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Cutting Edge: Expression of FcγRIIB Tempers Memory CD8 T Cell Function In Vivo

Gabriel R. Starbeck-Miller,* Vladimir P. Badovinac,*† Daniel L. Barber,‡ and John T. Harty*†§

During reinfection, high-affinity IgG Abs form complexes with both soluble Ag and Ag displayed on the surface of infected cells. These interactions regulate cellular activation of both innate cells and B cells, which express specific combinations of activating FcγRs (FcγRI, FcγRII, FcγRIIa, FcγRIII, FcγRIV) and/or the inhibitory FcγR (FcγRIIB). Direct proof for functional expression of FcγR by Ag-specific CD8 T cells is lacking. In this article, we show that the majority of memory CD8 T cells generated by bacterial or viral infection express only FcγRIIB, and that FcγRIIB could be detected on previously activated human CD8 T cells. Of note, FcγR stimulation during in vivo Ag challenge not only inhibited the cytotoxicity of memory CD8 T cells against peptide-loaded or virus-infected targets, but FcγRIIB blockade during homologous virus challenge enhanced the secondary CD8 T cell response. Thus, memory CD8 T cells intrinsically express a functional FcγRIIB, permitting Ag–Ab complexes to regulate secondary CD8 T cell responses. The Journal of Immunology, 2014, 192: 000–000.

Following acute infection with intracellular pathogens, Ag-specific CD8 T cells become activated, proliferate, and then contract in numbers to generate long-lived memory populations (1–4). By virtue of their enhanced numbers, immediate effector functions and capacity to undergo secondary proliferation, memory CD8 T cells can play a pivotal role in host protection against reinfection (2, 5, 6).

B cell populations activated by infection also promote protective immunity by maintaining high levels of circulating high-affinity IgG Ab (7–9). When Abs complex with soluble Ag or with Ag displayed on the surface of infected cells, the Fc fragment regulates the activation status and effector functions of nearby cells that bear FcRs. In mice, there are four FcRs for IgG: FcγRI, FcγRII, FcγRIII, and FcγRIV (10), which are classified based on their ability to regulate cellular activation. Activating FcγRs (FcγRI, FcγRIIa, and FcγRIII), which can be expressed by a variety of innate immune cell populations, contain intracellular ITAMs and have been shown to increase phagocytosis, release of proinflammatory cytokines, and facilitate Ab-dependent, cell-mediated cytotoxicity (11–13). In contrast, FcγRIIB, which is thought to be restricted to innate immune cells and B cells, contains an intracellular ITIM and is important for negatively impacting the signaling capacity of activating FcγRs on innate effector cells (11) and B cells, and also tempering BCR-mediated signaling (14).

Although FcγRs play a crucial role in regulating the activation of both innate cells and B cells during reinfection, their role in CD8 T cell biology is unclear and remains controversial. It has been suggested that T cells do not intrinsically express FcγRs (10) but in some instances can acquire FcγRs after intercellular transfer from an FcγR-bearing cell (15, 16). We recently showed by microarray analyses that FcγR2b mRNA, but not mRNA for any other FcγR, is upregulated in memory CD8 T cells generated after Listeria monocytogenes infection (17). In this article, we address both the protein expression and in vivo function of FcγRIIB in memory CD8 T cells generated by bacterial and viral infection.

Materials and Methods

Human blood, mice, bone marrow chimera, virus, and bacteria

Whole blood was acquired from anonymous donors that had consented for blood donation at the DeGowin Blood Center at the University of Iowa. Consent forms were approved by the University of Iowa’s Institutional Review Board. C57BL/6 (Thy1.2,CD45.2 and CD45.1) were obtained from the National Cancer Institute (Frederick, MD). TCR transgenic (Tg) OT-I (Thy1.1) and P14 (Thy1.1) mice have been described previously (18, 19). FcγRIIB knockout (KO) mice were obtained from Jackson Laboratories (Bar Harbor, ME). Wild type (WT):FcγRIIB KO bone marrow chimera mice were generated as previously described (20). LCMV Armstrong (LCMV Arm) and LCMV Clone 13 were propagated according to standard protocols. LCMV Arm (2 × 10⁶ PFU) was injected i.p., whereas LCMV Clone 13 (2 × 10⁶ PFU) was injected i.v. Attenuated aceA-deficient L. monocytogenes expressing OVA257 (att LM-OVA) or GP33 (att LM-GP33) were propagated and injected i.v. at 1 × 10⁷ CFU as described previously (21–23).

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Abbreviations used in this article: Arm, Armstrong; att LM-GP33, aceA-deficient L. monocytogenes expressing GP33; att LM-OVA, aceA-deficient L. monocytogenes expressing OVA257; CTV, CellTrace Violet Cell Proliferation Kit; KO, knockout; LCMV, lymphocytic choriomeningitis virus; p.i., postinfection; Tg, transgenic; WT, wild type.
Cell lines, Abs, peptides, MHC class I tetramers

CH12 B cells were provided by Dr. Gal Bishop (University of Iowa, Iowa City, IA). Abs for FACS analysis were used with the indicated specificity and the appropriate combinations of fluorochromes. For FcyRIIB/FcyRIIB staining, biotinylated-2.4G2 (BD Bioscience, San Jose, CA) and streptavidin-allophycocyanin (Invitrogen, Carlsbad, CA) were used. MHC class I tetramer H-2Kb/OVA257–264 and H-2Dp/GP33–41 were prepared as described previously (24–26). Ab treatment during LCMV rechallenge was 400 μg of either rat IgG (Fischer Scientific, Pittsburgh, PA) or 2.4G2 (prepared in-house) for three consecutive days following secondary infection.

Adaptive transfer and quantitative/phenotypic analysis of pathogen-specific CD8 T cells

A total of 10^5 OT-I (OVA257–264-specific) or 10^4 P14 (GP33–41-specific) TCR Tg CD8 T cells (Thy1.1; unless otherwise stated) from the spleen or blood of naive mice were transferred into naive B6 (Thy1.2) hosts as previously described (27). Recipient mice were then challenged with an LM-OVA or LCMV Arm. The magnitude of pathogen-specific CD8 T cell response was determined by either tetramer staining of endogenous CD8 T cells (28), staining for Thy1.1-expressing Tg cells (27), or evaluating changes in CD11a expression (29).

RNA purification and RT-PCR

Cells were sorted based on Thy1.1 (OT-I) and NK1.1 (splenic NK cells) to ~99% purity, and RNA from three independent pools of purified cells was extracted using the RNeasy kit (Qiagen). Approximately 50–100 ng RNA template was converted to cDNA and amplified using Script One-Step RT-PCR Kit with SYBR Green according to manufacturer’s protocol (Bio-Rad, Hercules, CA). The following oligonucleotides were used to analyze expression of the following transcripts: 5'-CCCTGGGAACTCTTCTACCC-3' and 5'-CAGCAGCCAGTCAGAAATCA-3' for 2.4G2, and 5'-CCTCATGGAC-GATTATGGACA-3' and 5'-TATGTCCCGGGCTAT-3' for Hprt. PCR was carried out using ABI PRISM 7700 Sequence. Expression of transcripts was normalized to controls groups as indicated.

In vivo cytotoxicity assay

Splenocytes from CD45.1 mice were harvested and stained with CFSE or CellTrace Violet Cell Proliferation Kit (CTV; Life Technologies, Carlsbad, CA). CFSEHIGH cells were stained with 1 μM CFSE, CFSELOW cells were stained with 0.04 μM CFSE, CTVHIGH cells were stained with 2.5 μM CTV, and CTVLOW cells were stained with 0.25 μM CTV. After 15 min at 37°C, staining was quenched 1:1 with FCS; then cells were washed three times with RPMI 1640 containing 10% (v/v) FCS. Stained cells were then coated with the following cocktails at 37°C in an orbital shaker for 1 h: CTVHIGH cells were incubated with 1 μM GM1a and α-H-2Kb, CTVLOW cells were coated with α-H-2Kb, CFSEHIGH cells were coated with 1 μM GP33–41, and CFSELOW cells were left uncoated. After washing, cells were mixed 1:1:1:1 and transferred into CD45.2 hosts that were either naive or ~60 d after LCMV Arm. Recipient mice were pretreated with 400 μg rat IgG or 2.4G2 mAb, or 20 min before target cell transfer. After 1 h, percentage GP1-M1-H2-DP–specific lysis was assessed by comparing the presence of CTVHIGH versus CTVLOW or CFSEHIGH versus CFSELOW using the following formula: %Specific lysis = [1 − (naive transfer ratio/infected transfer ratio)] × 100 (30). For in vivo cytotoxicity assays using LCMV Arm–infected target cells, splenocytes were harvested from naive CD45.1 mice (stained CTVLOW) and from CD45.1 mice that had been infected with LCMV Arm 4 d prior (stained CTVHIGH), mixed 1:1, and injected into LCMV Arm immune or naive CD45.1 mice. Percentage specific lysis was determined 1 h after transfer. FACS analysis using anti-LCMV nucleoprotein (113 hybridoma) was as described previously (31).

Statistics

Statistical analysis was performed using two-tailed Student t tests or ANOVA as indicated.

Results

Ag-specific CD8 T cells express FcyRIIB postinfection

A transcriptional profiling study performed in our laboratory revealed consistent upregulation of Fggr2b mRNA, but not other FcγR transcripts, in memory CD8 T cell populations generated by one or more Ag stimulations compared with naive CD8 T cells (17). To confirm these microarray data, we performed RT-PCR for Fggr2b mRNA in memory OT-I CD8 T cells, as well as a NK cells (which do not express Fggr2b) and CH12 B cells (which express Fggr2b) (10), and normalized expression relative to naive OT-I CD8 T cells. Expression of Fggr2b mRNA in NK cells did not differ from naive OT-I cells, whereas both CH12 B cells and memory OT-I cells expressed significantly (p < 0.01) more Fggr2b mRNA compared with naive OT-I cells (Supplemental Fig. 1A).

To determine whether Fggr2b mRNA expression by memory CD8 T cells resulted in protein production, we measured FcyRIIB protein expression with the 2.4G2 mAb on the surface of Ag-specific CD8 T cells at memory time points following LM-OVA and LCMV infection. Indeed, ~60% of memory CD8 T cells stained positive with 2.4G2 (Fig. 1A). 2.4G2 is known to detect (and block) both FcyRIII and FcyRIIB (32). To determine the specificity of 2.4G2 reactivity, we compared 2.4G2 binding on memory CD8 T cells from WT and FcyRIIB KO mice. As predicted based on lack of mRNA expression for FcγRIII (17), 2.4G2 failed to react with memory CD8 T cells from FcyRIIB KO mice (Fig. 1A), thus indicating that 2.4G2 exclusively detects surface FcyRIIB and not FcγRIII on memory CD8 T cells.

To determine whether expression of FcyRIIB is a general feature of memory CD8 T cells, we generated memory CD8 T cells specific for LCMV Arm or att LM-OVA in WT and FcyRIIB KO mice. Indeed, the majority of GP33–41- or OVA257–264-specific WT memory CD8 T cells also reacted with 2.4G2, and thus express FcyRIIB (Fig. 1A). Importantly, surface expression of FcyRIIB by memory CD8 T cells was not due to trogocytosis, because 2.4G2 failed to react with FcyRIIB KO Ag-specific CD8 T cells in WT: FcyRIIB KO bone marrow chimeric mice (Supplemental Fig. 1B). Together, these data indicate that in contrast with previous suggestions that T cells do not express FcyR (10, 11), memory CD8 T cells generated by both bacterial and viral infection intrinsically express surface FcyRIIB protein.

To address the kinetics of FcyRIIB expression by CD8 T cells postinfection (p.i.), we seeded mice with naive P14 or OT-I CD8 T cells and then gave them LCMV Arm or att LM-OVA infection, respectively. FcyRIIB protein expression was detected on a minority of effector CD8 T cells as early as day 7 p.i., and the fraction of FcyRIIB-expressing CD8 T cells continued to increase until ~21 d p.i. (Fig. 1B). At this
point, FcγRIIB-expressing CD8 T cells stabilized at 60–80% and were maintained as late as 200 d post–LCMV Arm or –att LM-OVA infection (Fig. 1B). As predicted by mRNA expression analysis by Wirth et al., memory CD8 T cells do not express either FcγRI or FcγRIV protein, whereas both receptors were readily detectable on CD3−CD11b+ cells (Supplemental Fig. 1C). These data suggest that some effector CD8 T cells express FcγRIIB shortly after activation, but that the majority of memory CD8 T cell population maintains high FcγRIIB expression while not expressing other FcγRs.

Because FcγRIIB expression can be regulated by cytokine stimulation alone in some immune cell types (e.g., IL-4 and TGF-β [33–36]), it is possible that TCR-mediated activation may not be required for FcγRIIB expression by CD8 T cells during infection. To determine whether FcγRIIB expression was limited to activated CD8 T cells, we evaluated the expression of FcγRIIB on “Ag-experienced” and “naive” polyclonal CD8 T cells during the course of infection, distinguishing these populations using surrogate activation markers: CD11aHIGHCD86LOW (Ag-experienced) and CD11aINT/CD86HIGH (not Ag-experienced) (29). Of note, only CD11aHIGHCD86LOW T cells from either LCMV Arm or att LM-OVA–infected mice expressed FcγRIIB (Fig. 2A). Interestingly, FcγRIIB expression could also be detected on 5–17% of CD45RO+ (activated/memory) human CD8 T cells from normal donors, whereas their CD45RO− (naive) counterpart did not exhibit FcγRIIB expression (Supplemental Fig. 1D, 1E). Based on these results, FcγRIIB expression is restricted to Ag-experienced CD8 T cells in both mice and humans.

Next, we determined whether FcγRIIB expression was limited to specific memory CD8 T cell subsets (T effector memory or T central memory). FcγRIIB staining with 2.4G2 reveals bimodal expression on memory CD8 T cells (Fig. 2B). Importantly, LCMV-specific memory CD8 T cells with low or high expression of FcγRIIB had similar expression profiles of CD62L, CD127, and KLRG-1 (Fig. 2B). Thus, FcγRIIB expression does not correspond to previously described T effector memory or T central memory CD8 T cell subsets.

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Characteristics of FcγRIIB-expressing memory CD8 T cells. (A) At day 65 p.i., FcγRIIB protein expression was evaluated on the surface of blood-derived CD11aHIGHCD86LOW/CD11aINTCD86HIGH/Ty1.2+ cells (dotted black line) and CD11aINTCD86HIGH/Ty1.2+ cells (solid black line) as compared with isotype (filled gray). FACS plots and histograms are representative of two independent experiments with at least three mice with each group. (B) At day 55 post–LCMV Arm infection, 2.4G2HIGH (dotted black line) and 2.4G2LOW (solid black line) H2-D^β/GP3.41−Tetramer+ CD8 T cells were assessed for CD62L, CD127, and KLRG-1 expression. Filled gray histogram represents isotype controls. Histograms are representative of two independent experiments with at least three mice in each group per experiment.

Because FcγRIIB is known to have a negative impact on B cell activation (37), we tested whether this FcR was capable of dampening the response of memory CD8 T cells when coengaged with TCR. To address this question, we used LCMV Arm immune mice (>60 d p.i.) for an in vivo cytotoxicity assay (GP33−41/H2-Db-specific) that compared the susceptibility of Ag-pulsed targets to lysis when left uncoated or coated with mouse-derived IgG Ab (anti-H2-Kb). The target populations were generated by surface staining and pulsing CD45.1 spleenocytes with the following combinations of reagents to compare these scenarios: 1) CFSEHIGH = un pulsed; 2) CFSEHIGH = pulsed with GP33−41; 3) CTVLOW = un pulsed with anti–H-2Kb; and 4) CTVMHIGH = anti–H2-Kb + pulsed with GP33−41. Four populations were then mixed into a 1:1:1:1 ratio (Supplemental Fig. 2A) and transferred into either naive CD45.2 hosts or an LCMV Arm immune CD45.2 hosts at day 70 p.i. The percent specific lysis of GP33−41−coated targets in the

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** FcγRIIB inhibits the cytotoxicity memory CD8 T cells. (A) CD45.2 mice were injected with P14 CD8 T cells and subsequently infected with LCMV Arm. At least 70 d p.i., hosts were treated with rat IgG or 2.4G2 and then subjected to in vivo GP33−41/H2-Db−specific cytotoxicity assay. Separate CD45.1 target populations were stained and coated in the following combinations: CFSEHIGH (GP33−41), CFSELOW (uncoated), CTVHIGH (GP33−41 + anti–H2-Kb), CTVLOW (anti–H2-Kb). Targets were mixed 1:1:1:1 and then injected into CD45.2 pretreated mice that were either naive or LCMV Arm immune. In vivo cytotoxicity was assessed by calculating percentage GP33−41/H2-Db−specific lysis (CFSELOW versus CFSEHIGH; CTVLOW versus CTVHIGH). Displayed data are compiled from two independent experiments, each with three mice per group. Statistical analysis was done using Student t test. *p < 0.05, **p < 0.01, not significant (ns): p > 0.05.

**FcγRIIB inhibits both cytotoxicity and secondary expansion of memory CD8 T cells**

Because FcγRIIB is known to have a negative impact on B cell activation (37), we tested whether this FcR was capable of dampening the response of memory CD8 T cells when coengaged with TCR. To address this question, we used LCMV Arm immune mice (>60 d p.i.) for an in vivo cytotoxicity assay (GP33−41/H2-Db−specific) that compared the susceptibility of Ag-pulsed targets to lysis when left uncoated or coated with mouse-derived IgG Ab (anti-H2-Kb). The target populations were generated by surface staining and pulsing CD45.1 spleenocytes with the following combinations of reagents to compare these scenarios: 1) CFSEHIGH = un pulsed; 2) CFSEHIGH = pulsed with GP33−41; 3) CTVLOW = un pulsed with anti–H-2Kb; and 4) CTVMHIGH = anti–H2-Kb + pulsed with GP33−41. Four populations were then mixed into a 1:1:1:1 ratio (Supplemental Fig. 2A) and transferred into either naive CD45.2 hosts or an LCMV Arm immune CD45.2 hosts at day 70 p.i. The percent specific lysis of GP33−41−coated targets in the
absence of anti–H2-K\textsuperscript{b} Ab coating was ~55% in LCMV Arm immune mice treated with control rat IgG. Strikingly, specific lysis of cells that were coated with anti–H2-K\textsuperscript{b}*GP\textsubscript{33-41} was decreased to ~20% (Fig. 3A), suggesting that the anti–H2-K\textsuperscript{b} Ab on target cells provided an inhibitory signal. In addition, if immune mice were treated with 2.4G2 mAb to block FcγRIIB stimulation (34, 38) before transfer of target cells, the specific lysis of cells coated with anti–H2-K\textsuperscript{b}*GP\textsubscript{33-41} returned to the level of cells pulsed with GP\textsubscript{33-41} alone (Fig. 3A). Finally, when LCMV Arm immune mice (day > 70 p.i., when neutralizing IgG Abs have developed) (39) were challenged with LCMV Arm–infected targets (Supplemental Fig. 2B) in the presence of control IgG or 2.4G2 treatment, specific lysis of infected targets was enhanced by 2.4G2 pretreatment compared with IgG control–treated mice (Fig. 3B). Therefore, these data demonstrate that FcγRIIB negatively regulates the cytotoxicity of memory CD8 T cells upon encountering mouse IgG-coated targets.

To further examine a function for FcγRIIB expression on memory CD8 T cells, we determined whether FcγRIIB played a role in regulating the expansion of memory CD8 T cells during homologous challenge in hosts with circulating levels of pathogen-specific high-affinity IgG Abs. Blocking FcγRIIB with 2.4G2 significantly (p < 0.01) enhanced the accumulation of memory P14 CD8 T cells after high-dose challenge with LCMV Clone 13 (Fig. 4A, 4B). However, treatment of LCMV Arm immune mice with 2.4G2 did not significantly enhance secondary accumulation of memory CD8 T cells when the mice were challenged with att LM-GP33, a scenario where the host lacks IgG Abs to the challenge pathogen (Fig. 4A). Thus, FcγRIIB tempts memory CD8 T cell responses to reinfection in hosts with pre-existing IgG Abs to the challenge pathogen.

**Discussion**

Expression of FcγR by T cells has been questioned in the literature (10). In this article, we confirm that both viral and bacterial infections specifically promote the expression of FcγRIIB, and not other FcγRs, by previously activated human and mouse CD8 T cells. Previous studies have indicated that FcγR expression can be endowed upon CD8 T cells by intercellular transfer from FcγR-bearing cells in vitro, often termed trogocytosis (15, 16). Because FcγR mRNA expression is elevated in memory CD8 T cells, and FcγRIIB KO Ag-specific CD8 T cells do not acquire FcγRIIB expression p.i. of mixed-bone marrow chimeras, we conclude that the presence of FcγRIIB on the surface of memory CD8 T cells is cell intrinsic. Acquisition of FcγR expression on CD8 T cells by trogocytosis has yet to be associated with a functional consequence in vivo (15). In contrast, our results indicate that FcγRIIB plays a discernible role in dampening not only the cytotoxicity of memory CD8 T cells upon encountering Ab/Ag-coated cells and virus-infected cells in vivo, but also by limiting the expansion of memory CD8 T cells during reinfection in hosts with an established IgG Ab response. These findings confirm that CD8 T cells do, in fact, express FcγRIIB, and this receptor can play an important role in tempering secondary responses during reinfection. This concept may be useful for future and current therapies that aim to optimally regulate memory CD8 T cell expansion after homologous booster vaccinations.

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**Disclosures**

The authors have no financial conflicts of interest.

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