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A Role for IFN-γ from Antigen-Specific CD8$^+$ T Cells in Protective Immunity to *Listeria monocytogenes*[1]

Kelly A. N. Messingham,* Vladimir P. Badovinac,* Ali Jabbari,* and John T. Harty[2]*†

Whether IFN-γ contributes to the per-cell protective capacity of memory CD8$^+$ T cells against *Listeria monocytogenes* (LM) has not been formally tested. In this study, we generated LM Ag-specific memory CD8$^+$ T cells via immunization of wild-type (WT) and IFN-γ-deficient (gamma knockout (GKO)) mice with LM peptide-coated dendritic cells and compared them phenotypically and functionally. Immunization of WT and GKO mice resulted in memory CD8$^+$ T cells that were similar in number, functional avidity, TCR repertoire use, and memory phenotype. The protective capacity of memory CD8$^+$ T cells from immunized WT and GKO mice was evaluated after adoptive transfer of equal numbers of WT or GKO cells into naive BALB/c mice followed by LM challenge. The adoptively transferred CD8$^+$ T cells from GKO donors exhibited a decreased ability to reduce bacterial numbers in the organs of recipient mice when compared with an equivalent number of Ag-matched WT CD8$^+$ T cells. This deficiency was most evident early (day 3) after infection if a relatively low infectious dose was used; however, transferring fewer memory CD8$^+$ T cells or increasing the LM challenge dose revealed a more pronounced defect in protective immunity mediated by the CD8$^+$ T cells from GKO mice. Our studies identified a decrease in Ag-specific target cell lysis in vivo by CD8$^+$ T cells from GKO mice as the mechanism for the decreased protective immunity after LM challenge. Further studies suggest that the lack of IFN-γ production by the Ag-specific CD8 T cells themselves diminishes target cell sensitivity to cytolysis, thereby reducing the lytic potency of IFN-γ-deficient LM-specific memory CD8$^+$ T cells. ***The Journal of Immunology,*** **2007, 179: 2457–2466.**

The CD8$^+$ T cell is critical for protective immunity to a variety of intracellular pathogens (1). This immunity is largely dependent on the diverse array of effector functions at the disposal of activated CD8$^+$ T cells, including the secretion of cytokines, such as IFN-γ and TNF (2, 3), and Ag-specific cytolysis of infected cells (3–5). In combination, it is thought that these effector functions produce a potent CD8$^+$ T cell response resulting in pathogen clearance. Historically, it has been difficult to estimate the contribution of each effector mechanism independently because of the complexity of the CD8$^+$ T cell response; however, experimental infection of gene knockout (KO)[3] mice has proven useful for this purpose (3, 4).

Initial studies examining protective immunity in IFN-γ-deficient KO (GKO) mice (6) determined that IFN-γ is critical for resistance to primary infection with virulent *Listeria monocytogenes* (LM). However, vaccination of GKO mice with an attenuated (*actA*-deficient (7)) strain of LM generated protective CD8$^+$ T cell immunity that was equivalent to that observed in vaccinated wild-type (WT) mice (6). This translates into a ~20,000-fold increase in resistance to challenge with virulent bacteria when compared with naive GKO mice (6). The conclusion drawn from these studies was that IFN-γ is not required for the development or elaboration of protective CD8$^+$ T cell-mediated immunity to LM. The caveat to this interpretation is that the protective immunity observed in vaccinated GKO mice occurred in the presence of dramatically elevated numbers of LM-specific memory CD8$^+$ T cells (9). Subsequent studies have defined a role for IFN-γ as a regulator of T cell homeostasis and attribute the increased memory CD8$^+$ T cell numbers in vaccinated GKO mice to a markedly protracted and reduced contraction phase of the T cell response observed in the absence of IFN-γ (8). Additionally, immunized GKO mice also display altered immunodominance hierarchies that have been attributed to the disparate influence of IFN-γ on presentation of discrete bacterial peptides by MHC class I molecules (9). Thus, it remains to be determined whether the protective immunity provided by IFN-γ-deficient CD8$^+$ T cells is equivalent to WT CD8$^+$ T cells on a per-cell basis or whether the protective immunity observed in LM-vaccinated GKO mice is due to the increased numbers of Ag-specific memory CD8$^+$ T cells.

The goal of the current study was to determine whether IFN-γ production by Ag-specific CD8$^+$ T cells contributes to protective immunity to LM. To address this question, we transferred LM Ag-specific CD8$^+$ T cells from dendritic cell (DC) peptide-immunized WT or GKO mice, into naive WT recipients and compared bacterial CFU reduction after challenge with virulent LM. In summary, memory CD8$^+$ T cells generated in the WT or GKO mice were similar when functional avidity, TCR repertoire use, and cell surface phenotype were compared. However, a decrease in the protective immunity provided by memory CD8$^+$ T cells from GKO mice was observed as early as day 3 after LM challenge, and was exacerbated when fewer Ag-specific CD8$^+$ T cells were transferred or when a higher bacterial challenge dose was given. Further functional analysis revealed diminished Ag-specific cytolysis in vivo by the IFN-γ-deficient memory CD8$^+$ T cells as a mechanism

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[3] Abbreviations used in this paper: KO, knockout; GKO, IFN-γ-deficient KO; LM, *Listeria monocytogenes*; WT, wild type; DC, dendritic cell; LCMV, lymphocytic choriomeningitis virus; LLO, listeriolysin O; NP, nuclear protein; ICS, intracellular cytokine staining; p.i., postinfection; MFI, mean fluorescence intensity; LOD, limit of detection.

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for their decreased per-cell protective capacity. Finally, our studies suggest that IFN-γ signals received by target cells from the memory $CD8^+$ T cells is essential for the most efficient killing in vivo. Thus, the absence of IFN-γ as a CD8 T cell effector molecule results in decreased killing efficiency and less effective protective immunity provided by these cells. We conclude that although IFN-γ does not appear to be required for the development or elaboration of $CD8^+$ T cell-mediated protective immunity, memory $CD8^+$ T cell production of IFN-γ contributes to optimal secondary resistance at early time points after LM infection of a vaccinated host.

Materials and Methods

Mice and bacteria

Eight- to 10-wk-old female BALB/c (Thy1.2, H-2^d MHC, WT) and C57BL/6 (Thy1.2, H-2^b) mice were purchased from the National Cancer Institute (Frederick, MD). BALB/c IFN-γ-deficient (Thy 1.2, H-2^d MHC, GKO) mice (6), BALB/c Thy1.1 mice (provided by R. Dutton, Trudeau Institute, Saranac Lake, NY), OT-I (10) (*C57BL/6 Tg(TcraTcrb) 1100Mjb/j*, Thy1.1; provided by Dr. T. L. Ratliff, University of Iowa, Iowa City, IA), and C57BL/6 IFN-γR KO (purchased from The Jackson Laboratory) mice were maintained by brother-sister mating and housed under specific pathogen-free conditions at the University of Iowa Animal Care Unit until initiation of experiments.

These studies used virulent and attenuated (*actA*-deficient) LM strains derived from XFL 303 (11), which expresses a secreted fusion protein containing the lymphocytic choriomeningitis virus (LCMV) $NP_{118-126}$ epitope (LM-nuclear proteins (NPs); Ref. 12), or *act*A-deficient LM-NPs (7) for immunization of BALB/c mice and *act*A-deficient LM expressing the OVA protein (LM-OVA) (13, 14) for immunization of C57BL/6 mice. Growth and maintenance of LM strains was as described (6). CFU of LM per spleen or per gram of liver were determined by plating serial dilutions of organ homogenates as described (4). Pathogen-infected mice were housed at the appropriate biosafety level. Experimental protocols were approved by the University of Iowa Animal Care and Use Committee.

DC immunization

$CD11c^+$ bone marrow-derived DCs were generated as previously described (15, 16). Briefly, RBC-depleted bone marrow was subjected to complement depletion after incubation with 50 μg/ml mAbs 3.168 (anti-CD8), 34-5-3 (anti-I-A^d), RL-172 (anti-CD4), and RA3-3A1/6.1 (anti-B220). Remaining cells were cultured with 1000 U/ml rGM-CSF (BD Pharmingen) and 25 U/ml rIL-4 (PeproTech) (DC medium) for 6 days with 80% medium replacement every other day. Next, cells were harvested and contaminating neutrophils were removed through complement depletion after incubation with the Ly-6-G-specific Ab RB6.8C5. The remaining cells were cultured overnight with in DC medium with LPS (500 ng/ml; Sigma-Aldrich) and 1 μM peptide was added to cultures 2 h before harvest, washing, and injection. The resulting cell populations consisted of 75–85% $CD11c^+$ and H-$2L^{d+}$, $B7.1^+$, $B7.2^+$, $CD8\alpha^-$, $CD4^-$, I-A^{d+}, and $CD11b^{high}$. For vaccination, $\sim 2.5 \times 10^5$ mature ($CD11c^+$) DCs were injected i.v. per mouse. After 35 days, a booster immunization ($0.8–1.0 \times 10^6$ DCs) was given. All experiments were conducted at least 35 days after the booster immunization.

Abs, peptides, and MHC class I tetramers

mAbs with the following specificities were used: TNF-α-PE or allophycocyanin (MP6-XT22), IL-2-PE (JES6-5H4), CD8-FITC or CyChrome (53-6.7), CD62L-PE (MEL-14), CD127-PE (A7R34), CD27-PE (LG.7F9), CD44-PE (Pgp-1), FITC-conjugated Vβ 2, 4,5.1/5.2, 8.1/8.2, and 10 (B20.6, KT4, MR9-4, MR5-2 and B21.5, respectively), Thy 1.2-FITC (53-6.7), CD107a-FITC (1D4B; BD Pharmingen), anti-human granzyme B-PE (Caltag Laboratories) and appropriately labeled IgG2a, IgG2b, and IgG1 isotype controls. All Abs were obtained from eBioscience unless noted. Synthetic LLO_{91-99}, $p60_{217-226}$ (both H-$2K^d$), $OVA_{257-264}$ (H-$2K^b$), and $NP_{118-126}$ (H-$2L^d$) peptides were obtained from BioSynthesis. MHC class I tetramers specific for $NP_{118-126}$, LLO_{91-99}, or $p60_{217-225}$ were obtained from the National Institute of Allergy and Infectious Diseases Tetramer Core (Atlanta, GA) or prepared using published protocols (17, 18).

T cell enrichment and adoptive transfer

Splenocyte cell suspensions were enriched for $CD8^+$ T cells by negative selection (Stem Cell Technologies) (4). Briefly, 8×10^7 splenic leukocytes per ml were labeled with a $CD8^+$ enrichment Ab mixture followed by conjugation to magnetic beads. $CD8^+$ T cells were obtained in the flow-through after magnetic separation (Macs LS Separation Column; Miltenyi Biotec). Alternatively, memory OT-1 cells were generated by transferring 5×10^4 naive OT-I (Thy1.1) $CD8^+$ T cells, enriched by negative selection, into naive B6 mice followed by infection with 10^6 *act*A-deficient LM-OVA (19). After ~35 days, mice were infected with 10^7 *act*A-deficient LM-OVA. Secondary memory OT-I T cells were purified with PE-conjugated anti-Thy1.1 (BD Pharmingen) and anti-PE magnetic beads (Miltenyi Biotec). All experiments used mice or memory cells obtained >35 days after secondary immunization. Enriched cells, routinely >93% pure by flow cytometry, were washed three times in sterile saline and adoptively transferred by i.v. injection.

To evaluate the percentage of $NP_{118-126}$, LLO_{91-99}, or $p60_{217-225}$-specific $CD8^+$ T cells present in the enriched population, a fraction of each donor cell pool (WT or GKO) was costained CD8 and LM peptide-specific MHC class I tetramers. The number of enriched cells from WT or GKO donors was adjusted so that equal numbers (noted in figure legends) of Ag-specific $CD8^+$ T cells were transferred i.v. into naive female BALB/c (Thy1.2 or Thy1.1) recipient mice. After transfer (24 h), an equal number (~10% of the transferred cells) of WT or GKO cells were observed in the spleens of recipient mice (data not shown). One to 2 days after adoptive transfer, T cell-recipient or naive control mice were challenged with virulent LM-NPs (1 LD_{50} = ~10,000 bacteria) as indicated.

Detection of Ag-specific $CD8^+$ T cells, T cell repertoire

The number of $CD8^+$ T cells specific for specific for $NP_{118-126}$, LLO_{91-99}, or $p60_{217-225}$ was determined using intracellular cytokine staining (ICS) for TNF-α after 5.5-h incubation with or without 200 nM peptide in brefeldin A (BD Pharmingen) (20). To detect CD62L on TNF^+ $CD8^+$ T cells, TAPI-2 (100 μM; a TACE inhibitor; Peptides International) was added 30 min before peptide addition (21). Nonspecific cytokine production (≤0.5%; no peptide control) was subtracted when the total number of Ag-specific $CD8^+$ T cells per spleen was calculated. The T cell repertoire was determined by flow cytometry after staining with LM peptide-specific MHC class I tetramers and Abs specific for various *Vβ* gene families (BD Pharmingen) (4).

In vivo cytotoxicity assay

The ability of WT or GKO memory $CD8^+$ T cells to lyse CFSE-labeled target cells was evaluated in the spleens of T cell-recipient mice by flow cytometry. Equal numbers (20×10^6 total) of peptide (1 μM LLO_{91-99}) coated or uncoated naive syngeneic splenocytes were labeled with 2.5 μM CFSE ($CFSE^{high}$) or 0.25 μM ($CFSE^{low}$), respectively, and injected into experimental mice i.v. (4).

To evaluate cytolysis of WT C57BL/6 vs IFN-γR-deficient (IFN-γR KO) targets, naive splenocytes of each type were incubated with or without $OVA_{257-264}$ peptide (1 μM) and the resulting populations—WT with OVA, WT without OVA, IFN-γR KO with OVA, IFN-γR KO without OVA—were washed and labeled with serial dilutions (1, 0.1, 0.01, or 0.001 μM, respectively) of CFSE for 12 min. Next, 1 ml of FCS was added for 1 min and then cells were washed extensively. All target cells were labeled uniformly (4 min) at 2×10^7 cells/ml with red cell linker in diluent C (PKH26; Sigma-Aldrich), an equal volume of FCS was added (1 min), and cells were washed three times in medium containing 10% FCS. The labeled cells were combined so that 2×10^6 of each target type (8×10^6 total) was injected i.v. into naive C57BL/6 mice or OT-I memory T cell (1.5×10^6) recipient mice. OVA peptide/TCR complexes on target cell populations were evaluated with optimized concentrations of mAb (25-D1.16) and strepavidin-FITC (22). In all cases, the percent killing was calculated as follows: 100 − (((percentage of peptide pulsed in T cell-recipient (or memory) mice/percentage of unpulsed in T cell-recipient (or memory) mice)/percentage of peptide pulsed in naive mice/percentage of unpulsed in naive mice) × 100) (5).

Degranulation assay

Spleen cells were stimulated in vitro with or without 100 ng/ml LLO_{91-99} peptide in 500 μl of RPMI 1640 containing 10% FCS and penicillin/streptomycin. FITC-conjugated anti-mouse CD107a or isotype control and monensin A (Sigma-Aldrich) was added to cells during the stimulation period (23).

Results

DC-LM peptide vaccination of WT and GKO mice

Although IFN-γ is not required for protective $CD8^+$ T cell immunity to LM (6), this cytokine plays a critical role in T cell

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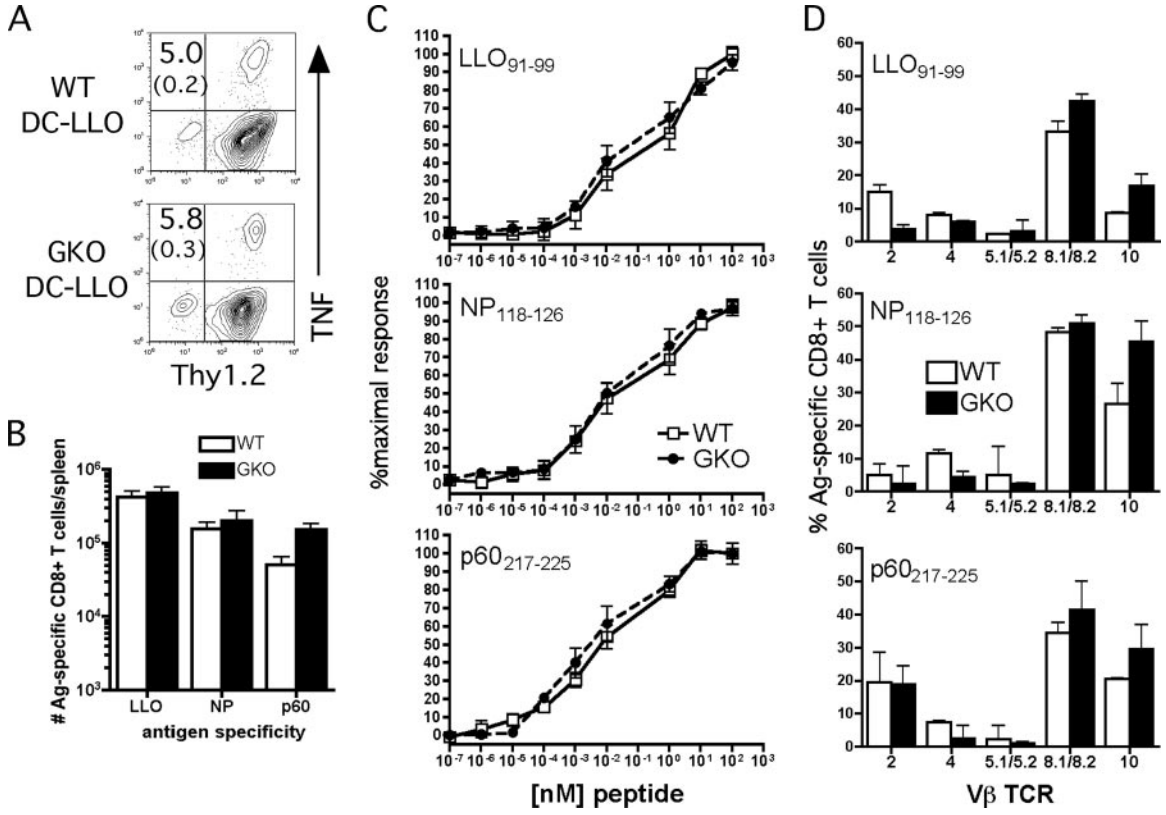

FIGURE 1. DC-peptide vaccination generates LM-specific memory $CD8^+$ T cells in GKO mice. $CD8^+$ T cell memory was generated in WT and GKO mice through i.v. immunization with DCs (2.5×10^5) coated with LM peptides (LLO_{91-99}, $NP_{118-127}$, or $p60_{217-225}$), and, 37 days later, followed by a booster injection (1×10^6 DCs coated with same peptide). Forty-five days after immunization, Ag-specific memory $CD8^+$ T cell levels were determined using ICS for TNF-α. Representative contour plots (*A*) are gated on $CD8^+$ T cells and show the percent Ag-specific $Thy1.2^+TNF^+$ after incubation with or without (number in parentheses) peptide (LLO_{91-99} in plots shown) followed by ICS. The number of LLO_{91-99}, $NP_{118-127}$, or $p60_{217-225}$-specific memory $CD8^+$ T cells/spleen (*B*) in immunized WT or GKO mice. Functional avidity (*C*) was evaluated by incubation (5.5 h) of splenocytes obtained from immunized WT or GKO mice with serial dilutions of cognate Ag followed by ICS for TNF-α. The Ag-specific $CD8^+$ TCR repertoire (*D*) was evaluated using MHC tetramer staining and Vβ-specific mAbs. Data shown are mean ± SD of three to six mice per group and are representative of two or more experiments.

homeostasis that is clearly evidenced by the altered immunodominance hierarchy and elevated numbers of memory $CD8^+$ T cells in LM-vaccinated GKO mice (8). The disparity in number, and altered hierarchy of, memory $CD8^+$ T cells in vaccinated GKO compared with WT mice precludes direct comparison of these mice to evaluate the role of $CD8^+$ T cell derived IFN-γ in protective immunity. The most valid approach to this question is comparison of the protective capacity of equal numbers of memory $CD8^+$ T cells, of identical Ag specificity, from WT or GKO mice. However, evidence from others suggests that sorting of Ag-specific $CD8^+$ T cells with conventional MHC class I-peptide tetramers can reduce protection by LM-specific $CD8^+$ T cells (24). To avoid this problem, we generated single epitope-specific memory $CD8^+$ T cells in WT and GKO mice using LM peptide-coated DCs for immunization.

Intravenous injection of relatively low numbers ($\sim 2 \times 10^5$) of LPS-matured LM peptide-coated DC into WT mice results in generation of Ag-specific memory $CD8^+$ T cells of similar number and protective capacity as observed after sublethal infection with LM (15). Moreover, these DC-stimulated Ag-specific memory $CD8^+$ T cell populations persist long-term and can be increased using a booster DC-peptide immunization (data not shown). To determine whether the absence of IFN-γ altered $CD8^+$ T cell responses to DC-peptide vaccination, we immunized WT and GKO mice i.v. with 2.5×10^5 WT DC coated with the defined $CD8^+$ T cell epitopes LLO_{91-99}, $NP_{118-126}$, or $p60_{217-226}$ followed 35 days later by a second immunization ($0.8–1.0 \times 10^6$ peptide-coated WT DCs) to increase memory cell numbers. LLO_{91-99} and $p60_{217-226}$ are well-characterized $CD8^+$ T cell epitopes from LM (18) and rLM have been generated that express the LCMV-derived $NP_{118-126}$ epitope as a secreted fusion protein (LM-NPs; Ref. 12). Forty-five days after the booster immunization, the number of Ag-specific memory $CD8^+$ T cells in the spleens of vaccinated mice was evaluated using peptide-stimulated ICS for TNF-α (Fig. 1, *A* and *B*). DC-peptide vaccination of WT or GKO mice resulted in similar numbers of $NP_{118-126}$, LLO_{91-99}, or $p60_{217-226}$- specific memory $CD8^+$ T cells. As previously shown for primary DC-peptide immunization of WT mice (15), the number of LLO_{91-99}-specific memory $CD8^+$ T cells was highest of the three epitope specificities examined; therefore, some of the current studies will focus on comparison of memory cells of this specificity.

Functional avidity, TCR repertoire use, and phenotypic analysis of LM-specific $CD8^+$ T cells from immunized WT and GKO mice

After Ag encounter, peptide sensitivity is modulated in responding T cells resulting in increased sensitivity to low-dose Ag when compared with naive cells (26). Likewise, the diversity of the TCR repertoire is diminished (termed focusing) after secondary infection due to preferential outgrowth of highly responsive T cell clones (25). It is important to know whether Ag-specific $CD8^+$ T cells generated in the absence of IFN-γ exhibit similar functional

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avidity and/or repertoire use as those generated in WT hosts as these parameters could affect the protective capacity of the $CD8^+$ T cell populations. To evaluate functional avidity of Ag-specific memory $CD8^+$ T cells, splenocytes from immunized WT and GKO mice were incubated with 10-fold serial dilutions of LM peptides and the fraction of cells producing peptide-stimulated intracellular TNF-α was determined (Fig. 1*C*) (4, 26, 27). No differences in T cell functional avidity were observed when WT and GKO memory $CD8^+$ T cells were compared for all three ($LLO_{91–99}$, $NP_{118–126}$, or $p60_{217–225}$) epitopes examined. Depending on the Ag specificity, from 10^{-1} to 10^{-2} nM peptide was required to stimulate 50% of the maximum number of Ag-specific memory $CD8^+$ T cells from immunized WT or GKO mice to produce TNF-α.

To evaluate TCR repertoire use by Ag-specific $CD8^+$ T cells from WT and GKO mice, MHC class I tetramer$^+$ $CD8^+$ splenocytes were costained with a panel of Vβ-specific mAbs known to represent the majority of T cells responding to LM infection in WT BALB/c mice (28). No difference in Vβ segment usage was observed when WT and GKO memory $CD8^+$ T cells were compared within each Ag specificity where the majority of the $LLO_{91–99}$, $NP_{118–126}$, or $p60_{217–225}$ MHC class I tetramer$^+$$CD8^+$ cells expressed the Vβ8.1/8.2 and Vβ10 TCR chains (Fig. 1*D*). Thus, the absence of IFN-γ does not influence functional avidity or TCR repertoire use of secondary memory $CD8^+$ T cells specific for LM Ags.

The hallmarks of Ag-specific $CD8^+$ T cell memory are increased numbers, long-term persistence, vigorous expansion upon Ag re-exposure, and the ability to mediate protective immunity against pathogen rechallenge (29). Phenotypically, memory $CD8^+$ T cells remain a heterogeneous population that cannot be identified by a single cell surface marker. Recently, several markers indicative of Ag experience (CD44), homing potential (CD62L), costimulatory capacity (CD27), and cytokine responsiveness or expression (CD127 or IL-2/TNF/IFN-γ) have been used to distinguish and further characterize memory cell subsets (16, 19, 30). Expression of these surface markers is modulated after Ag stimulation as $CD8^+$ T cells progress from naive to memory cells, and a subset of these markers (CD62L for example) is differentially expressed on effector and central memory subsets (30). Therefore, combinations of these markers have been used to identify memory $CD8^+$ T cells with the highest proliferative potential and protective capacity (16, 19, 30). Flow cytometric evaluation of memory markers on Ag-specific $CD8^+$ T cells from WT and GKO mice (Fig. 2) 43 days after booster DC immunization revealed similar proportions of Ag-specific $CD8^+$ T cells that produce both TNF and IL-2 after stimulation with cognate peptide, and similar proportions of Ag-specific cells with expression of CD44, CD127, CD62L, and CD27 (T_{CM} phenotype). These finding indicate that the absence of IFN-γ during DC immunization does not alter the phenotype of the resulting memory $CD8^+$ T cells.

Protective immunity and expansion of LM-specific $CD8^+$ T cells from WT and GKO mice

DC-peptide immunization of WT mice results in $CD8^+$ T cell memory capable of providing sterilizing immunity to previously lethal doses of LM (15, 31). We next wished to evaluate the contribution of $CD8^+$ T cell-derived IFN-γ to protection against LM infection. However, challenge of the DC-immunized WT and GKO mouse strains was problematic due to potential variation in the number of Ag-specific $CD8^+$ T cells (Fig. 1*B*) and differences in innate resistance of the two strains (32). To overcome these limitations and evaluate the contribution of $CD8^+$ T cell-derived IFN-γ in protective immunity, equal numbers (3×10^5, determined by MHC class I tetramer staining) of LM Ag-specific ($LLO_{91–99}$, $NP_{118–126}$, or $p60_{217–226}$) memory $CD8^+$ T cells from the spleens of vaccinated WT or GKO mice (Thy 1.2) were transferred i.v. into naive WT-recipient mice (Thy1.1). One day later, T cell-recipient mice and naive controls were challenged with 2×10^4 (2 LD_{50} for naive mice) LM-NPs and protective immunity was evaluated on days 3 and 5 postinfection (p.i.) by determining CFU of LM in the spleens and livers (Table I). Regardless of epitope specificity, Ag-specific $CD8^+$ T cells from WT mice provided robust antilisterial immunity, as shown by a 2 $\log_{10}$ reduction in the bacterial counts in the spleens and 3 $\log_{10}$ reduction of CFU in the livers of recipient mice at 3 days after infection (Table I). In contrast, IFN-γ-deficient memory $CD8^+$ T cells exhibited decreased protective capacity resulting in only 1 $\log_{10}$ reduction in bacterial numbers in the spleens and 2 $\log_{10}$ reduction in CFU in the livers of GKO $CD8^+$ T cell-recipient mice. Thus, mice that received GKO memory $CD8^+$ T cells had ~1 $\log_{10}$ more bacteria in their organs 3 days after LM challenge than mice that received an equal number of WT memory $CD8^+$ T cells. Therefore, Ag-specific $CD8^+$ T cell-derived IFN-γ contributes to protective immunity mediated by memory $CD8^+$ T cells against LM infection. However, it seems that the role of IFN-γ is more critical very early after infection or the GKO cells can somehow compensate for their decreased protective function because the difference in number of bacterial CFU in the organs between groups is less pronounced by day 5 p.i.

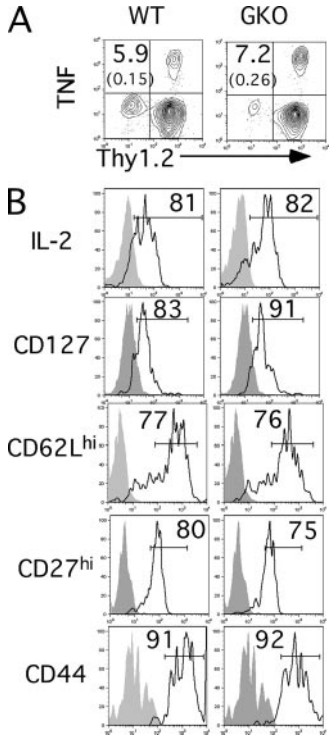

FIGURE 2. The absence of IFN-γ during DC immunization does not alter memory $CD8^+$ T cell phenotype. The frequency of Ag-specific memory $CD8^+$ T cells was determined 43 days after immunization in WT and GKO mice by ICS for TNF-α and memory cell phenotype was examined by costain for the indicated surface markers. Contour plots (*A*) are gated on $CD8^+$ cells and numbers indicate the percentage of TNF-α^+Thy1.2$^+$ cells/spleen after incubation of splenocytes with or without (number in parentheses) $LLO_{91–99}$ peptide. Histograms (*B*) representative of three independent experiments are gated on $CD8^+$TNF-α^+Thy1.2$^+$ cells and show the percent positive (open histogram) for the indicated marker based on isotype control staining (shaded histogram) of representative mice.

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Table I. *Log_{10} CFU protection from challenge with LM in WT or GKO T cell-recipient mice*[a]

	LLO_{91-99}[b]		$NP_{118-126}$[b]		$p60_{217-227}$[c]	
	Log_{10} CFU	Δ[d]	Log_{10} CFU	Δ	Log_{10} CFU	Δ
Day 3						
Spleen						
None[e]	6.90 ± 0.31		7.78 ± 0.11		7.50 ± 0.25	
WT[e]	4.77 ± 0.20	2.13	5.32 ± 0.15	2.46	5.61 ± 0.17	1.89
GKO[e]	5.6 ± 0.22	1.30	6.35 ± 0.14	1.43	6.60 ± 0.19	0.90
Liver						
None	6.8 ± 0.09		7.91 ± 0.27		7.33 ± 0.42	
WT	3.7 ± 0.37	3.10	5.27 ± 0.33	2.71	5.5 ± 0.26	1.83
GKO	4.8 ± 0.39	2.00	6.91 ± 0.19	1.01	6.5 ± 0.09	0.83
Day 5						
Spleen						
None	4.8 ± 0.40		5.96 ± 0.28		5.85 ± 0.28	
WT	3.26 ± 0.36	1.54	4.32 ± 0.24	1.66	4.19 ± 0.28	1.66
GKO	3.01 ± 0.39	1.79	4.66 ± 0.33	1.30	4.29 ± 0.53	1.56
Liver						
None	5.02 ± 0.56		6.29 ± 0.09		6.02 ± 0.17	
WT	2.75 ± 0.42	2.27	4.61 ± 0.18	1.69	4.94 ± 0.16	1.10
GKO	3.47 ± 0.31	1.55	5.28 ± 0.11	1.01	5.53 ± 0.13	0.50

[a] BALB/c WT or GKO mice were immunized with peptide-coated DCs. At least 35 days later, equal numbers LM Ag ($NP_{118-126}$, LLO_{91-99}, or $p60_{217-225}$)-specific $CD8^+$ T cells were transferred into naive recipient mice. The next day, T cell-recipient and naive control mice were challenged with 20,000 virulent LM-NPs. Three and five days later, portions of the spleen and liver were plated for CFU of LM. Log_{10} CFU reduction was calculated from naive control mice from each experiment.

[b] $n = 3–4$ mice/group from three independent experiments.

[c] $n = 3–4$ mice/group.

[d] The log_{10} protection (Δ) was determined by subtracting the mean log_{10} CFU of the test group from the mean log_{10} from the none (control) group.

[e] Donor T cell strain.

The remaining experiments will focus on a more detailed comparison of memory $CD8^+$ T cells from WT and GKO mice specific for the LLO_{91-99} epitope because this response is the largest (vs $NP_{118-126}$ or $p60_{217-225}$) after DC-peptide immunization, and protective immunity and expansion was similar for $CD8^+$ T cells specific for all three LM epitopes (Table I).

Ag-specifc $CD8^+$ T cells from GKO mice provide decreased protective immunity to high-dose challenge

Thus far, our data indicate that Ag-specific $CD8^+$ T cells from vaccinated GKO mice provide decreased early protection on a per-cell basis against a specific LM challenge dose. To extend this finding, we evaluated the protective immunity under conditions where we varied the number of memory cells transferred or the challenge dose of bacteria. First, we adoptively transferred fewer (1×10^5) Ag-specific $CD8^+$ T cells from WT and GKO donors and examined the rate of bacterial clearance in spleens and livers of naive and T cell-recipient mice challenged with LM (Fig. 3). Similar to our previous findings, transfer of $CD8^+$ T cells from WT mice resulted in 1–2 log_{10} reduction in bacterial numbers early after infection (day 3) compared with infected naive mice whereas equal numbers of GKO-derived Ag-specific $CD8^+$ T cells provided reduced protection. In contrast to the previous experiment in which more (3-fold) Ag-specific $CD8^+$ T cells were transferred, increased bacterial numbers were observed in the spleens and livers of GKO $CD8^+$ T cell-recipient mice on days 5 and 7 p.i. compared with WT T cell-recipient mice (Fig. 3). Over the course of 9 days, the infected naive mice were unable to clear LM while both WT and GKO $CD8^+$ T cell-recipient mice were able to clear the infection. The reduced bacterial clearance in GKO $CD8^+$ T cell-recipient mice confirms that loss of $CD8^+$ T cell-derived IFN-γ contributes to $CD8^+$ T cell-mediated protective immunity.

The infectious course of *L. monocytogenes* in an immune host is dependent on a balance between the number and function of Ag-specific $CD8^+$ T cells and the number of bacteria present (4). Successful sterilizing immunity occurs only when the potency of the Ag-specific CD8 T cell response is greater than the bacterial survival and proliferation. Therefore, we evaluated the number of LLO_{91-99}-specific $CD8^+$ T cells in the spleens of recipient mice 5 days after bacterial challenge, the peak of the memory response to this Ag (Fig. 3*C*) and identified a 3-fold increase in the number of LLO_{91-99}-specific $CD8^+$ T cells in the spleens of mice that received equal numbers Ag-specific cells from WT and GKO donors. A similar increase in the number of Ag-specific $CD8^+$ T cells in the spleens of recipient mice was observed when donor (Thy1.2) $CD8^+$ T cells specific for $NP_{118-126}$ or $p60_{217-226}$ LM epitopes were adoptively transferred into naive Thy 1.1 mice (Table I and data not shown). Thus, the transferred memory $CD8^+$ T cells from GKO mice are capable of robust Ag-induced proliferation. Additionally, it is likely that in some experimental scenarios the increased proliferation of IFN-γ-deficient $CD8^+$ T cells could compensate for reduced per-cell protective capacity, as has been shown in the absence of other effector molecules (4).

Next, we examined the protective immunity provided by adoptively transferred T cells to a 2.5-fold higher dose of bacteria (5×10^4 LM-NPs) and evaluated the kinetics of bacterial clearance on days 1–4 p.i. (Fig. 4*A*). Naive control mice had very high numbers (10^7–10^8) of bacteria in both organs throughout the experiment. The adoptive transfer of WT memory $CD8^+$ T cells resulted in 1–2 log_{10} fewer bacteria in both organs on days 1 and 2 p.i., whereas GKO memory $CD8^+$ T cell-recipient mice had intermediate levels of bacteria in both organs assayed at early time points. The deficiency in protective immunity exhibited by GKO $CD8^+$ T cells is most evident on days 3 and 4 p.i. when the number of bacteria in the organs of WT $CD8^+$ T cell-recipient mice declines but bacterial numbers in GKO $CD8^+$ T cell-recipient mice increase to levels similar to those observed in the naive control mice. Of additional interest is the observation that by day 4 p.i., some mortality was observed in both the naive (three dead) and GKO

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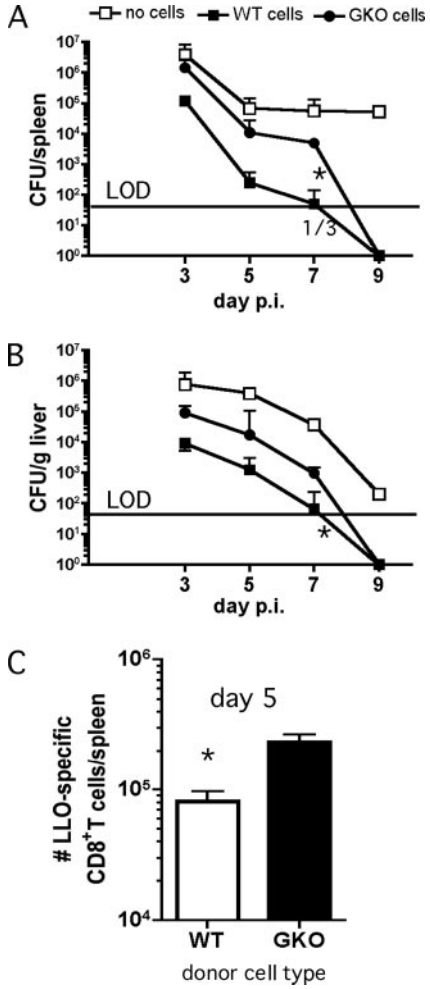

FIGURE 3. Adoptive transfer of IFN-γ-deficient memory $CD8^+$ T cells results in delayed bacterial clearance. $LLO_{91–99}$-specific memory $CD8^+$ T cells (1×10^5) were obtained from immunized WT or GKO donor mice and transferred i.v. into naive recipient mice. One day later, T cell-recipient and naive control mice were challenged with 2×10^4 LM-NPs. At various days after challenge, portions of the spleen (*A*) and liver (*B*) were plated to determine CFU of LM/spleen or /gram liver. The total number of Ag-specific $CD8^+$ T cells/spleen (*C*) was determined by ICS for TNF-α on day 5 p.i. Data are represented as mean ± SD of three to four mice/group; 1/3 indicates that one mouse had CFU below the LOD for this assay; *, $p \leq 0.05$ compared with GKO cell-recipient mice.

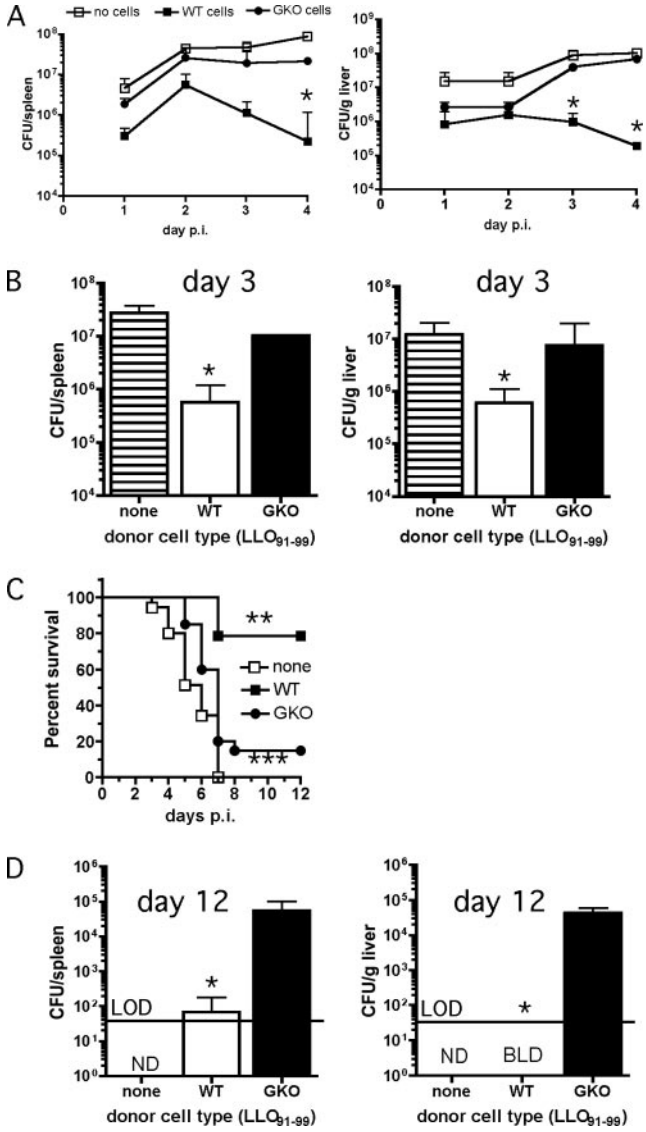

FIGURE 4. IFN-γ participates in protective immunity early after high-dose LM challenge. $LLO_{91–99}$-specific memory $CD8^+$ T cells (3×10^5 Thy 1.2^+) were obtained from immunized WT or GKO donor mice and transferred i.v. into naive recipient mice (Thy 1.1^+). One day later, T cell-recipient mice were challenged with ~5×10^4 LM-NPs. On various days after challenge, portions of the spleen and liver were plated to determine CFU of LM (*A*). In a separate experiment, bacterial clearance and survival was examined in T cell-recipient mice after i.v. transfer of (3×10^5 Thy 1.2^+) $LLO_{91–99}$-specific memory $CD8^+$ T cells and challenge with 5×10^4 LM-NPs. On day 3 after challenge, portions of the spleen and liver were plated to determine CFU of LM (*B*). Survival was evaluated until day 12 (*C*), when remaining mice were sacrificed and portions of the spleens and livers were plated for CFU of LM (*D*). Data are represented as mean ± SD of 3–4 (*A*, *B*, and *D*) and 10–12 (*C*) mice/group. Data are expressed as total CFU/spleen or CFU/gram liver. BLD, Below the level of detection for this assay; *, $p \leq 0.05$ from GKO T cell-recipient mice; ***, $p \leq 0.0001$ (log-rank test for trend) from WT T cell-recipient mice.

$CD8^+$ T cell-recipient (two dead) mice but all WT $CD8^+$ T cell-recipient mice survived this high-dose LM challenge dose.

Finally, we repeated a high-dose challenge (5×10^4 LM-NPs) experiment and evaluated bacterial numbers on days 3 and 12 p.i. in naive and T cell-recipient mice (Fig. 4, *B* and *D*). As previously observed on day 3 p.i., WT $CD8^+$ T cell-recipient mice had significantly fewer bacteria when compared with infected naive control mice whereas an equal number (3×10^5) of Ag-specific $CD8^+$ T cells from GKO mice did not provide any protection (Fig. 4*B*). Challenge with this dose of LM resulted in 0% survival of naive mice day 7 p.i. compared with 78 and 18% survival for WT and GKO $CD8^+$ T cell-recipient mice, respectively (Fig. 4*C*). When CFUs were evaluated on day 12 p.i. in the organs of remaining T cell-recipient mice (Fig. 4*D*), WT $CD8^+$ T cell-recipient mice had cleared bacteria from their spleens and livers to a level below the limit of detection (LOD). In contrast, adoptive transfer of an equal number of IFN-γ-deficient $CD8^+$ T cells were unable to effectively clear the infection and had nearly 5 $\log_{10}$ bacteria remaining in their organs on day 12 p.i.

Taken together, the data clearly demonstrate that the Ag-specific $CD8^+$ T cells obtained from vaccinated GKO mice exhibit decreased $CD8^+$ T cell-dependent protective immunity when compared with WT $CD8^+$ T cells on a per cell basis.

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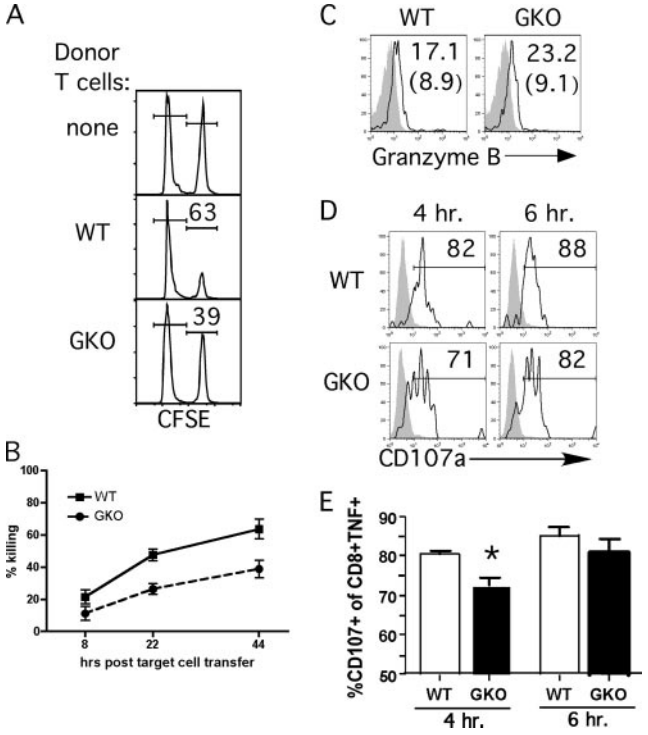

FIGURE 5. IFN-γ-deficient memory $CD8^+$ T cells exhibit decreased Ag-specific target cell lysis. In vivo cytolysis of peptide-coated ($CFSE^{high}$) or uncoated ($CFSE^{low}$) naive syngeneic lymphocytes was evaluated in WT or GKO memory $CD8^+$ T cell-recipient (4×10^5 $LLO_{91–99}$-specific) mice. Histograms are gated on $CFSE^+$ cells (*A*) and numbers represent the percentage of targets killed 44 h after target cell transfer. Percent killing over time (*B*). Granzyme B (*C*) or CD107a expression (*D* and *E*) on $CD8^+$ T cells from immunized WT and GKO mice after $LLO_{91–99}$ peptide stimulation. Histograms (*C*) are gated on $CD8^+TNF^+$ cells and numbers indicate the MFI of stained cells (open histogram) compared with the isotype control (in parentheses; shaded histogram). Histograms (*D*) are gated on $CD8^+TNF^+$ cells and numbers indicate the percent positive (open histogram) based on isotype control staining (shaded histogram). Mean percentage ± SD CD107a (*E*) staining from four mice per group and is representative of two independent experiments.

In vivo cytotoxicity

One of the main mechanisms by which Ag-specific memory $CD8^+$ T cells mediate protective immunity against LM is through lysis of infected target cells (4, 33). A deficiency in cytotoxicity could result in higher bacterial numbers within the infected host as we have observed when protective immunity mediated by memory $CD8^+$ T cells from GKO mice is compared with mice that received WT $CD8^+$ T cells. To address potential differences in cytolysis between WT and GKO-derived $CD8^+$ T cells, we used an in vivo cytotoxicity assay (5). Equal numbers (3×10^5) of $LLO_{91–99}$-specific memory $CD8^+$ T cells from DC-vaccinated WT and GKO mice were transferred into naive WT-recipient mice (Fig. 5). Next, a 1:1 ratio of $LLO_{91–99}$-coated ($CFSE^{high}$) and uncoated ($CFSE^{low}$) targets (syngeneic spleen cells) were injected i.v. into naive controls or the T cell-recipient mice and the relative elimination of the $CFSE^{high}$ peak (Ag-specific killing) was monitored in the spleens using flow cytometry. At 8, 22, and 44 h after target cell transfer, 24, 48, and 63% of the peptide-pulsed targets had been eliminated in mice that received WT-derived $CD8^+$ T cells (Fig. 5, *A* and *B*). In contrast, Ag-specific killing by an equal number of GKO-derived $CD8^+$ T cells was reduced at all time points, with a peak level of 38% cytotoxicity at 44 h post-target cell transfer. Further change in the percent cytotoxicity was not observed at later time points (data not shown) and may reflect loss of peptide/MHC stability on the target cells. In this experiment, memory $CD8^+$ T cells from WT or GKO mice were adoptively transferred into WT T cell-recipient mice so the deficiency is not due to a complete absence of IFN-γ in the system, but results from an inherent difference in function of memory $CD8^+$ T cells from GKO mice. Regardless, our studies are the first to identify a role for IFN-γ in protective immunity to LM and suggest that the absence of this cytokine results in inefficient Ag-specific cytolysis.

The primary mechanism of $CD8^+$ T cell-mediated target cell killing is through directed release of stored lytic granules containing perforin and granzymes into the target cell (34). To assess potential differences in cytotoxic potential, we evaluated level of granzyme B expression (Fig. 5*C*) in memory $CD8^+$ T cells from WT and GKO mice by flow cytometry. As is characteristic of memory cells (23), a low level of intracellular granzyme B was detected in Ag-specific $CD8^+$ T cells from vaccinated WT (mean fluorescence intensity (MFI) = 17.1 ± 1.1/isotype = 8.9 ± 0.5) and GKO mice (MFI = 23.2 ± 0.9/isotype = 9.0 ± 0.4) (Fig. 5*C*). Thus, the decreased target cell killing by memory $CD8^+$ T cells from GKO mice cannot be attributed to differences in granzyme B levels in stable memory populations.

An additional characteristic that can distinguish memory cell populations (23) is the rate of degranulation of Ag-specific $CD8^+$ T cells. We considered the possibility that delayed Ag-induced degranulation by GKO-derived memory $CD8^+$ T cells could contribute to reduced target cell killing in vivo. Recently, the presence of lysosomal-associated glycoproteins, molecules normally confined to the membrane of stored lytic granule, on the plasma membrane of T cells has been used as an indicator of degranulation (35). We evaluated the kinetics of degranulation as indicated by CD107a (lysosomal-associated glycoprotein-1) surface staining on the surface of memory $CD8^+$ T cells early after Ag stimulation (Fig. 5, *D* and *E*). Because 4 h of peptide stimulation was required to detect the maximal number of Ag-specific cells using ICS for TNF-α, we evaluated CD107a staining in memory $CD8^+$ T cells from vaccinated WT and GKO mice at 4 and 6 h after stimulation with $LLO_{91–99}$ peptide. A small decrease in the percentage of degranulated GKO cells was observed 4 h after Ag stimulation (GKO = 72.6 ± 3.4, WT = 80.5 ± 1.2), while no difference was observed by 6 h (GKO = 80.9 ± 5.1, WT = 85.2 ± 3.8) (Fig. 5, *D* and *E*). Although it is possible that a decrease in the rate of degranulation could influence the rate of cytolysis early after infection, a similar maximal degranulation is achieved within 6 h after Ag stimulation, so it is unlikely that inefficient degranulation could wholly account for the diminished protective immunity provided by memory $CD8^+$ T cells from GKO mice.

Ag-specific $CD8^+$ T cell-derived IFN-γ improves in vivo target cell killing

In a variety of in vitro experimental systems, IFN-γ can sensitize target cells to $CD8^+$ T cell-mediated lysis via the granule exocytosis pathway or death receptor engagement (36, 37). To determine whether IFN-γ production by memory $CD8^+$ T cells could influence cytolysis by directly acting on target cells in vivo, we used a C57BL/6 OT-1 adoptive transfer system in which the transferred WT OVA-specific memory $CD8^+$ T cells were evaluated for their ability to kill WT or IFN-γR-deficient (IFN-γR KO) targets. In this experiment, the sensitivity of the target cells to the direct effect of IFN-γ differs while the source of memory $CD8^+$ T cells remains the same. Each target cell population (WT or IFN-γR KO) was coated with (Ag specific) or without (nonspecific) 1 μM OVA peptide (Fig. 6*A*), and the resulting target cell populations (WT without OVA, WT with OVA, IFN-γR KO without OVA and

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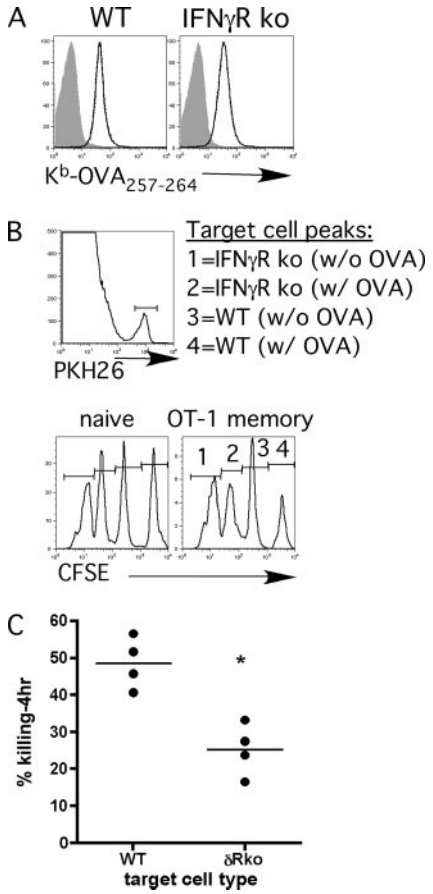

FIGURE 6. T cell-derived IFN-γ increases in vivo Ag-specific cytolysis. Lysis of peptide-coated WT or IFN-γR-deficient (IFN-γR KO) target cells was evaluated in naive B6 or memory OT-I (1.5×10^6) recipient mice. Histograms (*A*) indicate uniform peptide coating of WT or IFN-γR KO targets with K^b-OVA mAb (open histogram) or isotype control (shaded histogram). Target cells (*B*) were stained with PKH26 for uniform identification and serial dilutions of CFSE to identify target subpopulations as indicated and injected into naive or memory OT-I T cell-recipient mice. Percent killing (*C*) was determined 4 h post-target cell transfer by relative CFSE fluorescence of the $PKH26^+$ target cell population. *, $p \leq 0.05$ from WT targets.

IFN-γR KO with OVA) were labeled with 10-fold dilutions of CFSE (populations labeled 1–4 in Fig. 6*B*). Uniform identification of all target cell populations was achieved by combining equal numbers of each target cell population labeling with cell linker red dye (Fig. 6*B*). Targets (8×10^6 total/mouse) were injected i.v. into naive C57BL/6 mice or OT-I memory $CD8^+$ T cell-recipient mice (1.5×10^6 cells/mouse, i.v.) (Fig. 6*B*). Injection of all target cell populations into each experimental (and naive) mouse controlled for any possible mouse to mouse variations. The disappearance of the peptide-coated population relative to its uncoated control was monitored in the spleens of target cell-recipient mice and the percent killing was calculated.

At 4 h post-target cell transfer, significantly ($p < 0.05$) more (~48%) of the WT target cells had been lysed compared with the (~25%) IFN-γR KO target cells (WT 48.5 ± 8.06; GKO 23 ± 6.27) (Fig. 6, *B* and *C*). The difference was maintained 8 h post-target cell transfer and was not altered by incubation of target cells with 10-fold more peptide (data not shown). These findings suggest that IFN-γ derived from Ag-specific memory $CD8^+$ T cells can increase the susceptibility of target cells to lysis in vivo.

Discussion

Activated $CD8^+$ T cells possess an array of antimicrobial effector mechanisms capable of controlling pathogenic microbes, including production of proinflammatory cytokines (IFN-γ and TNF) and killing infected cells via the perforin-dependent granule exocytosis pathway. Interestingly, studies with GKO mice show that pathogen biology appears to dictate which effector pathways dominate in resistance to specific infection (reviewed in Ref. 2). For example, clearance of LCMV, a nonlytic viral infection, is absolutely dependent on perforin-mediated killing by $CD8^+$ T cells. In contrast, IFN-γ is essential for clearance of vaccinia, a rapidly lytic viral infection. Furthermore, granzyme A, but not granzyme B, is essential for clearance of ectromelia virus. Importantly, these above mentioned effector pathways are not unique to $CD8^+$ T cells, but are also deployed by other immune cells types (i.e., cytokine production by Th1 and NK cells, perforin-mediated killing by NK cells). This complicates interpretation of vaccine studies with GKO mice. In addition, the absence of at least some of these effector pathways can alter the homeostasis of Ag-specific $CD8^+$ T cells stimulated by infection or vaccination (8). Thus, determining the contribution of a specific effector function to $CD8^+$ T cell immunity is technically challenging. Additionally, these data suggest that the relevant $CD8^+$ T cell effector pathways for individual pathogens cannot be assumed but must be identified for each pathogen. This information is critical to vaccine design so that T cells with the correct array of effector mechanisms can be targeted.

LM is a facultative intracellular bacterium capable of infecting a wide array of eukaryotic cells and tissues and is extensively used as a model organism to evaluate innate and T cell immunity to infection. Previous studies from our laboratory revealed that perforin-dependent cytolysis is a major effector pathway for memory $CD8^+$ T cell-mediated protective immunity against LM infection (4). However, while perforin-deficient $CD8^+$ T cells exhibited only one-fifth the per-cell protective capacity of WT CD8 T cells, increasing the number of perforin-deficient $CD8^+$ T cells restored protective immunity against LM infection. Thus, other effector pathways contribute to $CD8^+$ T cell immunity against LM infection. In the current study, we used adoptive transfer of single Ag-specific $CD8^+$ T cells from WT and GKO mice to show that IFN-γ derived from memory $CD8^+$ T cells also contributes to protective immunity against LM infection.

IFN-γ plays a critical role in innate resistance to primary LM infection such that GKO mice succumb to as few as 10 virulent bacteria (6). Because activated $CD8^+$ T cells produce IFN-γ and secondary resistance to LM is dependent on $CD8^+$ T cells, it was assumed that this cytokine was also involved in adaptive protective immunity (38); however, the generation of protective immunity in vaccinated GKO mice proved otherwise (6). Careful examination of the LM Ag-specific $CD8^+$ T cell responses in vaccinated GKO mice revealed alterations in immunodominance hierarchies that persisted into the memory phase of the response and a substantial increase in the number of memory $CD8^+$ T cells due to lack of contraction (8, 39). Because of the changes in the number and hierarchy of memory $CD8^+$ T cells the previous studies were unable to determine whether $CD8^+$ T cell-derived IFN-γ contributed to $CD8^+$ T cell-mediated protective immunity against LM infection.

To overcome these limitations, we used an adoptive transfer system to compare the per cell protective capacity of memory $CD8^+$ T cells from WT or GKO mice of a single LM Ag specificity. The current studies determined that LM-epitope specific $CD8^+$ T cells from immunized GKO mice provided significantly decreased protective immunity early (days 1–3) after infection

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when compared with equal numbers of Ag-matched WT $CD8^+$ T cells. This finding was reproducible in a variety of experimental settings including those with fewer $CD8^+$ T cells transferred, increased challenge doses of LM, and at later time points when survival and bacterial clearance was examined.

How does $CD8^+$ T cell-derived IFN-γ contribute to antilisterial immunity? Ag-specific $CD8^+$ T cells generated by DC immunization in WT and GKO mice are phenotypically similar for a number of relevant memory cell markers (30) and produce similar amounts of important cytokines such as TNF and IL-2 after Ag stimulation. However, Ag-specific $CD8^+$ T cells from GKO mice exhibit a decreased ability to kill Ag-coated target cells in vivo. Our data suggest that this deficiency is due to the loss of IFN-γ as an effector molecule rather than a deficiency in the mechanisms of cytotoxicity such as granzyme B expression or a dramatic alteration in Ag-stimulated degranulation.

IFN-γ is a pluripotent cytokine capable of regulating >1000 genes in a variety of cell types including monocytes/macrophages, B cells, NK cells, and T cells as well as nonimmune cells. In our studies, the inefficient killing of IFN-γ-insensitive (IFN-$\gamma R^{-/-}$) peptide-coated targets identifies a need for this cytokine at the time of T cell engagement of the target cell. Several in vitro studies suggest that IFN-γ can increase the sensitivity of target cells to $CD8^+$ T cell lysis (36, 37). One potential mechanism by which IFN-γ could control the sensitivity of target cell lysis is through up-regulation of MHC class I gene expression with concurrent increase in number of stimulatory peptide-MHC class I complexes on the surface of infected cells (31). In the case of LM infection, this would aid in the detection of LM, an intracellular pathogen, by Ag-specific $CD8^+$ T cells.

Because the defect in cytolysis by the memory $CD8^+$ T cells from GKO mice occurred relatively quickly (4 h) after Ag stimulation (target injection), it is unlikely that increased class I Ag presentation is the only mechanism involved. To this end, IFN-γ has also been shown to exert apoptotic effects on immune cells. For example, IFN-γ can increase expression of Fas, FasL (40, 41), and TNF (42) receptors on target cells within hours after Ag stimulation and also increases the production of death-associated proteins, such as DAPI-5 (43). Moreover, Ag-specific $CD8^+$ T become rapidly insensitive to IFN-γ through receptor down-regulation (13, 44). Thus, during LM infection, localized IFN-γ production in the spleen or liver microenvironment could sensitize infected cells to Ag-specific destruction by activated $CD8^+$ T cells while the effector cells would remain insensitive to the proapoptotic effects of IFN-γ by virtue of their receptor down-regulation (13, 44). Even a small decrease in the efficiency of Ag-specific target cell killing in the early hours after infection would exponentially increase the number of infected cells (due in part to cell-cell bacterial spread) at later days after bacterial challenge (45).

Although perforin-dependent cytolysis is the major effector mechanism of Ag-specific $CD8^+$ T cells in resistance to LM infection (46), we conclude that the production of IFN-γ by effector T cells augments the efficiency of target cell killing. It remains to be determined whether other effector pathways such as TNF production also contribute to memory $CD8^+$ T cell protection against LM infection.

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Disclosures

The authors have no financial conflict of interest.

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