Development and Characterization of Monoclonal Antibodies Specific for Mouse and Human Fcγ Receptors


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FcγRs are key regulators of the immune response, capable of binding to the Fc portion of IgG Abs and manipulating the behavior of numerous cell types. Through a variety of receptors, isoforms, and cellular expression patterns, they are able to fine-tune and direct appropriate responses. Furthermore, they are key determinants of mAb immunotherapy, with mAb isotype and FcγR interaction governing therapeutic efficacy. Critical to understanding the biology of this complex family of receptors are reagents that are robust and highly specific for each receptor. In this study, we describe the development and characterization of mAb panels specific for both mouse and human FcγR for use in flow cytometry, immunofluorescence, and immunocytochemistry. We highlight key differences in expression between the two species and also patterns of expression that will likely impact on immunotherapeutic efficacy and translation of therapeutic agents from mouse to clinic. *The Journal of Immunology, 2015, 195: 000–000.

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The FcγRs are a highly related family of membrane receptors that bind the Fc regions of Abs of the IgG subclass, thereby linking Ag recognition with cellular response and effector function. The family consists of both high- and low-medium-affinity receptors, capable of binding monomeric and multimeric (immune-complexed) IgG, respectively (1). In humans, the FcγR are found on chromosome 1 with the genes encoding the low-medium-affinity receptors (FcγRIIA [CD32A], -IIB [CD32B], -IIC [CD32C], -IIIA [CD16A], and -IIIB [CD16B]) (2, 3) located together at the 1q23.3 locus and the high-affinity FcγRI (CD64) genes found more distally at 1q21 (FcγRIIIA and -C) (4) and 1p12 (FcγRIB). This places the FCGR1I gene family within a large pericentric linkage group that is conserved between humans and mice (5). It is unsurprising then that equivalent receptors are also found in similar locations in the mouse with the low–medium-affinity receptors (FcγRII [CD32], -III [CD16], and -IV) located together on chromosome 1 and FcγRI found on chromosome 3 near the end of the region that shows homology with human chromosome 1.

FcγR can be expressed on a wide range of cell types including key immune effectors such as macrophages, neutrophils, and NK cells, as well as epithelial and sinusoidal cells (6–9). Functionally, they can be broadly divided into three main types: activatory (human: FcγRI, -IIA, -IC, and -IIIA; mouse: FcγRII, -III, and -IV), which signal via an ITAM, inhibitory (human: FcγRIIB; mouse: FcγRII), which signal via an ITIM, and neutral, which bind Fe but do not directly signal (human FcγRIIIB).

The consequences of FcγR engagement vary from phagocytosis and cytotoxic granule release to cytokine production and release of inflammatory mediators (10). These outcomes depend on a number of factors, including Ab isotype, cell type, prior/current activation status, and the balance of activatory and inhibitory FcγR signaling (reviewed in Ref. 11).

It is clear that all of these factors contribute to shaping and refining the immunological response in vivo. In mouse models, the therapeutic and immune-stimulating activity of a range of mAb correlates with their isotype and associated FcγR binding profiles. Although a large body of data indicated that all mAb would become more effective with stronger engagement of activatory relative to inhibitory FcγR, this paradigm has recently been challenged (12–14, reviewed in Ref. 15). These findings clearly demonstrate that for mAb targeting the majority of TNF receptor superfamily members, stronger engagement of the inhibitory FcγR promotes their immunostimulatory activity. However, the data in terms of the FcγR...
requirements for immunomodulatory anti-cancer mAb activity are more complex, with some (CD40 and DR5) dependent on FcγRIIB-mediated cross-linking for immunostimulatory and optimal activity and others (CTLA-4, CD27, and glucocorticoid-induced TNR receptor) (16–19) likely depending on activatory FcγR to delete suppressive regulatory T cells, thereby generating effective CTL activity. Regardless of these complexities, it is clear that reagents capable of precisely/specifically staining and manipulating the individual FcγR are critical to further dissect the key mechanisms of action of therapeutic mAbs and to better define the central effector cells and FcγR involved in both induced and ongoing immune responses in normal and pathological settings.

Unfortunately, mAb capable of specifically and exclusively binding individual FcγR have been difficult to generate and/or obtain commercially. For example, the mAb 2.4G2, commonly used as an Fc block, binds both mFcγRII and -III, and AT10, KB6.1, and IV3 bind both hFcγRIIA and -B. In humans, this is largely due to an ancestral duplication event that replicated the low-affinity locus, resulting in the creation of highly homologous gene segments. During this process, an unequal crossover event between the 5’ part of FcγRIIB and the 3’ part of FcγRIIA is proposed to account for the creation of FcγRIIC (20), resulting in FcγRIIB and -IIc having identical extracellular domains that are indistinguishable by cell-surface staining alone.

We have therefore developed and characterized a series of reagents specific for either mouse or human FcγR (hereafter referred to as mFcγR or hFcγR, respectively, for clarity). To facilitate translation from mouse models to the clinic, we further characterized reagents capable of recognizing each FcγR using the widely available analytical tools of flow cytometry, immunofluorescence (IF), and immunohistochemistry (IHC) in both fresh and formalin-fixed, paraffin-embedded (FFPE) tissue.

Materials and Methods

Animals and cells

BALB/c and C57BL/6 mice (originally from Charles River Laboratories, Harlow, Essex, U.K. and Harlan Laboratories, Blackthorn, Oxfordshire, U.K., respectively) and Wistar rats were bred and maintained in local facilities. mFcγRIIIB, IIc, IIIc, γ-chain−/− mice have been described previously (21, 22). Heterozygous mFcγRIIA mice were intercrossed to obtain mFcγRIIA−/− mice (23) and were intercrossed to obtain mFcγRIIA−/− by the Helmholtz Zentrum München (Munich, Germany) and Wistar rats were bred and maintained in local facilities. mFcγRIIIA−/− mice were generated from C57BL/6J (mFcγRIIIA−/−) backcrossed onto the BALB/c or C57BL/6J background for at least 10 generations. Heterozygous mFcγRIIIA mice were intercrossed to obtain mFcγRIIIA−/− by the Helmholtz Zentrum München (Munich, Germany) and Wistar rats were bred and maintained in local facilities. mFcγRIIIA−/− mice were generated from C57BL/6J (mFcγRIIIA−/−) backcrossed onto the BALB/c or C57BL/6J background for at least 10 generations. Heterozygous mFcγRIIIA mice were intercrossed to obtain mFcγRIIIA−/− by the Helmholtz Zentrum München (Munich, Germany) and Wistar rats were bred and maintained in local facilities. mFcγRIIIA−/− mice were generated from C57BL/6J (mFcγRIIIA−/−) backcrossed onto the BALB/c or C57BL/6J background for at least 10 generations.

R Abs and reagents

All full-length FcγR and FcγR-ratCD4 (rCD4) fusion proteins were constructed in house. Briefly, FcγR were amplified from cDNA obtained from primary human leukocytes (24) or C57BL/6 splenocytes using specific primers. For FcγR-rCd4 fusion proteins, FcγR extracellular domains were cloned into the pcDNA3.1 vector N-terminal to rCD4 to form soluble fusion constructs (25) before being digested and ligated into the pcDNA3.1 expression vector (Invitrogen, Life Technologies). All full-length FcγRs and FcγR chains were cloned using the Zero Blunt TOPO PCR Cloning kit and recloned into the pcDNA3.1 expression vector (Invitrogen, Life Technologies). All constructs produced were tested for transient expression in a range of cell types. IgG was purified on Protein A with purity assessed by electrophoresis (Beckman EP system; Beckman Coulter, High Wycombe, Buckinghamshire, U.K.) and lack of aggregation determined by HPLC. F(ab)2 fragments were produced as described previously (26).

Rat IgGl and human IgGl derivatives of AT130-2 (m2a) were generated from the cloned V regions and subcloned into the rat or human FcγRI framework as previously described (27). All strains were also produced as a mIgGl N297A mutant to minimize FcγR interactions from its own Fc region (27). A full list of anti-mFcγR mAb isotypes, formats, and applications is contained in Table I. Abs used to detect human FcγR by flow cytometry were as follows: hFcγRI (10.1) and hFcγRIII (3G8) (Fab’2) were produced in house from mAb obtained from BioLegend (London, U.K.) and American Type Culture Collection, respectively. Allophycocyanin-labeled hFcγRIIA clone N92Q-2E08) or -B (clone N92Q-7C07) and IgGl isotype control (clone N92Q-FT18C) (28) were generated by BioInvent International (Lund, Sweden) using phage display technology (29). Abs used to detect human FcγR by IHC were as follows: hFcγRI (clone 3D3; Abcam, Cambridge, Cambridgeshire, U.K.); hFcγRIIA (clone EPR6658; Abcam); hFcγRIIB and -C (clone EP886Y; Abcam); and hFcγRIIA and -B (clone 2H7; AbD Serotec, Kidlington, Oxfordshire, U.K.). The Abs used to label human monocytes/macrophages or B cells in IHC double-staining were CD68 (clone PG-M1) and CD20cy (clone L26) (DakoCytomation, Agilent Technologies, Glostrup, Denmark), respectively. A full list of anti-hFcγR mAb isotypes, formats, and applications is contained in Table I.

Immunization procedures

Anti-mFcγRI, -II, -III, and -IV mAbs were raised using conventional hybridoma technology (16–19) likely depending on activatory FcγR to delete suppressive regulatory T cells, thereby generating effective CTL activity. Regardless of these complexities, it is clear that reagents capable of precisely/specifically staining and manipulating the individual FcγR are critical to further dissect the key mechanisms of action of therapeutic mAbs and to better define the central effector cells and FcγR involved in both induced and ongoing immune responses in normal and pathological settings.

We have therefore developed and characterized a series of reagents specific for either mouse or human FcγR (hereafter referred to as mFcγR or hFcγR, respectively, for clarity). To facilitate translation from mouse models to the clinic, we further characterized reagents capable of recognizing each FcγR using the widely available analytical tools of flow cytometry, immunofluorescence (IF), and immunohistochemistry (IHC) in both fresh and formalin-fixed, paraffin-embedded (FFPE) tissue.
mean fluorescence seen during the titration curve for each mAb, as follows: % maximum binding = observed binding/maximum binding × 100.

Ex vivo expression of mouse FcγR was determined as follows: blood, spleen, or bone marrow samples from C57BL/6 mice were stained on ice for 30 min with CD11b-PE, Ly6G-PE-Cy7, Ly6C-PerCP-Cy5.5, NK1.1-eFluor 450, CD19-allophycocyanin-Cy7, CD3–BD Horizon 500 (BD Biosciences or eBioscience, Hatfield, Hertfordshire, U.K.) and F4/80 APC (AbD Serotec) together with the relevant FITC-conjugated anti-mouse FcγR mAb (10–50 μg/ml). BCCs were lysed using RBC lysing buffer (AbD Serotec). For analysis, doublets were excluded based on forward light scatter area (FSC-A) vs. FSC width. Cell types were identified as follows: macrophages, CD11bint; neutrophils, CD11bintF4/80ly6G–ly6Cint; monocytes, CD11bF4/80ly6Gly6Cint; NK cells, NK1.1–CD3–; B cells, CD19–CD3+; and T cells, CD3+CD19+. The geometric mean fluorescence intensity (MFI) for each FcγR was determined.

Ex vivo expression of human FcγR was determined as follows: blood, spleen, or bone marrow samples were stained on ice for 30 min with human anti-CD3 PerCP, anti-CD56–PE, mouse IgG1-FITC, the isotype control (BioLegend), anti-CD19 APC-Cy7, or anti-CD14–Pacific Blue (BD Biosciences), or eFluor 450, CD19-allophycocyanin–Cy7, CD3–BD Horizon 500 (BD Biosciences or eBioscience, Hatfield, Hertfordshire, U.K.) and F4/80 APC (AbD Serotec) or biotinylated anti–follicular dendritic cells (FDC; BD Biosciences), or fluorescently conjugated F4/80 (AbD Serotec), rat anti-CD45R (B220; BD Biosciences), rat anti-follicular dendritic cells (FDC; BD Biosciences), or fluorescently conjugated wheat germ agglutinin (WGA; Vector Laboratories) followed by Alexa Fluor 488–conjugated streptavidin or anti-IgG, as required. Sections were mounted in Vectashield Hardset (Vector Laboratories).

Images were collected using a CXX41 inverse microscope equipped with a CDD color camera running under Cell software, using Plan Achromat 10× objective, and a CLSM 5 laser fluorescence system equipped with a CC12 Color Camera running under Cell (see Table I).

### Table I. Mouse and human anti-FcγR panels

<table>
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<tr>
<th>Application</th>
<th>Flow Cytometry</th>
<th>IHC/IF</th>
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<td>Clone</td>
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<td></td>
<td>hFcγRIIIA/B</td>
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**IHC and IF**

**Anti-mouse FcγR.** Fresh spleen and liver tissue was embedded in OCT (CellPath, Newton, Powys, U.K.) and frozen in isopentane on a bed of dry ice. Sections (8 μm) were cut, air-dried (overnight), fixed in 100% acetone (10 min), and blocked with 2.5% normal goat serum before incubation with mFcγR Abs. In order to carry out double IF staining with other rat Abs, human anti-mFcγRII (AT130-2) was used (see Table I).

For IHC, peroxidase inhibitor (Pierce, Thermo Fisher Scientific) was applied for 10 min before incubation with an HRP-conjugated anti-rat IgG polymer (30 min) followed by 3,3′-diaminobenzidine (5 min) and counterstained with hematoxylin (all from Vector Laboratories, Peterborough, Cambridgeshire, U.K.). For double IF, mFcγR Abs were detected (45 min) with Alexa Fluor 568– or 488–conjugated anti-IgG (Life Technologies) or DyLight594–conjugated goat anti-human IgG (Abcam). When using a second rat primary Ab, sections were then incubated with rat IgG (30 μg/ml, 30 min, prepared in house). Abs used for double labeling were FITC-conjugated F4/80 (AbD Serotec), rat anti-CD45R (B220; BD Biosciences), rat anti-follicular dendritic cells (FDC; BD Biosciences), or fluoscently conjugated wheat germ agglutinin (WGA; Vector Laboratories) followed by Alexa Fluor 488–conjugated streptavidin or anti-IgG, as required. Sections were mounted in Vectorshield Hardset (Vector Laboratories).

Images were collected using a CXX41 inverted microscope with reflected fluorescence system equipped with a CDD color camera running under Cell software, using Plan Achromat 10× objective, and a CLSM 5 laser fluorescence system equipped with a CC12 Color Camera running under Cell (see Table I).

### Analysis

#### Statistical analysis

Expression levels of FcγRIIB on different B cell subsets were compared using Wilcoxon matched-pairs comparison.

#### Results

**Generation and characterization of anti-mouse FcγR mAb**

Conventional immunization and hybridoma technology was used to raise a panel of mAb specific to each mFcγR, with the most...
promising selected for further characterization. ELISA assays using mFcγR fusion proteins confirmed that each mAb bound only a single FcγR (Fig. 1A): AT152-9 was specific for mFcγRI, AT154-2 for mFcγRIII, and mFcγRIV were cotransfected with mFcRγ into to 293F cells, whereas mFcγRII was transfected alone. At 48 h after transfection, cells were harvested and stained with the indicated anti-mFcγR before analysis by flow cytometry. Representative data from three independent experiments. Error bars indicate SD.

Table II. Specificity and affinity of anti-mFcγR mAb

<table>
<thead>
<tr>
<th>mAb</th>
<th>mFcγR</th>
<th>Isotype</th>
<th>$k_a$ ($\times 10^5$ mol$^{-1}$s$^{-1}$)</th>
<th>$k_d$ ($\times 10^{-4}$ s$^{-1}$)</th>
<th>$K_D$ (nmol)</th>
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<td>Rat IgG2a</td>
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</table>
surface plasmon resonance, which additionally provided affinity constants for binding (Supplemental Fig. 1, Table II). To minimize possible Fc–FcγR-mediated interactions with other FcγR, mAb were subsequently produced as either F(ab’)2 or mIgG1-N297A variants (see Supplemental Fig. 1B for example SPR data for AT130-2 mIgG2a, mIgG1, and N297A variants). mIgG1 was selected as the backbone, as it does not bind to mFcγRI or -IV; the NA mutation also removes the ability to bind to FcγRIII.

Staining of mFcγR by flow cytometry

Having confirmed mAb binding specificity in the model systems above, we subsequently used the panel of mAb to determine the cell and tissue expression of each mFcγR, comparing blood and sec-

**FIGURE 2.** FcγR expression on mouse leukocyte subsets. Murine spleen, blood, or bone marrow tissue were isolated and dissociated before being labeled with Abs; CD19, NK1.1, CD3, CD11b, Ly6G, Ly6C, F4/80, irrelevant F(ab’); (Irr), or anti-mFcγR. n = 3 representative of at least two independent experiments. (A) Representative histogram overlays of mFcγRs (black) on leukocyte populations (highlighted by circular gate on accompanying dot plot) compared with the irrelevant control (clear). (B) Comparison of mFcγR expression on various leukocyte populations in spleen (black), blood (gray), and bone marrow (white) from WT mice. The hashed bar indicates staining levels with an irrelevant control mAb. Details of Ab clones and formats used are contained in Table I.
ondary lymphoid tissue (spleen and bone marrow). Initial validation of specific binding was performed by comparing results from wild-type (WT) versus mFcγR-null splenocytes on cell types with established FcγR expression patterns; namely macrophages, monocytes, neutrophils, B cells, and NK cells (Fig. 2A; also reviewed in Ref. 33). AT152-9 F(ab′)2 (mFcγRI) staining was confirmed as positive on macrophages and monocytes, AT130-2 N297A (mFcγRII) was positive on B cells, AT154-2 F(ab′)2 (mFcγRIII) was positive on monocytes, neutrophils, and NK cells, and AT137 F(ab′)2 (mFcγRIV) was positive on macrophages and neutrophils. In each case, staining was completely absent on T cells and cells from the mFcγR-null (mFcγRI/II/III/IV2/2) mice, further confirming their fine specificity (data not shown).

Largely equivalent staining patterns were also seen on cells from BALB/c mice, indicating these mAb are not detecting polymorphic differences in mFcγR present in the most commonly used genetically distinct mouse strains (Supplemental Fig. 2A).

Expression of mFcγR in blood, spleen, and bone marrow determined by flow cytometry

We next compared mFcγR expression patterns on monocytes, neutrophils, B cells, and NK cells from bone marrow, spleen, and blood (Fig. 2B); macrophages were not included as their frequency in blood and bone marrow was too low. In general, expression levels were similar in each compartment with the possible exception of cells in the bone marrow, where mFcγRI on monocytes and mFcγRII on B cells were lower than that observed in the blood and spleen. B cell subset expression of mFcγRII is shown in Supplemental Fig. 2B and demonstrates that mFcγRII expression is largely similar throughout B cell development in the bone marrow, with pro-pre, immature, and mature B cells expressing equivalent levels of mFcγRII. In the spleen, T1 and T2/follicular B cells appeared to express equivalent levels of mFcγRII to those in the bone marrow, whereas marginal zone and GC B cells tended to express slightly higher levels.

Immunohistochemical analysis of mFcγR expression

We subsequently explored the mFcγR staining pattern obtained with these mAbs in situ in the spleen and liver using conventional single-color IHC (Fig. 3) and multicolor IF (Fig. 4). Importantly, none of the mAbs gave staining of the spleen or liver from mFcγR−/− mice (Fig. 3).

In mouse spleen, all four mFcγR were expressed with distinct patterns. mFcγRI and -III were expressed by the majority of red pulp (RP) macrophages as judged by their location, morphology (Fig. 3), and costaining with F4/80 (Fig. 4A). mFcγRII and -III were also expressed by cells within the periarteriolar lymphoid sheaths (PALS; Fig. 3, open arrows), which were F4/80 negative (Fig. 4A, open arrows).

mFcγRII was not detected on RP macrophages in the spleen but was expressed on follicular B cells (Fig. 4A, closed arrows, Fig. 4B) and particularly high on FDC in well-defined GCs (Figs. 3, 4A, 4B). In contrast, non-GC FDC did not express mFcγRII (Fig. 4B, open arrows). Interestingly, mFcγRII was more readily detectable on

![FIGURE 3. Distribution of FcγR expression in the mouse spleen and liver. Fresh, frozen spleen or liver was isolated from WT or mFcγR−/− mice, sectioned, and then stained for each of the mFcγR by IHC. mFcγRI, -III, and -IV+ cells are predominantly found in the RP of the spleen, with mFcγRII and -III also expressed on some cells within the splenic PALS (open arrows). In the spleen, mFcγRII is weakly expressed in the B cell follicles (closed arrows) with strong expression within GC. mFcγRII, -III, and -IV+ cells are scattered throughout the liver, whereas FcγRII is expressed on the sinusoidal lining cells. There was no detectable staining with any of the mFcγR Abs on the spleen or liver of mFcγR−/− mice with the respective single mFcγR removed. Details of Ab clones and formats used are contained in Table I. Scale bars, 100 μm.](http://www.jimmunol.org/)

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Follicular B cells in BALB/c spleens, which generally had no or smaller, less well-defined GCs than those of C57BL/6 mice (Supplemental Fig. 3A). mFcγRII was also expressed around splenic vessels (Fig. 4B, asterisk) and on putative RP stromal cells (Fig. 4A, 4B, arrowheads); however, this was variable between mice. mFcγRIV was expressed on a proportion of RP macrophages, but both the level of expression and cell types expressing this receptor were variable between mice (Figs. 3, 4A). For example, strong expression was sometimes observed on megakaryocytes, cells surrounding the splenic vasculature, and small cells scattered throughout the splenic follicles (Supplemental Fig. 3B).

**FIGURE 4.** Double IF of FcγR in the mouse spleen and liver. Fresh, frozen spleen or liver was isolated, sectioned, and then stained for each of the mFcγR by IF. (A) mFcγRI, -III, and -IV are expressed on macrophages in the splenic RP and Kupffer cells in the liver. The mFcγRI and III+ cells within the splenic PALS are not F4/80+ (red staining; open arrows). mFcγRII is not expressed by the Kupffer cells in the liver or by splenic macrophages (red staining) but could be seen in the B cell follicles of the spleen (closed arrows) and on elongated cells within the RP (arrowhead). mFcγRIV expression by RP macrophages was not as extensive as that of mFcγRI and III and was variable between mice. (B) In the spleen, mFcγRII was expressed on follicular B cells (B220; this pattern of expression was variable between mice, Supplemental Fig. 3A). mFcγRII was strongly expressed by FDC within well-formed GCs but not on non-GC FDC (open arrows). In the spleen, mFcγRII staining was also seen on cells lining the vasculature (asterisks) and on stromal cells within the RP (arrowhead); again, this pattern of expression was variable between mice. In the liver, mFcγRII was expressed on sinusoidal lining cells (stained with WGA). Colocalized signal is shown as yellow/orange. Details of Ab clones and formats used are contained in Table I. Scale bars, 100 μm and 50 μm for ×10 and ×40 original magnification images, respectively.
In the mouse liver, expression of mFcγRI, -III, and -IV was lower than that in the spleen; but mFcγRII was highly expressed. The activatory receptors mFcγRI, -III, and -IV but not the inhibitory mFcγRII were expressed on F4/80 + Kupffer cells in the liver (Figs. 3, 4A), with mFcγRII expressed on sinusoidal lining cells, as determined by costaining with WGA (Fig. 4B).

Characterization of anti-hFcγR mAb

We next identified a panel of anti-hFcγR mAb (Table I) and subjected these to a similar analysis before using them to determine the expression patterns of hFcγR in human tissues. To perform initial validation, we transiently expressed the individual hFcγR in 293F cells and assessed the binding of each hFcγR mAb to these cells by flow cytometry (Fig. 5A). 10.1 and 3G8 are commercial anti-hFcγRI and anti-hFcγRIII mAbs, respectively, and were confirmed to bind specifically to 293F cells expressing these receptors. 3G8 bound to both 158V and F alleles of hFcγRIIIA as previously reported (34). 2E08 and 7C07 were identified by screening the n-CoDeR scFv phage display library using fusion proteins encoding the extracellular domain of hFcγRIIA or hFcγRIIB as targets, respectively (28). Given that our hFcγR were transiently transfected, and some cells expressed supraphysiological levels of FcγR, we used Ramos cells stably transfected with either FcγRIIA or FcγRIIB to demonstrate the fine specificity of 2E08 and 7C07, respectively (Fig. 5B). Binding specificity was also confirmed by surface plasmon resonance, which additionally provided affinity constants for binding (Supplemental Fig. 4A, Table III). To minimize possible interactions with other FcγR, the hFcγRI (10.1) and hFcγRIII (3G8) mAb were digested to produce F(ab′)2 fragments and hFcγRII mAb (2E08 and 7C07) were subsequently produced as hIgG1-N297Q variants that cannot bind FcγR through their Fc domain (27). Having confirmed specificity of our panel, we then examined staining patterns in the blood and tissues.

Expression of hFcγR in blood, spleen, and tonsils determined by flow cytometry

We next compared hFcγR expression patterns on monocytes, neutrophils, B cells, and NK cells from blood, spleen, bone marrow, and tonsil. First we assessed PBMCs from the blood (Fig. 6A shows representative plots, with Fig. 6B indicating the MFIs for 42 individual healthy donors). Monocytes can be grouped into classical and

![Figure 5](http://www.jimmunol.org/)

**Table III.** Specificity and affinity of anti-hFcγR mAb used for flow cytometry

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<thead>
<tr>
<th>mAb</th>
<th>hFcγR</th>
<th>Isotype</th>
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FIGURE 6. Expression levels of FcγRs on human immune cells determined using flow cytometry. PBMCs from 42 healthy donors (A and B) and immune cells isolated from human spleen, bone marrow (C), and tonsil samples (D) were stained for hFcγRI (using clone 10.1), hFcγRIIA (using clone N297Q-2E08), hFcγRIIB (using clone N297Q-3C07), hFcγRIIIA (using clone 3G8), and isotype controls. (A) shows representative example histogram data (gated populations shown by elliptical gate on accompanying dot plots), and (B) shows the geometric mean for each hFcγR stained and normalized by subtracting the geometric mean value of the corresponding isotype control Ab staining (bars represent mean and SD) on a population of 42 healthy donor samples. The lymphocyte population was determined using FSC-A versus SSC-A, alongside CD14⁺ (monocytes), CD19⁺CD3⁻ (B cells), and CD56⁺CD3⁻ (NK cells) phenotypes. Monocytes were subdivided into CD14⁺CD14⁺ and CD14⁺⁰ and are highlighted in the relevant plots by the elliptical gate. NK cells display major (CD56⁺CD3⁻) and minor (CD56⁺⁰CD3⁻) subsets based upon CD56 intensity and are highlighted in the relevant plots by the elliptical gate. (C) Representative sample histograms from human tissues are shown, alongside their gating strategies (highlighted by elliptical (Figure legend continues)}
two populations. CD14 hi monocytes expressed both hFcRI and hFcRIIA and at higher levels than the less frequent CD14 lo monocytes. In contrast, CD14 lo monocytes expressed more hFcRIIB (albeit still at a relatively low level) than hFcRIIIA than those in the blood. CD14 hi bone marrow monocytes expressed equivalent levels of hFcRI to CD14 hi monocytes in the blood and spleen, but less hFcRIIA and little hFcRIIIA as seen in the blood. CD14 lo bone marrow monocytes expressed higher levels of hFcRI, similar levels of hFcRIIB, and lower levels of hFcRIIIA and IIIA compared with the CD14 lo monocytes in the spleen. The number of NK cells was extremely low in spleen and bone marrow. Gating on these rare cells, hFcRIIB was seen to be lower on the CD56 hi NK cells in the spleen than in the blood. In contrast, expression of hFcRIIIA on the CD56 lo NK cells in the bone marrow was equivalent to that in the blood. The number of CD56 lo NK cells observed in these tissues was too low to accurately define their phenotype.

hFcRIIB was expressed similarly on B cells from spleen and bone marrow compared with the blood, albeit a proportion of B cells stained negatively in the bone marrow. To explore the potential differences in expression of hFcRIIB in rarer B cell populations, we performed equivalent staining on samples that were not previously passed through Lymphoprep (Supplemental Fig. 4B). This analysis identified that granulocytes (predominantly neutrophils) in the blood expressed negligible hFcRI and -IIB, intermediate levels of IIIA, and high levels of hFcRIII, presumably hFcRIIIB (41). In the bone marrow granulocytes, expressed appreciable levels of hFcRI and hFcRIII (albeit hFcRIII was considerably lower than in the blood) and low to negligible levels of hFcRIIIA and IIB.

We next assessed the hFcR staining pattern of these cell types in a selection of other tissues including spleen, bone marrow (Fig. 6C), and tonsil (Fig. 6D). CD14 lo monocytes in the spleen showed similar hFcRI, -IIA, and -IIIA levels to those in the blood. In contrast, CD14 lo splenic monocytes displayed substantially higher hFcRIIB and somewhat lower hFcRIIIA than those in the blood. CD14 hi bone marrow monocytes expressed equivalent levels of hFcRI to CD14 hi monocytes in the blood and spleen, but less hFcRIIA and little hFcRIIIA as seen in the blood. CD14 lo bone marrow monocytes expressed higher levels of hFcRI, similar levels of hFcRIIB, and lower levels of hFcRIIIA and IIIA compared with the CD14 lo monocytes in the spleen. The number of NK cells was extremely low in spleen and bone marrow. Gating on these rare cells, hFcRIIB was seen to be lower on the CD56 lo NK cells in the spleen than in the blood. In contrast, expression of hFcRIIIA on the CD56 lo NK cells in the bone marrow was equivalent to that in the blood. The number of CD56 lo NK cells observed in these tissues was too low to accurately define their phenotype.

Expression of hFcR determined by IHC

Having established the expression patterns of hFcR by flow cytometry, we then attempted to investigate their expression patterns in situ by IHC. Unfortunately, the mAb detailed above for flow cytometry were not suitable for IHC with FFPE material and so alternative reagents were sought for this purpose (see Table I). We first confirmed the specificity of this panel of hFcR-specific mAb using stably transfected CHO-k1 cells, fixed and embedded as tissue blocks (Fig. 7).

Once validated, we used these Abs to examine the expression pattern of hFcR in spleen, tonsil, and liver using single-staining (Fig. 8) and double staining for B cells (Fig. 9A) and macrophages (Fig. 9B).

In the spleen, hFcRI, -IIA, and -IIIA expression was prominent in the RP with IIB highly expressed in the white pulp (WP) (Fig. 8). In the tonsil, overall the expression of IIB was much higher than that of the other hFcR. High expression of IIB was evident on cells in the mantle zone, within the follicles, and throughout the interfollicular areas, whereas hFcRI, -IIA, and

FIGURE 7. FcγR staining of hFcγR-transfected CHO-k1 cell lines. CHO-k1 cells stably transfected to express a single hFcγR were embedded in paraffin and stained by IHC using the same protocol as for human tissues. The staining pattern shows the clear monospecificity of each mAb for a single hFcγR. Details of Ab clones and formats used are contained in Table I. Scale bar, 20 μm.

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-III A staining was much more sparse and present, to varying degrees, on relatively few cells scattered throughout the interfollicular areas (Fig. 8) and on cells of the epithelium. hFcγRI was also present on some cells within the follicles (Fig. 8). In the liver, FcγRI, -IIA, -IIB, and -IIIA were all expressed although hFcγRIIA expression was much lower than that of the other hFcγR. FcγRI and -IIB in particular were expressed by sinusaloidal lining cells (Fig. 8, arrows).

Subsequent double-staining with CD20 (Fig. 9A) and CD68 (Fig. 9B) allowed us to distinguish expression patterns of FcγR on B cells and macrophages, respectively. This analysis revealed that FcγRIIB was expressed by B cells in the splenic WP with no expression in the PALS (Fig. 9A) and little expression on the RP macrophages, which, in contrast, were strongly positive for FcγRI, -IIA, and -IIIA (Fig. 9B). There were occasional FcγRI- and IIIA-positive macrophages within the WP of the spleen. In the tonsil, mantle zone B cells and those throughout the interfollicular tissue were strongly positive for FcγRIIB with a weaker expression on the GC B cells (Fig. 9A) in agreement with our flow cytometry data (Fig. 6D). There were few hFcγRI- , -IIA-, and -IIIA-positive cells within the tonsil; however, there was strong expression of all three by the Langerhans cells of the tonsil epithelium (Fig. 9B, arrowheads). NB: these areas in Fig. 9B were selected to best highlight this epithelial staining. In the liver, hFcγRII, -IIA, and -IIIA, but not IIB, were shown to be expressed by the liver macrophages, Kupffer cells (Fig. 9B; arrows).

Discussion

In this study, we have generated, developed, and/or characterized panels of mAb specific for h- and mFcγR. The human low-affinity FcγR locus developed by segmental duplication and so features a high level of homology between the various family members. For example, hFcγRIIA and -IIB are 93% identical in their extracellular domain. Nonetheless, we have developed and characterized reagents capable of discriminating these receptors by both flow cytometry and IHC/IF.

The anti-mFcγR described in this study were validated using a comprehensive panel of FcγR fusion proteins, transfected cells, and FcγR+/− mice. The anti-hFcγR mAb were validated through use of a combination of hFcγR fusion proteins, transfected cells, and assessment of any unexpected binding in complex cell mixtures (blood, spleen, tonsil, etc.). Typically, the anti-hFcγR reagents used for flow cytometry did not work in IHC applications with FFPE-fixed material. Therefore, we assessed a second panel of mAb and validated them using FcγR transfecteds embedded into FFPE blocks. Although flow cytometry reagents are extremely useful, for most retrospective analysis, such as clinical trial biomarker assessment, only FFPE material is available, and so this fully validated panel of mAb allows the confident analysis of the expression patterns of FcγR in this setting.

A panel of validated reagents is extremely valuable in assessing the importance of individual FcγR expression levels in diseases in which they are proposed to be modulated. We recently demonstrated that expression of the inhibitory hFcγRIIB accelerates the internalization of rituximab (42) and other mAb (43) from the surface of B cells in a dose-dependent manner that limits their therapeutic efficacy (42, 44–46). A careful assessment of hFcγRIIB levels in the blood of patients receiving a therapeutic mAb may be an important biomarker of prognostic value. Furthermore, only with reagents capable of specifically recognizing hFcγRIIA or -B will the activation status of cells that express both receptors, such as macrophages and monocytes, be determined, once again raising the potential for prognostic value in disease settings. For example, in addition to its role in regulating the activity of rituximab...
so provide a background on which to assess potential differences in this context.

Although expression patterns were generally similar between the strains, some reproducible differences were observed. In BALB/c spleens, mFcγRI* and -II* cells were present around the central arterioles and extended through the PALS toward the RP. These cells were not positive for F4/80, CD11b, or CD14 and were not prevalent in C57BL/6 mice. We speculate that they may be fibroblast reticular cells and their origins are the focus of our ongoing studies. There were also marked strain differences with respect to the inhibitory mFcγRII with expression by the sinusoidal lining cells of the liver strong in C57BL/6 mice but variable in BALB/c, and, in the spleen, mFcγRII was more strongly expressed on follicular B cells in BALB/c than C57BL/6 mice. As BALB/c are more Th2 prone, developing strong humoral immunity with high levels of IgG, this may reflect a requirement for more negative feedback to maintain immune homeostasis.

Expression of inhibitory mFcγRII and hFcγRIIB was broadly similar between mouse and human cells, predominantly expressed on B cells and monocytes (at a low level), but not NK cells and on the sinusoidal lining cells of the liver. However, mFcγRII was strongly expressed on the FDC present in well-formed GCs in the mouse spleen (particularly in C57BL/6 mice), whereas we could not detect hFcγRII on FDC in the GCs of the tonsil (data not shown). Furthermore, GC-FDC were much more tightly packed than in human secondary lymphoid tissue. This site has the potential to be highly favorable for providing cross-linking of opsonized target cells and their receptors in the context of mAb immunotherapy. As FDC are also a source of captured Ag, they may represent an ideal cell type for augmenting further Ag presentation and processing for subsequent T cell stimulation. This observation may therefore relate to the potent immune stimulating activity of mAb targeting TNF receptor super family members such as CD40 and DR4/5 (12–14, 50). In contrast to direct targeting mAb such as rituximab, which are impaired by mFcγRII and hFcγRIIB, these receptors are now known to be an important component of immunomodulatory mAb activity, where they serve to further cross-link the target receptor for optimal signaling (20–25, reviewed in Ref. 26). Although in mice it appears the FDC of the spleen may offer the greatest potential for this activity, in humans, this capacity may be afforded by the tonsil and spleen, where subsets of B cells and potentially other cells are expressing high levels of hFcγRIIB. When viewed at low-power magnification (Fig. 8), the environment of the tonsil appears less inflammatory than the spleen, with lower activatory hFcγR expression overall and with high levels of hFcγRIIB within the tissue; how this might affect responses remains to be seen.

Concerning further species differences, our flow cytometric and IHC analysis indicates that GC B cells in humans express low-negligible levels of hFcγRIIB in contrast to direct targeting mAb such as rituximab, which are impaired by mFcγRII and hFcγRIIB, these receptors are now known to be an important component of immunomodulatory mAb, particularly given their juxtaposition to the T cell compartments.

Expression patterns of the other FcγR were broadly similar between mouse and human with both mouse and human activatory FcγR present on RP macrophages in the spleen and on Kupffer cells in the liver. However, there were also some substantial differences, perhaps most notable in NK cells. Mouse NK cells express low levels of mFcγR both in the blood and tissue (spleen and bone marrow), whereas monocytes and neutrophils express considerable amounts of mFcγRII in these locations with measurable

FIGURE 9. Double IHC of FcγR in the human spleen, tonsil, and liver. (A) Double staining of hFcγRIIB (brown) and CD20 (red) by IHC. hFcγRIIB is strongly expressed on B cells in the splenic follicles (open arrow) and on mantle zone (MZ) B cells in the tonsil. There is weak expression of hFcγRIIB by GC B cells in the tonsil. (B) Double staining of hFcγRI (brown) and CD68 (red), hFcγRI and -IIA are expressed by the majority of RP macrophages in the spleen. A smaller number of RP macrophages express hFcγRIIA, and very few express hFcγRIIB. In the tonsil, CD68+ Langerhans cells in the epithelium are positive for hFcγRI, -IIA, and -IIIA (arrowheads). Similar to the spleen, hFcγRI and -IIA are expressed by the majority of CD68+ Kupffer cells in the liver, with a smaller proportion expressing hFcγRIIA (closed arrows). Kupffer cells did not express hFcγRIIB. Details of Ab clones and formats used are contained in Table I. Scale bars, 200 μm and 20 μm for ×4 and ×20 original magnification images, respectively.

(42, 44, 46), hFcγRIIB levels have previously been proposed to be important in regulating metastasis (47) (reviewed in Ref. 48), and hFcγRII and/or hFcγRIIA versus hFcγRIIB expression might be important in distinguishing macrophage activation status in vivo, for example, in the tumor setting in which M1/M2 skewing has been proposed.

A major aim of this work was to allow comparison between FcγR expression patterns of mouse and human (summarized in Table IV) and between the two most commonly used mouse strains (C57BL/6 and BALB/c). C57BL/6 and BALB/c are proposed as prototypical Th1 and Th2 strains, respectively (49), and
amounts of mFcγRI and IV on monocytes and neutrophils, respectively. In contrast, the majority of human NK cells (CD56\(^b\)) express high levels of hFcγRIIIA in the blood and bone marrow, with potentially less in the spleen. As FcγR are key regulators of the immune response, this indicates a potentially fundamental difference in NK cells between the species, with human but not mouse NK cells primed to respond. However, it should be noted that humans also express a minor population of CD56\(^b\) NK cells, expressing lower levels of FcγRIII, which may more closely mimic the population in mouse. Therefore, studies of immunotherapy and immune response relating to NK cells should be treated with caution when extrapolating between the species.

Anatomical location is also known to be an important determinant of Ab activity, and our own data indicate that the same therapeutic/immunostimulatory mAb can differ in its FcγR dependency accordingly. For example, whereas the optimal immune-stimulating, antitumor isotype for anti-CD40 mAb when injected i.v. is an mIgG1, due to its ability to bind avidly to mFc\(\gamma\)R, this property is influenced by FcγR engagement patterns in each location and their affinity for a particular mAb isotype, but certainly comprehensive knowledge of FcγR expression patterns in each location will help in the fine-tuning and design of mAb reagents requiring FcγR engagement for specific functions.

Knowledge of FcγR expression patterns in each location is also of relevance when attempting to understand and better predict cytokine release syndromes, such as that observed with TGN1412 (52). Although initially proposed to be related solely to CD28 expression on a particular subset of T cells and independent of hFcγR expression, it is now evident that hFcγR and in particular hFcγRIIB is critical for this response, at least in predictive in vitro assays (53, 54) and our own observations (55). However, what is not yet resolved is where the hFcγR interaction occurs in vivo to cause the resulting, rapid, cytokine storm. Based upon the data presented in this study, we speculate that the secondary lymphoid tissue offers ideal sites for provoking cytokine release syndromes, being readily accessed, and containing numerous B cells expressing high levels of hFcγRIIB, capable of efficiently engaging therapeutic mAbs and eliciting target receptor signaling in close proximity to T cells. As with immunodulatory mAb, whether the highest expressing B cell subsets or other cells are involved remains to be determined.

In conclusion, we have described the development and characterization of panels of Abs specific for m- and hFcγR in flow cytometry and IF/IHC. We have used these reagents to highlight differences in both the levels and patterns of expression between the two species that will likely impact on immunotherapeutic efficacy and translation of therapeutic agents from mouse to human.

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