum, and for all animal experiments, principles of laboratory animal care (NIH publication No. 85-23, revised 1985) were followed.

Cells

Human SK-OV-3 (ovarian carcinoma) cells were obtained from the American Type Culture Collection (Rockville, Md.), and were maintained in complete RPMI-1640 medium (Gibco, BRL Life Technologies, Inc., Grand Island, N.Y.) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Intergen Company, Purchase, N.Y.), containing penicillin/streptomycin (JRH Biosciences, Lenexa, Kan.), insulin (Eli Lilly and Company, Indianapolis, Ind.), L-glutamine, gentamicin, HEPES, and bicarbonate (Gibco).

Reagents

The fluorescein-isothiocyanate (FITC)- and phycoerythrin (PE)-labeled mAb 2B4-FITC [anti-(murine NK cell)] [16], NK1.1-PE [anti-(murine NK cell)] [19]; 3G8-FITC and 3G8-PE (anti-hFcγRIII) [12, 41]; anti-(mouse αβTCR)-FITC, γδTCR-biotin and TCR-PE, and anti-(mouse CD3ε)-FITC; CD4-FITC; CD8α-FITC; CD19-FITC; streptavidin-FITC; anti-(murine CD71)-PE (C2; murine transferrin receptor); unconjugated and PE-conjugated 2.4G2 [Fc block; anti-(mouse FcγRII/III)]; Ly-6G-FITC [Gr-1; RB6-8C5; anti-(murine granulocyte differentiation antigen)] [14, 29]; unconjugated anti-(mouse CD34) [26]; the isotypic controls

(mouse IgG1-FITC, mouse IgG2a-PE, mouse IgG2b-FITC, rat IgG2a-FITC, rat IgG2b-FITC and IgG2b-PE, hamster IgG-FITC and hamster IgG-PE) and the secondary antibody [polyclonal FITC-labeled goat anti-(rat Ig) (GAR-FITC)] were purchased from Pharmingen (San Diego, Calif.). The anti-macrophage mAb F4/80-FITC [1] was purchased from Harlan Bioproducts for Science (Indianapolis, Ind.). The anti-(murine mid-stage myeloid marker), ER-MP-20 (Ly-6C, CD59) [28], was purchased from BACHEM Bioscience Inc. (King of Prussia, Pa.). The isotypic control mouse IgG1-PE and secondary antibody polyclonal goat anti-(mouse Ig)-FITC (GAM-FITC) were obtained from Becton Dickinson (San Jose, Calif.). The unconjugated mouse IgG1 (MOPC-21) isotypic control was purchased from Sigma Chemical Co. (St. Louis, Mo.). Negative isotypic controls and their corresponding mAb, used in all of the experiments illustrated, were as follows: mouse IgG1-FITC and IgG1-PE for anti-hFcγRIII(3G8)-FITC and -PE, hamster IgG-FITC for anti-CD3ε-FITC and anti-αβTCR-FITC, hamster IgG-PE for anti-γδTCR-PE, rat IgG2a-FITC for anti-CD59 (Ly-6 C)-FITC, anti-CD19-FITC, anti-CD4-FITC and anti-CD8α-FITC, rat IgG2b-FITC for anti-F4/80-FITC and anti-Ly-6G-FITC, rat IgG2b-PE for anti-Ly6G-PE, mouse IgG2b-FITC for anti-2B4-FITC, mouse IgG2a-PE for anti-NK1.1-PE, rat IgG2a + GAR-FITC for anti-CD34 + GAR-FITC, and mouse IgG1-PE + GAM-FITC for 3G8-PE + GAM-FITC. Purified bsAb 2B1 was produced as previously described [15, 44–46, 49]. 2B1 binds to an epitope on the extracellular domain of HER-2/neu and to an epitope on the extracellular domain of hFcγRIII. Whole IgG and F(ab′)$_2$ fragments of 2B1, 520C9, 3G8, and recombinant human (rh)IL-2 (specific activity 18×10^6 IU/mg protein) were generous gifts from Chiron Corporation (Emeryville, Calif.). Propidium iodide was purchased from Molecular Probes Inc. (Eugene, Ore.). Dynabeads M-280/streptavidin were obtained from Dynal (Lake Success, N.Y.).

Collection of mouse peritoneal cells, PBL, splenocytes, bone marrow cells, peritoneal macrophages and peritoneal PMN

Peritoneal cells were harvested from mice by peritoneal lavage with PBS. The cells were then washed once with either fluorescence-activated cell sorting (FACS) buffer [Phosphate-buffered saline (PBS), 1% bovine serum albumin (BSA), and 0.1% sodium azide] or with PBS, 3% heat-inactivated FBS. Trypan blue dye exclusion was used to determine the viability of the peritoneal cells. Viability always exceeded 95%.

Peripheral blood was obtained from mice by retro-orbital bleeding into heparinized microcapillary tubes (Fisher Scientific Inc., Malvern, Pa.). Erythrocytes were depleted by hypotonic lysis with 0.2% (w/v) sodium chloride (Fisher Scientific Inc., Malvern, Pa.) and subsequent equilibration in 1.6% (w/v) sodium chloride. Cells were then washed with FACS buffer or PBS, 3% heat-inactivated FBS.

Splenocyte suspensions were obtained from freshly isolated spleens that were finely minced in PBS and passed through Falcon 70-μm nylon cell strainers (Fisher). Erythrocytes were removed by hypotonic lysis, as described above, and the splenocytes were washed with FACS buffer or PBS, 3% heat-inactivated FBS for FACS analysis or complete RPMI medium for cytotoxicity assays.

Bone marrow cells were flushed out of both femurs and tibiae using Becton Dickinson 1-ml tuberculin syringes fitted with 27-gauge needles, filtered through Falcon 70-μm cell strainers (Fisher), and washed in complete RPMI medium. Erythrocytes were lysed as described, and cells were washed with PBS, 3% heat-inactivated FBS for FACS analysis.

To induce macrophage accumulation in the peritoneal cavity of the animals, mice were injected i.p. with 1 ml 5% (w/v) thioglycollate (Sigma) [30], and peritoneal cells were harvested and washed as described above 3 days later. Mice also were injected i.p. with 2 ml 0.2% Casein (w/v) in PBS (pH 7.2) in order to stimulate intraperitoneal PMN accumulation. Cells were harvested and washed as described above 3 h after injection to obtain the greatest number of PMN [63].

Flow cytometry

Peritoneal cells, PBL, splenocytes, or bone marrow cells were incubated with 500 ng–1 μg appropriate mAb in FACS buffer or PBS containing 3% heat-inactivated FBS at 4 °C for 30 min. In all phenotyping experiments in which secondary anti-(rat Ig) Ab were not required, primary mAb incubations were preceded by a 5-min 4 °C preincubation with 2.4G2 (murine Fc block) to prevent non-specific binding via mFcγR. In some experiments, the cells were washed twice with FACS buffer and fixed in 1% paraformaldehyde (Electron Microscopy Sciences, Ft. Washington, Pa.) in PBS or incubated with the secondary GAR-FITC or GAM-FITC antibody conjugates or streptavidin-FITC, washed twice and then fixed. For live-cell FACS analyses, cells were washed twice with and resuspended in 100 μl PBS with 3% heat-inactivated FBS. Propidium iodide (10 μl, 10-μg/ml dilution) was subsequently added to stain and gate-out any dead cells.

One- to three-color analysis of mAb-labeled cell populations was performed using a FACScan flow cytometer interfaced with a Macintosh computer equipped with Consort 30 or CELLQuest software (Becton Dickinson, San Jose, Calif.), or with the Desk program [34] on a computer interfaced to a Digital Equipment Corporation VaxStation-4000/90 computer. Most cell populations were gated by forward and right-angle light scatter and, when applicable, propidium-iodide-stained dead cells were excluded from the analysis gates. Lymphocyte gates were set in peritoneal cells and splenocyte populations by forward and right-angle light scatter. PMN were gated using the PMN-specific anti-Ly-6G mAb. Macrophages were gated using the anti-macrophage F4/80 mAb. Fluorescence staining intensity was measured on a logarithmic scale. Between 10^4 and 2×10^4 cells were acquired per sample and background fluorescence of cells stained with corresponding isotypic controls was subtracted from all results.

Induction of hFcγRIII expression by IL-2

To examine the kinetics of IL-2 induction of hFcγRIII, hFcγRI-IIA/B transgenic mice were treated with 3×10^5 IU rhIL-2 for 5 consecutive days (days 0–4). Cohorts of three mice were sacrificed for the baseline measurement, and then on days 2, 3, and 5. Splenocytes and peritoneal cells were collected and analyzed for the expression of hFcγRIII by flow cytometry using the PE-conjugated 3G8 mAb as described previously in this report.

Cytotoxicity assay

This assay was performed as previously described [67]. Briefly, SK-OV-3 (HER-2/neu-expressing) tumor cell targets were labeled with 100 μCi ^{51}Cr (sodium chromate; NEN Life Science Products, Boston, Mass.)/10^6 cells, washed twice and resuspended in complete RPMI medium. ^{51}Cr-labeled target cells (1×10^4 cells) were added to varying numbers of effector cells to yield E:T ratios ranging from 50:1 to 1:1 in 96-well flat-bottomed plates (Costar, Cambridge, Mass.). All tests were performed in triplicate. Effector cells and target cells were incubated in a total assay volume of 200 μl in the presence or absence of 1 μg/ml 2B1 bsAb, 2B1 F(ab′)$_2$ bsAb, 520C9, 520C9 F(ab′)$_2$, 3G8, or 3G8 F(ab′)$_2$ mAb. Plates were centrifuged at 300 g for 3 min and incubated 4 h at 37 °C. Following incubation, the plates were again centrifuged at 300 g for 3 min, and 100 μl (1/2 of total assay volume) supernatant was removed from each well and counted on a Beckman Gamma 4000 counter (Fullerton, Calif.). Cytotoxicity was calculated as:

$$\text{Lysis (\%)} = 100 \times \frac{\text{experimental release (cpm)} - \text{spontaneous release (cpm)}}{\text{total added radioactivity (cpm)}/2 - \text{spontaneous release (cpm)}}$$

Depletion of γδ T cells

hFcγRIIIA mice (four mice, two per experiment) were sacrificed on day 6 following i.p. treatment with 3×10^5 IU rhIL-2 for 5 consecutive days, and splenocytes were collected as described above. Half of the total cells harvested were retained for pre-depletion flow cytometry and cytotoxicity assays. The remainder was γδT-cell-depleted by incubating the cells for 30 min at 4 °C with biotinylated anti-(mouse γδTCR) (1 µg/10^6 cells). The cells then were washed twice with cold PBS, 1% BSA to remove unbound antibody. Dynabeads-M-280/streptavidin were added to establish a 4:1 bead:target cell ratio and incubated for 30 min at 4 °C on a bidirectional rotator in PBS, 0.1% BSA. Dynabead-γδTCR-positive cell rosettes were removed via four rounds of magnetic depletion. Depleted cell populations were washed and resuspended in FACS buffer for flow cytometry or complete RPMI medium for ^{51}Cr-release assays.

Flow internalization assay and confocal microscopy

Thioglycollate-induced peritoneal cells from C57BL/6JNIcr and hFcγRIIIA/B mice were stained and processed for flow cytometry as described above. All cell samples except for those stained for mFcγRII/III were preincubated for 5 min with 2.4G2 to block mFcγR binding by the Fc portions of IgG. Following the last wash of primary or secondary antibody with PBS, 3% heat-inactivated FBS, tubes were divided into four groups: 0 min ± acid wash and 60 min or 120 min ± acid wash. Samples in the 0 min − acid wash group were fixed for FACS analysis. The cells in the 0 min + acid wash group were acid washed twice with 1 ml 1:1 mix of 100 mM glycine, pH 2.5 (Sigma) and 1 M sodium chloride (Fisher). The samples were washed one final time with PBS, 3% heat-inactivated FBS and fixed. The remaining samples were incubated at 37 °C for either 60 min or 120 min and processed in an identical manner to the 0 min ± acid wash samples. All fixed cell suspensions were then collected and analyzed by flow cytometry. The percentage internalization at each assay time was calculated by employing the following formula:

$$\text{Internalization (\%)} = 100 \times \frac{\text{mean fluorescence index (MFI) of acid-washed sample} - \text{MFI of acid-washed isotypic control sample}}{\text{MFI of untreated control sample} - \text{MFI of untreated isotypic control sample}}$$

Aliquots (50 µl) of the fixed suspensions (approximately 2×10^5 cells) were used to make cytospin preparations using a Cytospin 2 (Shandon Inc., Pittsburgh, Pa.) for 5 min at 400 rpm on Corning microslides (Fisher). Slides were rinsed twice with equilibration buffer for 5 min, SlowFade Light reagent in 50% glycerol (v/v) (Molecular Probes, Eugene, Ore.) was applied, and the samples were mounted with coverslips that were then sealed with Permount solution (Fisher). Cytospin slides were analyzed by confocal microscopy with a 60× lens on a Bio-Rad MRC 600 laser scanning confocal microscopy system, and images were rendered with VoxelView ultrasoftware on a Silicon Graphics work station.

Results

Breeding strategy profiles of hFcγRIII A/B and hFcγRIIIA-scid mouse strains

A true-breeding colony of mice possessing the C57BL/6JNIcr background that express both isoforms of hFcγRIII is currently in its sixth generation. Table 1 displays a summary of flow cytometry screenings of these mice and of the hFcγRIIIA/B progeny that were not derived from the hFcγRIIIA/B and C57BL/6JNIcr

Table 1 hFcγRIIIA-C.B17/Icr-scid and hFcγRIIIA/B mouse progeny screenings. All progeny were screened by flow cytometry for the presence of the A isoform of hFcγRIII expressed by peritoneal macrophages (anti-F4/80-FITC/anti-CD16-PE$^+$ cells) and the B isoform expressed by peripheral blood polymorphonuclear neutrophils (anti-Ly-6G-FITC/anti-CD16-PE$^+$ cells). For hFcγRIIIA/B crosses, only mice abundantly expressing both isoforms were mated, and only mice expressing the A isoform and testing positive for scid (no membrane Ig secretion by ELISA) were used for hFcγRIIIA-scid matings. For the hFcγRIIIA/B progeny, included are data from animals that were mated in order to segregate for the C57BL/6JNIcr H-2 haplotype and those that were not. – Not available

Mouse strain	No. tested	Positive for		
		hFcγRIIIA (%)	hFcγRIIIB (%)	hFcγRIIIA/B (%)
hFcγRIIIA-scid				
F1	10	50.0		
F2	32	53.1		
F3	38	89.5		
F4	60	96.7		
F5	105	97.1		
F6	29	100		
hFcγRIIIA/B				
F1	27	–	–	22.2
F2	60	68.3	61.7	48.3
F3	50	78.0	70.0	60.0
F4	68	89.7	85.3	79.4
F5	101	99.0	100	99.0
F6	34	100	100	100

cross. Progeny were deemed to be hFcγRIIIA$^+$ if their peritoneal cells contained hFcγRIII$^+$ macrophages, hFcγRIIIB$^+$ if their PBL contained hFcγRIII$^+$ PMN, and hFcγRIIIA/B$^+$ if these two subpopulations coexisted. All of the F6 generation were hFcγRIIIA/B$^+$. The F6 mice have a C57BL/6JNIcr haplotype by hemagglutination assay (data not shown).

Similarly, 100% of the F6 immunodeficient transgenic (hFcγRIIIA$^-$C.B17/Icr-scid) mice expressed hFcγRIIIA, as shown in Table 1. These mice were confirmed to have the SCID phenotype by the lack of membrane Ig secretion in serum by ELISA (data not shown).

Expression of hFcγRIII by peritoneal macrophages, splenic NK cells and peripheral blood PMN of hFcγRIII transgenic mice

The expression of hFcγRIII was determined by FAC-Scan analysis as depicted in Fig. 1A–C. In the hFcγRIIIA mice, hFcγRIII was detected in 93% of the peritoneal macrophage population (Fig. 1A, left panel) and was found on 33% of splenic NK cells (Fig. 1A, right panel). Peripheral blood PMN of hFcγRIIIA mice (Fig. 1A, center panel) were devoid of hFcγRIII ex-
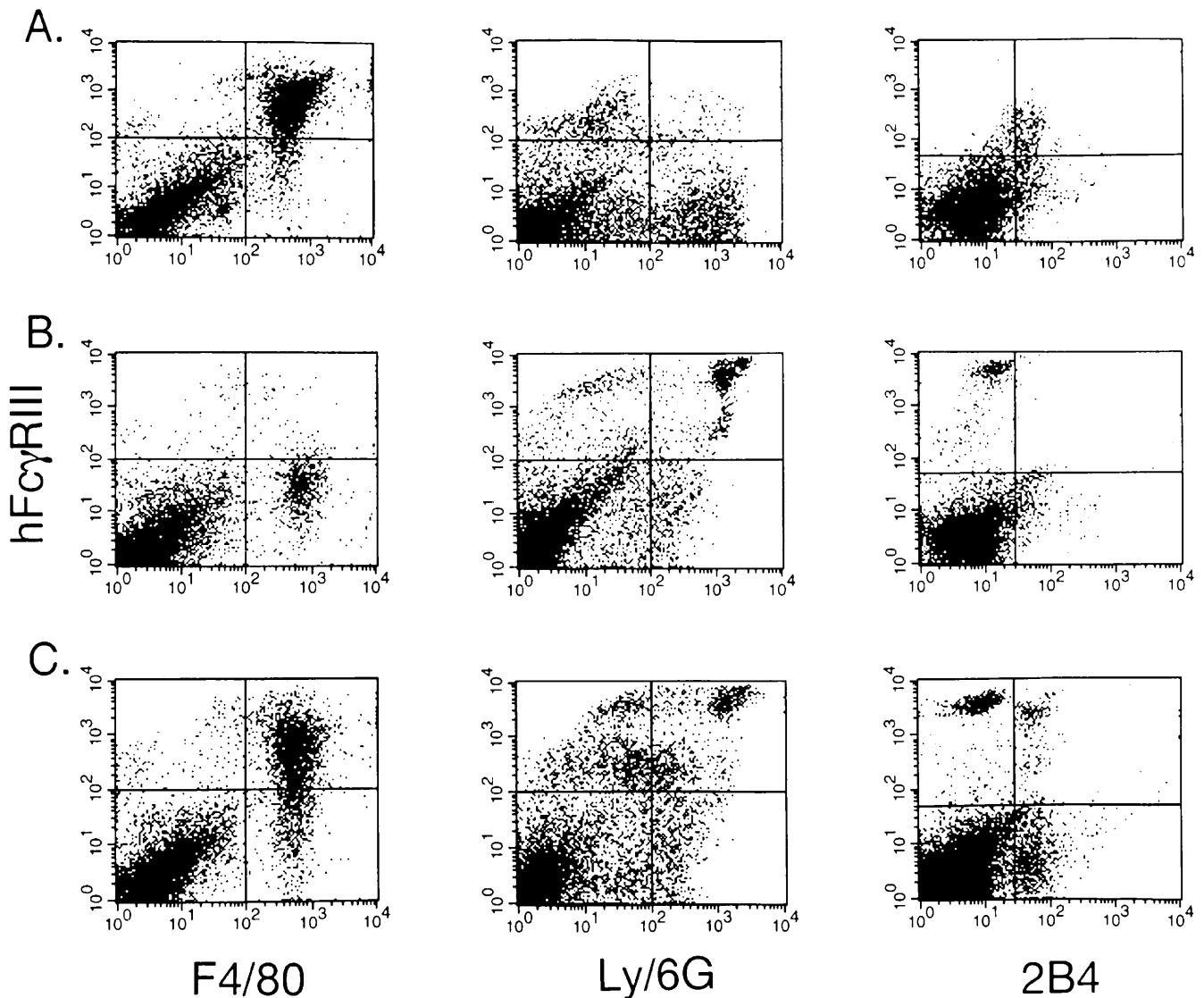
Fig. 1A–C hFcγRIII isoform expression on peritoneal macrophages, peripheral blood polymorphonuclear nuetrophils (PMN) and splenic natural killer (NK) cells in hFcγRIII transgenic mice. Cells were preincubated 5 min with 500 ng 2.4G2 to block non-specific binding to mFcγR and subsequently incubated with 500 ng fluorescein-isothiocyanate(FITC)-conjugated F4/80 [anti-(mouse macrophage antigen); *left panels*], Gr-1 [anti-(mouse Ly-6G, mouse PMN differentiation marker); *middle panels*], or 2B4 [anti-(murine NK cell receptor); *right panels*] and phycoerythrin(PE)-conjugated 3G8 (anti-hFcγRIII; *all panels*) mAb. Cells were washed twice and analyzed on a flow cytometer with CELLQuest software. Total gates are shown for all three cell types and exclude any propidium-iodide-stained cells. Each dot plot is representative of at least ten experiments. **A** Expression of hFcγRIIIA isoform in hFcγRIIIA mice. **B** Expression of hFcγRIIIB isoform in hFcγRIIIB mice. **C** Expression of hFcγRIIIA and B isoforms on peritoneal macrophages, splenic NK cells, and peripheral blood PMN of hFcγRIIIA/B mice

pression (0.6%). In contrast, hFcγRIII expression by hFcγRIIIB mice was restricted to peripheral blood PMN (Fig. 1B, center panel), with 5.8% of the total PBL population positive for both Ly-6G and hFcγRIII. Typical of several experiments, the PMN population

from these mice expressed high levels of hFcγRIII per cell (MFI = 3791). Peritoneal macrophages (Fig. 1B, left panel) and splenic NK cells (Fig. 1B, right panel) did not express hFcγRIIIB. hFcγRIIIA/B F5 generation mice expressed hFcγRIII on all three cell populations: on 79% of peritoneal macrophages (Fig. 1C, left panel), on 59% of peripheral blood PMN (Fig. 1C, center panel), and on 22% of NK cells in the spleen (Fig. 1C, right panel).

Expression of hFcγRIII by NK cells in untreated and IL-2-treated transgenic mice

The pattern of hFcγRIII expression on NK cells was examined in IL-2-untreated F2 hFcγRIIIA and C57BL/6JNIcr mice (Table 2). NK cells (2B4⁺) were found in the peripheral blood and spleens of hFcγRIIIA mice, 10.8% and 8.0% respectively. C57BL/6JNIcr control mice had similar percentages of NK cells in peripheral blood and spleens. NK cells expressing both 2B4 and

Table 2 Expression of hFcγRIII by natural killer (*NK*) cells in peripheral blood lymphocytes (*PBL*) and splenocytes of hFcγRIIIA mice. All interleukin-2 (*IL*-2)-treated mice were given i.p. injections of IL-2 (3×10^5 IU) daily for 5 days prior to PBL or splenocyte harvest. Analyses were performed on lymphocyte-gated populations. Results show SEM in parentheses

Treatment	Cell source	Mouse strain	Number of mice	2B4⁺ (%)	2B4/hFcγRIII dual positive (%)
None	PBL	hFcγRIIIA	4	10.8 (3.7)	2.5 (0.5)
		C57BL/6JNIcr	4	13.9 (1.8)	0.2 (0.1)
	Spleen	hFcγRIIIA	8	8.0 (1.6)	1.4 (0.5)
		C57BL/6JNIcr	4	9.4 (0.8)	<0.1
IL-2	PBL	hFcγRIIIA	4	21.6 (6.7)	7.7 (4.0)
		C57BL/6JNIcr	4	23.0 (3.1)	0.4 (0.2)
	Spleen	hFcγRIIIA	10	15.0 (1.4)*	4.9 (0.8)***
		C57BL/6JNIcr	6	14.4 (1.6)**	<0.1

* Splenic NK cells in IL-2-treated hFcγRIIIA mice increased significantly ($P < 0.006$) compared to those from untreated hFcγRIIIA mice
** Splenic NK cells in IL-2-treated C57BL/6JNIcr mice increased significantly ($P < 0.03$) compared to those from untreated C57BL/6JNIcr mice
*** Splenic NK cells expressing hFcγRIII in IL-2-treated hFcγRIIIA mice increased significantly ($P < 0.003$) compared to those from untreated hFcγRIIIA mice

hFcγRIII constituted 2.5% and 1.4% of the cell populations in the peripheral blood and spleens respectively. NK cells from C57BL/6JNIcr mice did not express both 2B4 and hFcγRIII.

To induce in vivo expansion of NK cells, hFcγRIIIA and C57BL/6JNIcr mice received five consecutive daily i.p. injections of 3.0×10^5 IU rhIL-2 prior to the harvest of PBL or splenocytes. IL-2 treatment increased the percentage of NK cells in the peripheral blood and significantly increased the percentage of NK cells in the spleens of both hFcγRIIIA and C57BL/6JNIcr mice ($P < 0.006$ and $P < 0.03$; Table 2). Similarly, IL-2-treated hFcγRIIIA mice showed an increase in the percentage of splenic hFcγRIII⁺ NK cells ($P < 0.003$; Table 2), constituting 33% of the total splenic NK cells (Table 2).

hFcγRIII expression profile in the bone marrow of hFcγRIII transgenic mice

To determine the approximate stage in myeloid cell development in which the hFcγRIII transgene is expressed, bone marrow cells were isolated from hFcγRIIIA, hFcγRIIIB and hFcγRIIIA/B mice and phenotyped by flow cytometry (Fig. 2). CD34⁺ cells, which comprised about 3% of the bone marrow cell population, did not express hFcγRIII. However, the mid-stage myeloid cell precursors, CD59 or Ly-6C⁺, were strikingly positive for hFcγRIII in the hFcγRIIIB (40.2%) and hFcγRIIIA/B (52.2%) mouse strains. hFcγRIIIA mice did not express hFcγRIII on these same cell populations. There was dual staining for CD59 and the granulocyte differentiation marker, Ly-6G in 65.3% of hFcγRIIIA, 44.0% of hFcγRIIIB, and 50.4% of hFcγRIIIA/B bone marrow cells. hFcγRIII⁺ PMN (Ly-6G⁺/hCD16⁺ cells) comprised a large percentage of the bone marrow cells in hFcγRIIIB mice (39.4%) and hFcγRIIIA/B mice (55.0%). However, the PMN in hFcγRIIIA mouse bone marrow did not express hFcγRIII. B and T cells in the bone marrow were negative for hFcγRIII expression in all three transgenic mouse strains.
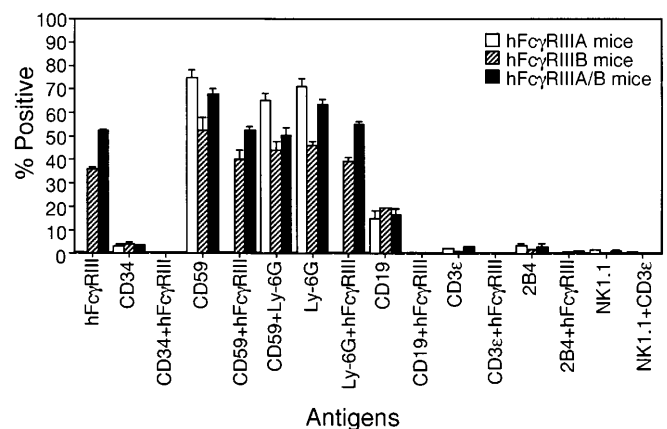

Fig. 2 hFcγRIIIA, hFcγRIIIB, and hFcγRIIIA/B bone marrow cell expression of hFcγRIII. *Bars* represent mean percentage of positive cells of the total live cell population minus isotypic control values. Bone marrow cells were preincubated for 5 min with 2.4G2 (Fc block) to prevent non-specific binding by mAb Fc portions to mFcγR, stained for 30 min with 500 ng–1 µg mAb specific for the antigens listed, and washed twice. For detection of unconjugated primary mAb, 500 ng secondary antibody, goat anti-(rat Ig), FITC-labeled, was added for 30 min and the cells were washed twice. Cells from four hFcγRIIIA, two hFcγRIIIB, and four hFcγRIIIA/B mice were tested. *Error bars* SEM

Expression of hFcγRIII in splenocyte subpopulations of naive and IL-2-treated hFcγRIII transgenic mice

An expanded panel of mAb was used to characterize the splenocytes of hFcγRIIIA, hFcγRIIIB and hFcγRIIIA/B mice by flow cytometry. hFcγRIII was expressed in 2.4%, 4.2%, and 5.4% of splenocytes of naive hFcγRIIIA, hFcγRIIIB, and hFcγRIIIA/B mice respectively (Fig. 3A, left panel). After 5 days of i.p. IL-2 treatment the percentages increased to 5.5% in hFcγRIIIA, 5.6% in hFcγRIIIB, and 11.7% in hFcγRIIIA/B mice (Fig. 3A, right panel) ($P = 0.04$, 0.07, and 0.00005, respectively). Neither αβ nor γδ splenic T cells from all three strains expressed significant levels of hFcγRIII with or without IL-2 treatment (Fig. 3A).

Figure 3B displays hFcγRIII expression on myeloid lineage cells and macrophages. Mid-stage myeloid pre-

Fig. 3A–C Expression of hFcγRIII by murine spleno-cytes. Mean percentages of positive cells from a total splenocyte gate from each mouse strain are shown. Gates exclude propidium-iodide-stained cells. *Left panels* sple-nocytes from untreated mice; *right panels* splenocytes from mice treated for 5 days i.p. with 3×10^5 IU recombinant human interleukin-2 (rhIL-2). Cells were preincubated 5 min with Fc block (2.4G2) to prevent non-specific binding of primary mAb and then incubated for 30 min with FITC- and PE-conjugated mAb to the various murine antigens listed as well as to hFcγRIII. Negative isotypic control values were subtracted from all results. These data reflect the results from two hFcγRIIIA, two hFcγRIIIB, and four hFcγRIIIA/B mice. *Error bars* SEM. **A** hFcγRIII and T cell marker expression in splenic mononuclear cells of transgenic hFcγRIII mice. **B** hFcγRIII and myeloid/mac-rophage marker expression in transgenic murine splenocytes. **C** hFcγRIII and NK marker expression by splenic cells in transgenic mice
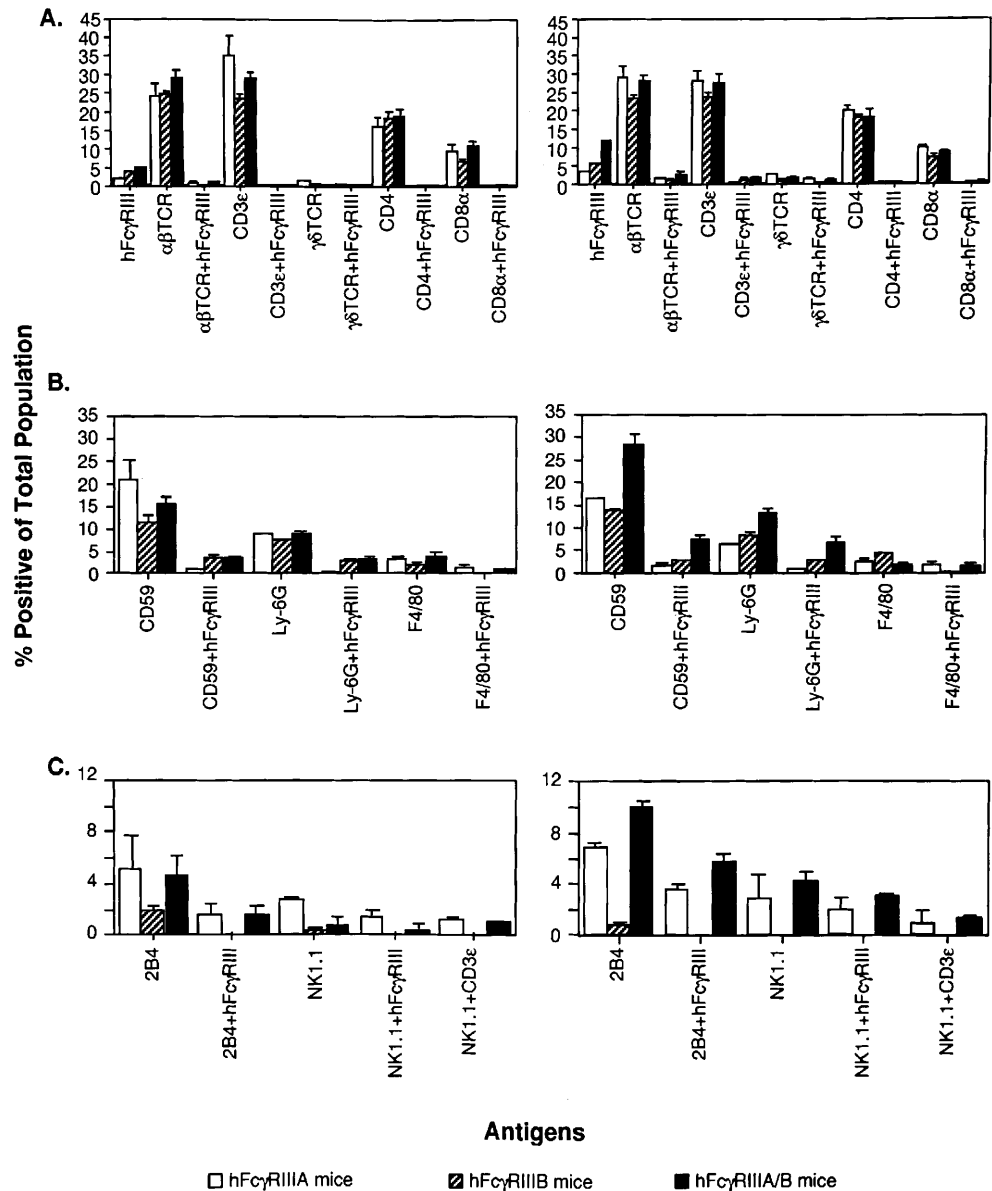
cursor cells staining positive for CD59 and hFcγRIII in the spleens of hFcγRIIIA/B mice were 3.6% in un-treated mice and 7.3% in IL-2-treated hFcγRIIIA/B animals ($P = 0.07$). hFcγRIIIB mouse splenic macro-phages did not express hFcγRIII.

Figure 3C illustrates the NK cell subpopulations in the spleens of hFcγRIII transgenic mice, and the dis-tribution of hFcγRIII on these cells. Splenic NK cell expansion by in vivo IL-2 therapy was only evident in the hFcγRIIIA/B mice, with an increase in $2B4^+$ cells from 4.7% to 10.1% ($P = 0.04$). The percentage of $2B4^+/hFc\gamma RIII^+$ NK cells increased only in the hFcγRIIIA/B strain following IL-2 treatment (from 1.6% to 5.9%, $P = 0.02$). Both hFcγRIIIA and hFcγRIIIA/B mouse spleens possessed $CD3\varepsilon^+$ NK cells. This population of cells constituted approximately 1% of the total splenocyte populations in both strains with and without IL-2 treatment.

Kinetics of IL-2 induction of hFcγRIII expression

Cohorts of three mice were sacrificed at various times following 5 consecutive days of i.p. injections of 3×10^5 IU rhIL-2. Results are shown in Fig. 4A, B. The percentage of peritoneal cells expressing hFcγRIII in-creased from 35% at baseline to a peak of 69.2% on day 3, but then declined to 48.7% on day 5. The percentage of splenocytes expressing hFcγRIII was 18.1% at base-line and increased to 41.2% on day 2, with sustained expression through day 5.

hFcγRIII-mediated ADCC in transgenic hFcγRIII mice

The ability to trigger ADCC via hFcγRIII by hFcγRIII transgenic mouse splenocytes was examined using whole IgG and F(ab')₂ fragments of the bsAb 2B1 to determine
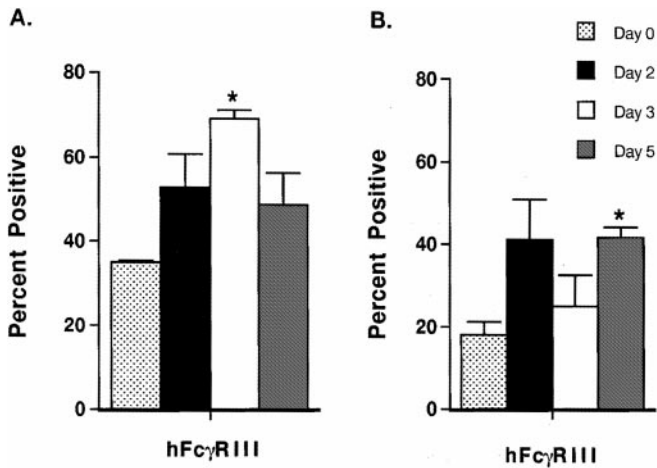
Fig. 4A, B The kinetics of IL-2 induction of hFcγRIII expression on (**A**) hFcγRIIIA/B mouse peritoneal cells and (**B**) hFcγRIIIA/B mouse splenocytes. Cohorts of three hFcγRIIIA/B transgenic mice were treated with 3×10^5 IU rhIL-2 from days 0 to 4 and sacrificed at the times shown for fluorescence-activated cell sorting analysis of hFcγRIII expression. Results shown are mean values ± SEM. * Difference from baseline ($P < 0.05$). **A** The day-5 hFcγRIII$^+$ percentage is artificially low because a significant portion of the hFcγRIII$^+$ peritoneal cell population was so bright it was off the logarithmic FL1/FL2 scale

the functional capacity of this transgenic receptor. Figure 5 illustrates a series of cytotoxicity studies using naive and IL-2-treated C57BL/6JNIcr (Fig. 5A), hFcγRIIIA (Fig. 5B), hFcγRIIIB (Fig. 5C), and hFcγRIIIA/B (Fig. 5D) mouse splenocytes as effector cells. Incubation of untreated C57BL/6 splenocytes and HER-2/*neu*-overexpressing SK-OV-3 tumor cells with 2B1 resulted in no antibody-promoted enhancement of lysis in the absence (Fig. 5A, left panel) or presence of IL-2 (Fig. 5A, right panel). Splenocytes from hFcγRIIIA mice exhibited 2B1-enhanced, specific tumor cytotoxicity in the absence and presence of IL-2. At 25:1 ratios, the addition of 2B1 enhanced cytotoxicity from 0.9% to 5.3% ($P = 0.05$) in the absence of IL-2 (Fig. 5B, left panel), and from 6.6% to 18.1% when the mice were treated with IL-2 ($P = 0.006$; Fig. 5B, right panel). Comparable results were obtained with either whole IgG 2B1 or F(ab')$_2$ fragments of 2B1 (data not shown) using hFcγRIIIA mouse IL-2-treated splenocytes. Incubation of these cells with the parental IgG1 mAb of 2B1 had differing effects on cytotoxicity. Whole IgG 520C9 (anti-HER-2/*neu*) produced equivalent results to 2B1 F(ab')$_2$, giving 25.8% lysis of target cells compared with 26.3% lysis when 2B1 F(ab')$_2$ was used at 50:1 E:T ratios (data not shown). Incubation of a 50:1 dilution of IL-2-treated hFcγRIIIA mouse splenocytes with F(ab')$_2$ 520C9, MOPC-21, and whole IgG 3G8 produced results similar to those seen when no mAb was used (data not shown). Splenocytes from IL-2-treated C57BL/6JNIcr mice showed no ADCC (data not shown).

FcγRIII-mediated cytotoxicity was also observed in hFcγRIIIB and hFcγRIIIA/B mice (Fig. 5C, D). At a 25:1 E:T ratio, naive hFcγRIIIB splenocytes promoted ADCC – from −0.7% without mAb to 4.8% with 2B1 ($P = 0.04$) – of SK-OV-3 cells (Fig. 5C, left panel). This effect was more pronounced when IL-2-treated splenocytes were used, with cytotoxicity of 1.9% without mAb and 15.2% with the addition of 2B1 at a 25:1 E:T ratio ($P = 0.009$; Fig. 5C, right panel).

Lysis potentiation also was observed with the addition of 2B1 to hFcγRIIIA/B splenocytes (Fig. 5D). With hFcγRIIIA/B naive splenocytes at 25:1 E:T ratios, cytotoxicity increased from 0% to 4.5% when 2B1 was added ($P = 0.01$; Fig. 5D, left panel). Similarly, with IL-2 treatment, hFcγRIIIA/B mouse splenocytes at identical E:T ratios promoted more tumor lysis in the presence of 2B1 (13.4%) than in the absence of mAb (2.1% lysis, $P = 0.07$; Fig. 5D, right panel).

To ascertain whether FcγRIII-mediated cytotoxicity was confined to γδTCR/CD3ε-hFcγRIIIA subpopulations, IL-2-treated hFcγRIIIA mouse splenocytes were depleted of γδ T cells. 2B1-promoted cytotoxicity was maintained following γδ T cell depletion. At 50:1 and 25:1 E:T ratios, the pre-depleted population of hFcγRIIIA splenocytes promoted 13.0% and 8.5% lysis respectively; following depletion the cells produced cytotoxicity of 18.4% and 10.5% respectively (data not shown). The splenocyte populations in these experiments contained few cells dually positive for γδTCR/hFcγRIII before depletion.

hFcγRIII-mediated internalization by hFcγRIII transgenic mouse peritoneal macrophages

The functional properties of hFcγRIII were further investigated in the hFcγRIIIA/B transgenic mouse strain to determine whether the receptor was capable of internalization as well as ADCC. Results are displayed in Table 3. hFcγRIIIA/B and C57BL/6JNIcr mice were treated with thioglycollate, and freshly isolated peritoneal cells were incubated with PE-conjugated mAb to murine (m) TfR (anti-CD71), mFcγRII/III (2.4G2) and hFcγRIII (3G8) as well as their respective PE-conjugated isotypic controls. All samples (except 2.4G2-PE) were preincubated with unconjugated 2.4G2 to block non-specific binding by the Fc portions of the IgG to mFcγRII/III. Antibodies to mTfR, mFcγRII/III, and hFcγRIII were all internalized by hFcγRIIIA/B transgenic mouse peritoneal macrophages. When crosslinked with the secondary GAM-FITC conjugate, almost 50% of the anti-hFcγRIII mAb was internalized (Table 3).

Internalization was demonstrated on cytospin preparations of the flow cytometry samples by confocal microscopy (Fig. 6). 3G8-PE localized to the extracellular membranes of hFcγRIIIA/B peritoneal macrophages at 0 min (Fig. 6A). At that time, acid-washed cells showed little to no fluorescence due to 3G8-PE (Fig. 6B); however, the PE label could be demonstrated intracellularly after a 60-min incubation at 37 °C, followed by an acid-wash step (Fig. 6C).

Fig. 5A–D Cytotoxicity of ^{51}Cr-labeled SK-OV-3 target cells by (**A**) C57BL/6JNIcr, (**B**) hFcγRIIIA, (**C**) hFcγRIIIB, and (**D**) hFcγRIIIA/B mouse effector cells. Freshly isolated murine splenocytes were diluted to 50:1, 25:1 and 5:1 E:T ratios in cell culture medium and incubated for 4 h at 37 °C with ^{51}Cr-labeled SK-OV-3 cells in the absence or presence of 2B1 bsAb. One-half of the supernatant was removed from each well, and radioactive label released into the supernatant was measured (cpm) on a gamma counter. *Left panels* results using naive mouse splenocytes; *right panels* results obtained using splenocytes obtained from mice treated with 3×10^5 IU rhIL-2 i.p. for 5 days. 2B1 IgG was added at a final concentration of 1 µg/ml. Percentage lysis was determined by dividing the radioactivity released in the test well (cpm) by half of the total radioactivity (cpm) originally added. Spontaneous release of ^{51}Cr by ^{51}Cr-SK-OV-3 cells alone was subtracted. All points with error bars are mean values ± SEM (**A**, **C**, $n = 3$; **B**, **D**, $n = 4$)
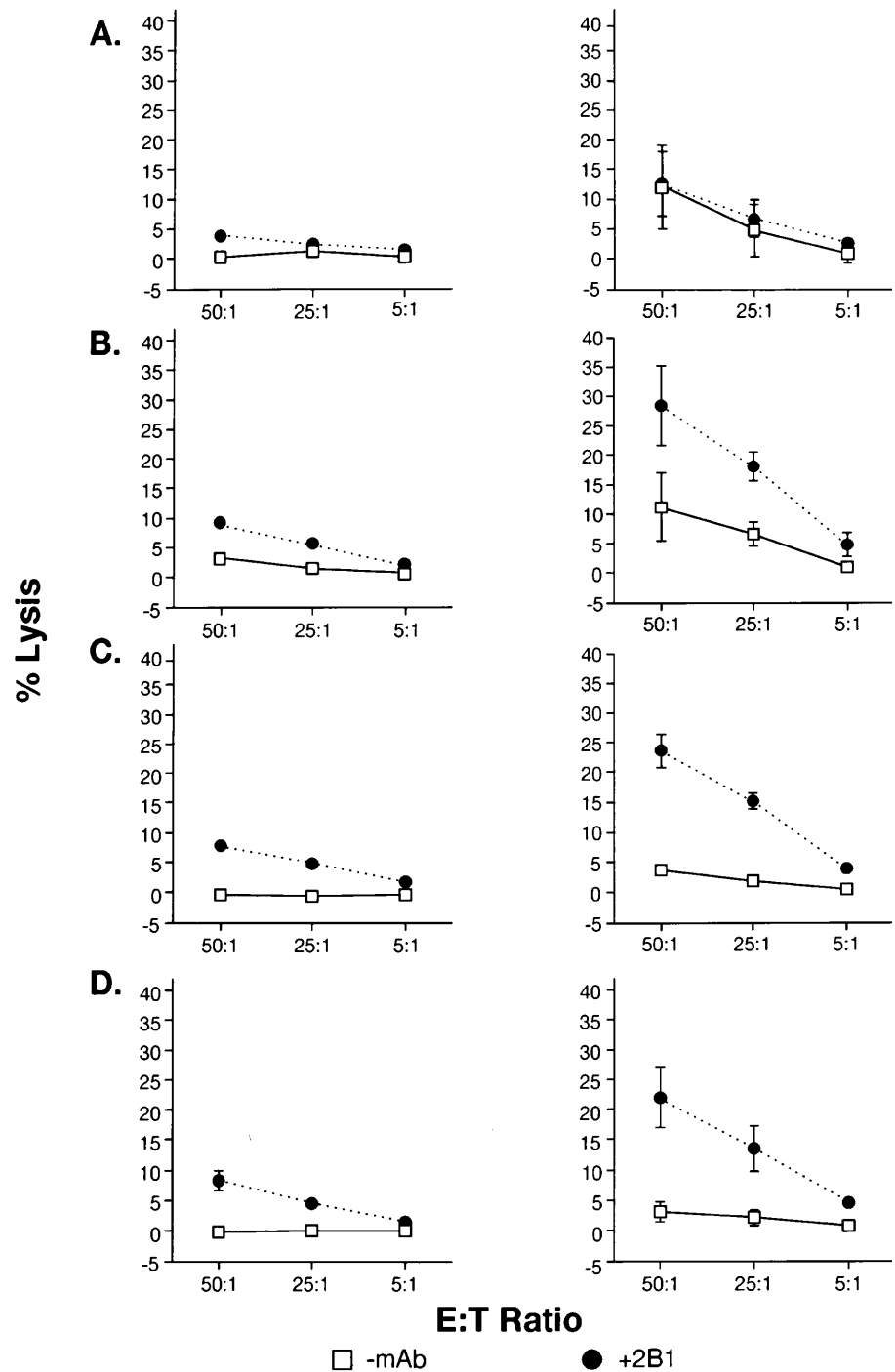
Discussion

This is the first demonstration of the leukocyte expression patterns of hFcγRIII in hFcγRIIIA/B transgenic mice. In hFcγRIIIA mice, hFcγRIII is expressed on the majority of peritoneal macrophages, on approximately 30%–50% of IL-2-treated peripheral white blood cells and splenic NK cells (Table 2), and on 74% of the splenic macrophage population (Fig. 3B). The expression of hFcγRIII in hFcγRIIIB mice is restricted to PMN in the peripheral blood and in the spleen (Fig. 3). Peritoneal macrophages and splenic NK cells from hFcγRIIIB mice did not express hFcγRIII (Fig. 1). Transgenic mice that possess both the A and B isoforms of hFcγRIII express this receptor on peritoneal macrophages, splenic NK cells, and peripheral blood PMN (Fig. 1). The expression patterns of hFcγRIII in the hFcγRIIIA, hFcγRIIIB, and hFcγRIIIA/B transgenic mice support tissue-specific isoform expression of hFcγRIII in these mice.

The developmental expression of hFcγRIII in the bone marrow of transgenic mice (Fig. 2) is similar to

Table 3 Receptor-mediated internalization by hFcγRIIIA/B transgenic murine peritoneal macrophages. Percentage internalization was determined by a flow-cytometry-based assay. Briefly, thioglycollate-elicited peritoneal macrophages were incubated with PE-conjugated mAb to the receptors listed, washed, and then subjected to fixing or acid washing at 0 min or incubated for 120 min at 37 °C and processed in the same manner. Fluorescence-activated cell sorting (FACS) analysis was performed using a macrophage gate. Mean results with hFcγRIIIA/B mice are from three experiments for mTfR and hFcγRIII, and two experiments for mFcγRII/III and crosslinked hFcγRIII. The SEM did not exceed 27% of any mean value. Negative isotypic control values for each corresponding mAb were subtracted. Results from one C57BL/6JNIcr experiment are shown. hFcγRIII was crosslinked via a separate incubation with FITC-labeled goat anti-(mouse Ig), for 30 min at 4 °C, and the samples were then processed at 0 min and 120 min with and without acid washing for FACS analysis

Target	Internalization (%)	
	$t = 0$	$t = 120$ min
hFcγRIIIA/B mice		
mTfR	14.6	40.5
mFcγRII/III	8.5	34.1
hFcγRIII	10.7	30.0
hFcγRIII-crosslinked	23.9	47.5
C57BL/6JNIcr mice		
MTfR	13.3	44.4
mFcγRII/III	9.1	46.4
hFcγRIII	Not bound[a]	Not bound[a]
hFcγRIII-crosslinked	Not bound[a]	Not bound[a]

[a] The mean fluorescence indices of these samples were within background range, thus the mAb were not bound

that seen for this receptor in human bone marrow. The percentages of hFcγRIII⁺ bone marrow cells from hFcγRIIIB and hFcγRIIIA/B strains of mice and the lack of hFcγRIII expression in the bone marrow of hFcγRIIIA mice concur with the percentages observed in normal human bone marrow and results showing that human bone-marrow-derived macrophages were negative for hFcγRIII expression [2, 25]. In addition, the expression of the hFcγRIII transgene at later stages of myeloid cell differentiation is in agreement with reports illustrating that hFcγRIII is first expressed at the metamyelocyte stage in human bone marrow [13, 54].

NK cell populations represent less than 10% of the peripheral blood leukocyte population. Therefore, functional characterization of the hFcγRIII transgene in hFcγRIIIA and hFcγRIIIA/B transgenic mouse cells required expansion of this cell population. The expression of hFcγRIII by mouse leukocytes is dependent in part on IL-2 induction in a manner similar to that seen in humans [37, 38, 57]. IL-2 treatment resulted in both a total NK cell increase and an increase in the hFcγRIII⁺ NK cell population, similar to that seen with human NK cell cytokine priming. IL-2-driven regulation of hFcγRIIIA expression increased the percentage of hFcγRIIIA⁺ NK cells in the total NK cell population from approximately 25% to 33% in PBL, and from 17% to 33% in splenocytes (Table 2). Similar increases were seen in hFcγRIIIA/B mouse splenocytes (Fig. 3C). The induction of hFcγRIII expression in hFcγRIIIA/B mice

Fig. 6A–C Confocal microscopy of hFcγRIIIA/B mouse peritoneal macrophages. Thioglycollate-elicited peritoneal macrophages were harvested, pre-incubated 5 min with 2.4G2 to block mFcγR binding (except for cells stained for mFcγRII/III), and incubated with PE-labeled mAb to mTfR, mFcγRII/III and hFcγRIII for 30 min at 4 °C. Cells were then either washed and fixed or acid-washed at 0 min, or were allowed to incubate at 37 °C for 60 min, and then processed with or without acid washing for flow cytometry to calculate the percentage of mAb internalized. From these samples, cytospin preparations were made and subsequently viewed with a Bio-Rad MRC600 laser scanning confocal microscopy system with a 60× lens. The images were rendered on a Silicon Graphics workstation with VoxelView software. The images are representative of two experiments (the first was performed with a 120-min assay time). **A** 3G8-PE, 0 min, with no acid washing; **B** 3G8-PE, 0 min, following acid washing; **C** 3G8-PE, 60 min, with acid washing

by IL-2 treatment was seen 2 days following initiation of therapy and was sustained throughout the treatment period. Effects were seen on hFcγRIII expression in peritoneal cells (Fig. 4A), splenocytes (Fig. 4B), and peripheral blood cells (not shown). The expression pattern of hFcγRIII by the NK cells of hFcγRIIIA and hFcγRIIIA/B mice could serve as a model for the further in vivo study of the development and maturation of FcγRIII$^+$ NK cells.

The transgene is functional and can be triggered to promote ADCC. Splenocytes from IL-2-treated hFcγRIIIA, hFcγRIIIB and hFcγRIIIA/B mice exhibited enhanced cytotoxicity towards human HER-2/neu-overexpressing SK-OV-3 tumor targets in the presence of the bsAb 2B1 (Fig. 5B–D) or its F(ab')$_2$ fragments (data not shown). Splenocytes from C57BL/6JNIcr control mice did not exhibit any augmented cytotoxicity in the presence of 2B1 (Fig. 5A). The enhanced cytotoxicity observed with 2B1 IgG, 2B1 F(ab')$_2$ fragments, and 520C9 IgG, but not 520C9 F(ab')$_2$, suggests that ADCC can be mediated through the triggering of hFcγRIIIA by 2B1 IgG or F(ab')$_2$. Previous work by our laboratory supports this conclusion [67]. In those studies, it was shown that 520C9 IgG, or 2B1 IgG, augmented the cytotoxicity of IL-2-treated human PBL, and that 2B1-promoted cytotoxicity was preserved in the presence of competing human IgG [67]. In hFcγRIIIA transgenic mice, NK cells appear to be primarily associated with the observed ADCC. It could not be demonstrated that γδ TCR/CD3ε$^+$ cells expressed hFcγRIIIA. Depletion of the γδ TCR/CD3ε$^+$ subset from splenocytes did not diminish cytotoxic activity.

Splenocytes from the hFcγRIIIB strain of transgenic mice were found to mediate hFcγRIII-directed ADCC efficiently and specifically, possibly because of the heterologous association of the transgenic receptor with intracellular signaling proteins or mFcγRII/III. Further studies are required to elucidate the mechanisms underlying this observation. This result may be due to the association of this transgenic receptor with murine Fc receptor signaling chains, since it is known that the association of hFcγRIIIB with hFcγRIIA enhances hFcγRIIA-mediated functions [6, 47]. In addition, complement receptor 3 is known to act as a signaling partner for glycosyl phosphatidyrinositol-linked hFcγRIIIB and is able to thus mediate antibody-dependent phagocytosis [27, 53].

We investigated the functional status of the transgenic receptor of hFcγRIIIA/B mice by testing the internalization by hFcγRIII$^+$ peritoneal macrophages of the 3G8 mAb, which specifically engages the extracellular domain of this receptor. As expected, the anti-(murine Fc receptor) mAb, 2.4G2, and the anti-(murine tranferrin receptor) (CD71) mAb were efficiently internalized by these cells, as was 3G8 (Table 3, Fig. 6). The specificity of this effect for the transgenic protein was demonstrated by the absence of 3G8 internalization by C57BL/6JNIcr peritoneal macrophages (Table 3).

In summary, we have characterized the in vivo distribution of hFcγRIII$^+$ leukocytes in hFcγRIIIA/B transgenic mice. hFcγRIII$^+$ NK cells from the spleens of the hFcγRIIIA/B transgenic mouse strains mediate FcγRIII-directed ADCC towards human tumor cells and hFcγRIII$^+$ transgenic murine peritoneal macrophages are capable of mediating receptor-directed internalization. hFcγRIIIA/B transgenic mice thus can serve as models to evaluate agents designed to exploit the therapeutic potential of FcγRIII-directed leukocyte function. Transgenic mouse strains with hFcγRIII$^+$ peritoneal macrophages that efficiently internalize antibody-receptor complexes permit the study of FcγRIII-targeted immunization strategies [18, 31]. These observations provide a basis for using hFcγRIIIA/B transgenic mice to evaluate novel cancer immunotherapeutic strategies preclinically that involve hFcγRIII. In addition, hFcγRIIIA/B transgenic mice will provide in vivo model systems that can be employed to address mechanistic questions regarding leukocyte migration in such preclinical studies.

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