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Heather D. Marshall, Amanda L. Prince, Leslie J. Berg and Raymond M. Welsh

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IFN-αβ and Self-MHC Divert CD8 T Cells into a Distinct Differentiation Pathway Characterized by Rapid Acquisition of Effector Functions

Heather D. Marshall,[1] Amanda L. Prince, Leslie J. Berg, and Raymond M. Welsh

Nonvirus-specific bystander CD8 T cells bathe in an inflammatory environment during viral infections. To determine whether bystander CD8 T cells are affected by these environments, we examined P14, HY, and OT-I TCR transgenic CD8 T cells sensitized in vivo by IFN-αβ–inducing viral infections or by polyinosinic:polycytidylic acid. These sensitized cells rapidly exerted effector functions, such as IFN-γ production and degranulation, on contact with their high-affinity cognate Ag. Sensitization required self-MHC I and indirect effects of IFN-αβ, which together upregulated the T-box transcription factor Eomesodermin, potentially enabling the T cells to rapidly transcribe CTL effector genes and behave like memory cells rather than naive T cells. IL-12, IL-15, IL-18, and IFN-γ were not individually required for sensitization to produce IFN-γ, but IL-15 was required for upregulation of granzyme B. These experiments indicate that naive CD8 T cells receive signals from self-MHC and IFN-αβ and that, by this process, CD8 T cell responses to viral infection can undergo distinct differentiation pathways, depending on the timing of Ag encounter during the virus-induced IFN response. ***The Journal of Immunology*****, 2010, 185: 1419–1428.**

Many viral infections induce robust CD8 T cell responses that result in the lysis of virus- infected cells, secretion of antiviral cytokines, and the clearance of the virus. Virus-specific CD8 T cells undergo a programmed pathway of differentiation that is tightly coupled to proliferation (1, 2). After several rounds of division, effector CD8 T cells gain the ability to secrete cytokines and chemokines, including IFN-γ, MIP-1β, and Rantes, and acquire the ability to lyse virus-infected or peptide-pulsed target cells after differentiating into CTLs (2–4). Effector functions of CTLs are tightly regulated to diminish the potential immune pathology associated with inflammatory cytokines and cytolysis. Naive CD8 T cells normally require ~3 d to start expressing IFN-γ, whereas effector and memory CD8 T cells can rapidly turn on *IFN-γ* transcription as early as 1 h after re-encountering their cognate ligands (4–6). Importantly, virus-specific CTLs do not continuously produce IFN-γ during infections, but they turn on its expression when re-encountering Ag in a local environment of infected tissue (7).

IFN-γ gene transcription, like that of other CTL effector genes, such as *granzymes* and *perforin*, is regulated by chromatin accessibility and the expression of appropriate transcription factors. The T-box transcription factors T-bet and Eomesodermin (Eomes) are the principal transcription factors regulating CTL effector gene transcription (8–13), and the expression of these transcription factors is low in naive CD8 T cells. They are both upregulated in effector CTLs, with T-bet being the prominent transcription factor present, whereas Eomes is upregulated further in memory CD8 T cells (10, 14). Knockout (KO) and knockdown studies of both transcription factors revealed partially overlapping and compensatory functions in CD8 T cells (8, 11–13, 15). Thus, CD8 T cells lacking both transcription factors have the most pronounced defect in effector function capabilities; lymphocytic choriomeningitis virus (LCMV)-specific CD8 T cells lacking T-bet and Eomes express very little IFN-γ, perforin, and granzyme B (GrzB), and instead express an aberrantly high amount of IL-17 (9).

It was once highly debated whether bystander T cells contributed to the large pool of CD8 T cells at the peak of an immune response; however, for the most part, sophisticated techniques and models to detect virus-specific and bystander T cells have quelled this theory (16–18). Despite the lack of direct participation of bystander T cells during antiviral immune responses, it was conceivable that these cells still received signals by the inflammatory milieu of cytokines and chemokines or by nonviral peptide–MHC–TCR interactions. Additionally, virus-specific T cells recruited later in the immune response, or "latecomer" T cells, may also be affected by inflammatory signals prior to Ag stimulation, and it is likely that a combination of T cells with different signaling sequences from cytokine receptors and TCR constitutes the total T cell response to pathogens.

We sought to understand how bystander and, thus, possibly latecomer CD8 T cells, were affected by ongoing antiviral immune responses by generating several in vivo models using P14 (LCMV glycoprotein-specific), HY (male Ag-specific), and OT-I (OVA-specific) transgenic CD8 T cells. We found that, during acute viral infections or after stimulation with type 1 IFN (IFN-αβ) inducers, some bystander CD8 T cells were sensitized to upregulate GrzB in vivo and immediately exert effector functions, such as IFN-γ production and degranulation, upon stimulation with high-affinity cognate Ag in vitro. Sensitization of naive CD8 T cells required

Department of Pathology, Program in Immunology and Virology, University of Massachusetts Medical School, Worcester, MA 01655

[1]Current address: Department of Immunobiology, Yale University School of Medicine, New Haven, CT

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Address correspondence and reprint requests to Dr. Raymond M. Welsh, Department of Pathology, University of Massachusetts Medical School, S2-244, 55 Lake Avenue North, Worcester, MA 01655. E-mail address: Raymond.Welsh@umassmed.edu

Abbreviations used in this paper: CPE, cytopathic effect; Eomes, eomesodermin; GrzB, granzyme B; KO, knockout; LCMV, lymphocytic choriomeningitis virus; N/A, not applicable; poly(I:C), polyinosinic:polycytidylic acid; PV, Pichinde virus; UMMS, University of Massachusetts Medical School; VSV, vascular stomatitis virus; VV, vaccinia virus; WT, wild-type.

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self-MHC I and indirect effects of IFN-αβ, whereas IL-12, IL-18, and IFN-γ were not individually required. IL-15 was not required for the rapid expression of IFN-γ, but it was required for upregulation of GrzB. Sensitized naive CD8 T cells upregulated the T-box transcription factor Eomes, which can regulate these rapid effector functions.

Materials and Methods

Mice

C57BL/6J, B6.129P2-B2m tm1Unc/J (β2m KO), B6.129S7-IFNg < tm1Ts > /J (IFN-γ KO), and B6.129P2-*Il18*^{tm1Aki}/J (IL-18 KO) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6-H-2Kb < tm1 > H-2Db < tm1 > (K^bD^b KO), C57BL/6-H-2Kb < tm1 > (K^b KO), C57BL/6-H-2Db < tm1 > (D^b KO), C57BL/6NTac-IL15 < tm1 > N5 (IL-15 KO) (19) and TCR-LCMV P14/Rag2 KO (P14) mice were purchased from Taconic Farms (Germantown, NY). B6.SJL (Ly5.1$^+$) male and female mice were purchased from Taconic Farms or bred within the Department of Animal Medicine at the University of Massachusetts Medical School (UMMS). TCR transgenic mice [P14 (20), HY (21), and OT-I (22)], IFN-αβR KO mice (IFN-αβR KO) (23), and B6.129-IL-12β (IL-12 p40 KO) mice were bred at UMMS. TCR transgenic P14 mice were crossed to IFN-αβR KO mice and were screened via surface expression of Vα2$^+$ TCR on CD8 T cells and genomic PCR for the KO IFN-αβR locus. All mice were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee of UMMS.

Virus stocks and inoculations

LCMV, strain Armstrong; Pichinde virus (PV), strain AN3739; and vesicular stomatitis virus (VSV), strain Indiana; were propagated in baby hamster kidney cells (BHK21), as previously described (24, 25). Vaccinia virus (VV), strain WR, was propagated in NCTC 929 cells and purified over a sucrose gradient (26). Mice were inoculated i.p. with 5×10^4 PFU LCMV, 1.5×10^7 PFU PV, or 1×10^6 PFU VV. To induce IFN-αβ in vivo, mice were inoculated with 200 μg polyinosinic:polycytidylic acid [poly (I:C)] (InvivoGen, San Diego, CA) i.p., unless otherwise described. To deplete NK cells, mice were inoculated i.v. with 25 μg anti-NK1.1 or IgG2a (Cl.18.4) isotype control (BioXcell, West Lebanon, NH), and cells were stained with NK1.1 (PK136; BD Pharmingen, San Diego, CA) to assess depletion. To deplete CD4 T cells, mice were inoculated i.v. with 100 μg anti-CD4 (GK1.5) Ab or IgG2b (LTF-2) isotype control (BioXcell), and cells were stained with anti-CD4 (RM4-4; BD Pharmingen) to assess depletion.

Adoptive transfers

Spleens were harvested from TCR transgenic mice (P14, HY, or OT-I), and single-cell suspensions were prepared. RBCs were lysed with a 0.84% NH_4Cl solution, and lymphocytes were washed with HBSS. Where described, cells were labeled with the fluorescent dye CFSE by incubation in 2 μM CFSE in HBSS (Invitrogen, Carlsbad, CA) at 37°C for 15 min. A total of 5×10^5–1×10^6 TCR transgenic CD8 T cells were injected i.v. into congenic recipient mice.

Synthetic peptides

Synthetic peptides were used to stimulate T cell responses. All peptides were purchased from 21st Century Biochemicals (Marlboro, MA) and were purified with reverse phase-HPLC to 90% purity. For ex vivo stimulations, P14 transgenic T cells were stimulated with the LCMV epitope $GP_{33–41}$ (KAVYNFATC) (20), HY transgenic T cells were stimulated with the Y-chromosome–encoded Smcy epitope (KCSRNRQYL) (27), and OT-I transgenic T cells were stimulated with $OVA_{257–264}$ (SIINFEKL) (22).

Intracellular cytokine and effector molecule staining

Cytokine production was evaluated after stimulation with peptides using the Cytofix/Cytoperm Kit Plus (with GolgiPlug; BD Pharmingen). Spleen leukocytes ($2–4 \times 10^6$) were plated in replicates (as many as 10 wells/spleen) in 96-well plates with 5 μM synthetic peptide, 10 U/ml human rIL-2, and 0.2 μl GolgiPlug (BD Pharmingen) for 5 h at 37°C. For positive controls, splenocytes were stimulated with 1 μg purified anti-mouse CD3ε mAb (145-2c11; BD Pharmingen). Following stimulation, splenocytes were washed in Flow Cytometry Buffer (2% FCS in HBSS) and blocked with α-Fc (2.4G2; BD Pharmingen) for 15 min at 4°C. Splenocytes were then stained with a combination of fluorescently labeled mAbs specific for CD8 (53-6.7; BD Pharmingen), Ly5.2/CD45.2 (104; BD Pharmingen), Ly5.1/CD45.1 (A20; eBioscience, San Diego, CA or BioLegend San Diego, CA), Thy1.2/CD90.2 (53-2.1; BD Pharmingen), Thy1.1/CD90.1 (H1S51; eBioscience), Vα2 TCR (B20.1; eBioscience), HY TCR (T3.70; eBioscience), CD44 (IM7; BD Pharmingen), CD122 (TM-β1; BD Pharmingen), CD62L (MEL-14; BD Pharmingen), and CD43 (1B11; BioLegend) for 20 min at 4°C. Subsequent fixation and permeabilization were performed via Cytofix/Cytoperm for 20 min at 4°C. Following permeabilization, cells were stained with fluorescently labeled mAbs specific for IFN-γ (XMG1.2; BD Pharmingen or eBioscience), TNF (MP6-XT22; BD Pharmingen), and/or GrzB (GB11; Invitrogen). Eomes protein was stained with anti-mouse/human Eomes (Dan11mag; eBioscience) after fixation and permeabilization with the FoxP3 staining buffer kit (eBioscience), as per the manufacturer's instruction. To assay the ability of CD8 T cells to undergo Ag-driven degranulation, splenocytes were stimulated with synthetic peptides, as stated above, with the addition of 0.5 μl/well anti-CD107a (1D4B) and anti-CD107b (ABL-93) FITC-labeled Abs and 0.2 μl/well GolgiStop (all from BD Pharmingen).

Flow cytometry

Freshly stained and previously fixed samples were acquired using an LSRII (BD Biosciences, San Jose, CA) with FACS Diva software and analyzed with FlowJo software (Tree Star, Ashland, OR). To analyze enough bystander TCR transgenic CD8 T cells, the threshold for acquisition was set to CD8$^+$ events only, and the storage and stoppage gates were set on CD8$^+$ events only. By setting these parameters, FACS Diva ignored all other

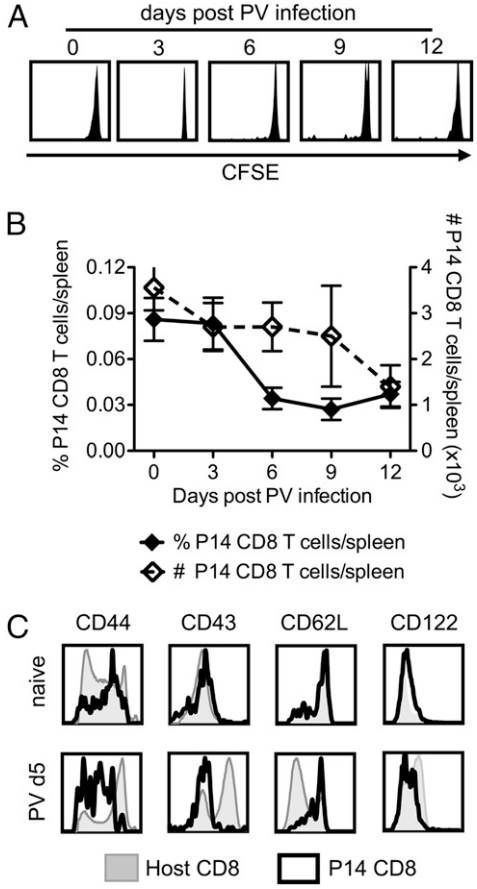

FIGURE 1. P14 CD8 T cells do not divide or proliferate and remain phenotypically naive during PV infection. P14 transgenic CD8 T cells were adoptively transferred into naive congenic recipients followed by infection with 1.5×10^7 PFU PV i.p. *A*, CFSE profiles of donor P14 CD8 T cells at 0, 3, 6, 9, and 12 d post-PV infection from individual mice representative of >10 mice from three independent experiments. *B*, Frequency (*left axis*, ♦) and number (*right axis*, ◇) of P14 CD8 T cells during PV infection, average of three mice/group, representative of three independent experiments. *C*, At days 0 (naive) and 5 post-PV infection, splenocytes were harvested and stained for the surface markers shown. Host CD8 T cells and P14 CD8 T cells are overlaid from the same host (representative >10 independent experiments).

($CD8^-$) events that ran through the cytometer and allowed for the acquisition of up to ~3–4 × 10^6 CD8 T cell events/sample. For most experiments in this study, 3–6 × 10^5 $CD8^+$ T cell events were collected/sample.

Functional IFN bioassay

Functional IFN-αβ was measured using a standard virus inhibition bioassay (28). Briefly, sera collected from mice and control human rIFN-α (PBL Interferon Source, Piscataway, NJ) were serially diluted (2-fold) across a 96-well flat-bottom plate. Each well was seeded with 2 × 10^4 L-929 cells (NCTC clone 929). The following day, cells were infected with 7.5 × 10^5 PFU VSV. Cell morphology and cytopathic effects (CPEs) were monitored 2 d postinfection, and the amount of functional IFN was measured as the last dilution of serum or control rIFN-α to provide ~50% protection from VSV-mediated CPEs. Because of the 2-fold serial dilutions, the $\log_2$ of the reciprocal of the serum dilution that provided 50% protection from VSV-mediated CPE was graphed.

RNA isolation and quantitative real-time PCR

P14 transgenic CD8 T cells (7-AAD^-, $CD8^+$, $V\alpha2^+$, and congenic $marker^+$) were sorted to 93–99% purity on a MoFlo sorter (Beckman Coulter, Fort Collins, CO). RNA was isolated from sorted P14 CD8 T cells with an RNeasy kit (Qiagen, Valencia, CA) and evaluated spectrophotometrically at 260 nm to determine concentration. cDNA was generated using the SuperScript III first-strand synthesis system (Invitrogen) on a PTC-200 Thermo Cycler (MJ Research, Cambridge, MA) at 25°C for 10 min followed by 50°C for 50 min. Relative mRNA concentrations were determined by quantitative real-time PCR using SYBR Green PCR core reagent kit (Applied Biosystems, Foster City, CA) on an iCycler iQ (Bio-Rad, Hercules, CA). The following primers were used: *18S rRNA* sense 5′-TGGTGGAGGGATTTGTCTGG-3′ and antisense 5′-TCAATCTCGGGTGGCTGAAC-3′, *eomesodermin* sense 5′-TGAATGA ACCTTCCAAGACTCAGA-3′ and antisense 5′-GGCTTGAGGCAAAGTGTTGACA-3′, and *T-bet* sense 5′-TTCCCATTCCTGTCCTTCACC-3′ and antisense 5′-TGCCTTCTGCCTTTCCACAC-3′. For the generation of standard curves, cDNA clones of *18S rRNA*, *eomesodermin*, and *t-bet* were used.

Statistical analyses

Where appropriate, Student *t* tests were calculated using GraphPad InStat software (GraphPad Software, La Jolla, CA). Significance was set at $p < 0.05$. All results are expressed as mean ± SD.

Results

Naive bystander CD8 T cells are transiently sensitized to exert rapid effector functions during acute viral infection or after poly(I:C) treatment

To study how bystander and latecomer CD8 T cells are affected by acute viral infections, we developed in vivo models to track and specifically activate bystander CD8 T cells. We tested three TCR transgenic CD8 T cell types and defined bystander as a transgenic CD8 T cell population that did not divide or proliferate (lose CFSE or increase in cell number) or alter the expression of activation markers (CD44, CD43 [1B11], CD62L, and CD122) during the viral infection. An example of this bystander phenotype is shown in Fig. 1 for P14 transgenic CD8 T cells during PV infection. Fig. 1*A* shows that there is no loss of CFSE, Fig. 1*B* shows no increase in frequency or cell number, and Fig. 1*C* shows the expression of activation Ags on P14 CD8 T cells and the host polyclonal CD8 T cells, which includes PV-specific CD8 T cells in the PV-infected mice. We also used these phenotypes to define P14 cells as bystander cells during VV infection. HY and OT-I transgenic CD8 T cells were similarly defined as bystander cells during LCMV, PV, and VV infections (data not shown).

Once the in vivo bystander CD8 T cell models were established, we asked how naive P14 CD8 T cells would respond when activated with cognate peptide $GP_{33–41}$ during the early acute phase of PV infection. Spleen leukocytes from P14-implanted mice were harvested at day 5 post-PV infection and stimulated with $GP_{33–41}$ for 5 h ex vivo. As predicted, naive P14 CD8 T cells isolated from untreated mice produced very little IFN-γ in response to $GP_{33–41}$ stimulation. However, a substantial frequency of the P14 CD8 T cells from the PV-infected mice rapidly expressed IFN-γ after $GP_{33–41}$ stimulation (Fig. 2*A*). Likewise, P14 cells isolated from mice 1 d after poly(I:C) inoculation also rapidly synthesized IFN-γ upon $GP_{33–41}$ stimulation (Fig. 2*A*). The PV- or poly

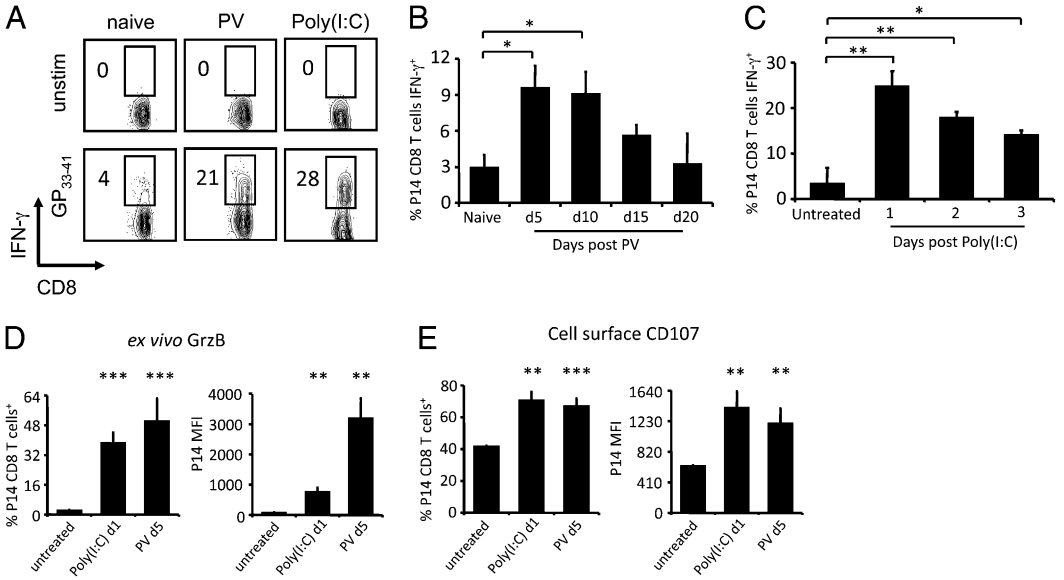

FIGURE 2. PV infection and poly(I:C) treatment transiently sensitize bystander P14 CD8 T cells to rapid effector functions upon cognate Ag stimulation. P14 transgenic CD8 T cells were adoptively transferred into naive congenic recipients followed by infection with 1.5 × 10^7 PFU of PV or i.p. inoculation with 200 μg poly(I:C). *A*, At day 5 of PV infection or day 1 after poly(I:C) treatment, splenocytes were stimulated with $GP_{33–41}$ peptide ex vivo. P14 transgenic CD8 T cells were gated, and intracellular accumulation of IFN-γ was assessed. *B*, At days 0 (naive), 5, 10, 15, and 20 post-PV infection, splenocytes were stimulated with $GP_{33–41}$ peptide ex vivo, and the frequency of P14 CD8 T cells producing IFN-γ was assessed. *C*, At days 0 (untreated), 1, 2, and 3 post-poly(I:C) inoculation, splenocytes were stimulated with $GP_{33–41}$ peptide ex vivo, and the frequency of P14 CD8 T cells producing IFN-γ was assessed. At day 0 (untreated), day 1 post-poly(I:C), or day 5 post-PV, GrzB expression was assessed by intracellular staining directly ex vivo (*D*) and degranulation (CD107 surface expression) was assessed after $GP_{33–41}$ peptide stimulation (*E*). The frequency of P14 CD8 T cells staining positive and mean fluorescence intensity for both molecules were graphed. Representative experiments with three to five mice/group are shown; experiments were independently performed >10 times. $*p < 0.05$; $**p < 0.005$; $***p < 0.0005$.

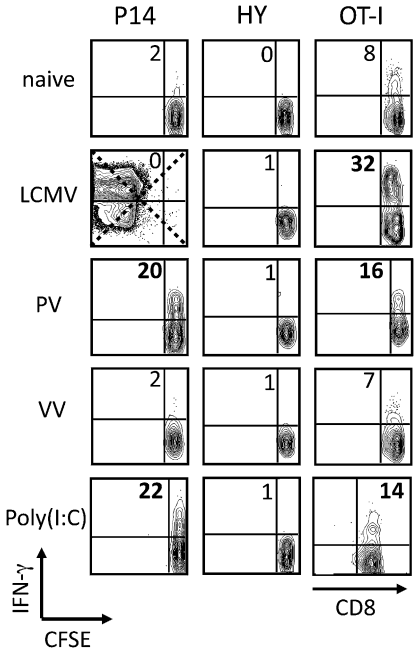

FIGURE 3. Not all virus infections or proinflammatory stimuli sensitize bystander CD8 T cells. P14, HY, or OT-I transgenic CD8 T cells were adoptively transferred into naive congenic recipients followed by i.p. inoculation with 5×10^4 PFU LCMV, 1.5×10^7 PFU PV, 1×10^6 PFU VV, or 200 μg poly(I:C). At days 0 (naive) and 5 postinfection or day 1 post-poly(I:C), splenocytes were stimulated with $GP_{33–41}$, Smcy, or SIINFEKEL peptides ex vivo. Each transgenic population was gated, and the ability of the transgenic CD8 T cells to produce IFN-γ in response to their cognate peptide was assessed. IFN-γ versus CFSE is shown from representative mice from eight independent experiments. The numbers depict the frequency of $CFSE^{hi}$ $IFN\text{-}\gamma^+$ events, except for OT-I + poly(I:C), which shows IFN-γ versus CD8.

(I:C)-induced sensitization to rapid IFN-γ expression was transient: the ability to rapidly express IFN-γ in response to cognate Ag was decreased at day 15 and had returned to background levels by day 20 post-PV infection (Fig. 2*B*); it was similarly decreased by 2–3 d after poly(I:C) inoculation (Fig. 2*C*).

We next questioned whether these naive bystander CD8 T cells would also have the ability to be cytolytic. We measured their expression of GrzB immediately ex vivo and their ability to undergo Ag-driven degranulation in vitro by staining for the surface expression of lysosome-associated membrane proteins 1 and 2 (CD107a and CD107b) in response to $GP_{33–41}$ stimulation. In the absence of exposure to cognate $GP_{33–41}$ ligand, GrzB was induced in P14 CD8 T cells after poly(I:C) treatment in terms of the frequency of P14 cells expressing GrzB and the relative amount of GrzB per cell (mean fluorescence intensity) (Fig. 2*D*). The ability of P14 CD8 T cells to undergo Ag-driven degranulation, as measured by surface expression of CD107, was also significantly enhanced for P14 CD8 T cells after poly(I:C) treatment and PV infection (Fig. 2*E*). Thus, PV infection and poly(I:C) inoculation can sensitize naive P14 CD8 T cells such that they upregulate GrzB prior to cognate Ag stimulation in vivo and prime

Table I. *Not all virus infections or proinflammatory stimuli sensitize bystander CD8 T cells*

TCR Tg	Virus Infection or Proinflammatory Stimulus			
	LCMV	PV	VV	Poly(I:C)
P14	N/A	5.8 ± 4	1.1 ± 0.09	5.8 ± 5
HY	0.93 ± 0.13	0.96 ± 0.04	0.5 ± 0.5	0.66 ± 0.3
OT-I	2.9 ± 2	1.7 ± 0.6	1.2 ± 0.4	2.1 ± 0.2

P14, HY, or OT-I transgenic CD8 T cells were adoptively transferred into naive congenic recipients followed by i.p. inoculation with 5×10^4 PFU LCMV, 1.5×10^7 PFU PV, 1×10^6 PFU VV, or 200 μg poly(I:C). At day 5 postinfection or day 1 post-poly(I:C) inoculation, splenocytes were stimulated with $GP_{33–41}$, Smcy, or SIINFEKL peptides ex vivo. Each transgenic population was gated, and the ability of the transgenic T cells to produce IFN-γ in response to its cognate peptide was assessed. Data represent the mean (± SD) ratio of IFN-γ production from infected/treated mice over IFN-γ production from naive/untreated mice stimulated with cognate peptide from >30 total experiments.

N/A, not applicable.

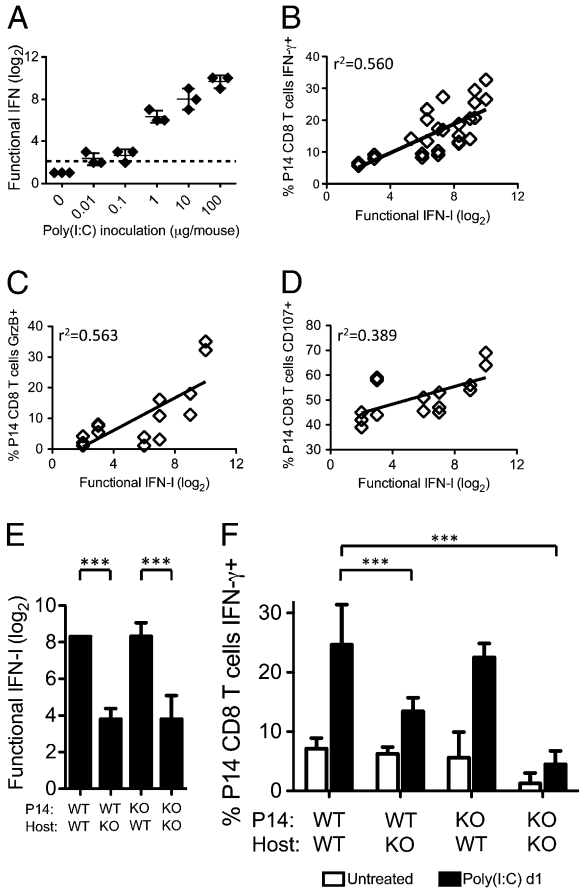

FIGURE 4. Indirect role for IFN-αβ in the sensitization of P14 CD8 T cells after poly(I:C) treatment. P14 CD8 T cells were adoptively transferred into congenic recipients followed by i.p. inoculation with 0–100 μg poly(I:C). One day post-poly(I:C) inoculation, serum was collected for bioassay, and splenocytes were collected for stimulation. *A*, Functional IFN for each individual mouse was assessed via VSV-induced CPE bioassay, and the $\log_2$ of the reciprocal of the serum dilution that provided 50% protection was graphed. Dashed line depicts the level of detection for the assay. *B*, The level of functional IFN was plotted against the ability of P14 T cells in each individual mouse to express IFN-γ in response to GP33–41 peptide stimulation from two independent experiments ($n = 34$ mice; $r^2 = 0.560$; $p < 0.005$). *C*, The level of functional IFN was plotted against the expression of GrzB in P14 T cells in each individual mouse ($n = 18$ mice; $r^2 = 0.563$; $p < 0.005$). *D*, The level of functional IFN was plotted against the ability of P14 T cells in each individual mouse to degranulate in response to GP33–41 peptide stimulation ($n = 18$ mice; $r^2 = 0.389$; $p < 0.05$). *E* and *F*, WT or IFN-αβR KO P14 CD8 T cells were adoptively transferred into WT or IFN-αβR KO congenic recipients, followed by i.p. inoculation with 200 μg poly(I:C). At days 0 (untreated) and 1 post-poly(I:C) inoculation, serum was collected for bioassay, and splenocytes were stimulated with $GP_{33–41}$ peptide ex vivo. *E*, Functional IFN in all poly(I:C)-treated groups was graphed. *F*, The ability of P14 CD8 T cells to rapidly express IFN-γ was assessed. Cumulative data from five independent experiments are depicted. ***$p < 0.0005$.

cells for IFN-γ production and degranulation upon ligand exposure in vitro.

Sensitization varies with the virus and the transgenic T cell

To determine whether other viral infections could sensitize P14 CD8 T cells and whether TCR transgenic CD8 T cells of other specificities could be sensitized, we examined the sensitization of P14, HY, and OT-I transgenic CD8 T cells by LCMV, PV, VV, and poly(I:C). Examples from eight independent experiments depicting the IFN-γ production of cognate peptide-stimulated TCR transgenic CD8 T cells from all of these models are shown in Fig. 3. The cumulative data generated from >30 experiments using all of these models are listed in Table I, which depicts the average ratio of cognate peptide-induced IFN-γ production for TCR transgenic CD8 T cells in infected/treated mice over the IFN-γ production for transgenic CD8 T cells from control mice. P14 CD8 T cells were sensitized to rapidly express IFN-γ in response to cognate peptide stimulation by PV and poly(I:C) but not by VV. HY transgenic CD8 T cells were not sensitized by any of the stimuli, and OT-I CD8 T cells were sensitized well by LCMV and moderately by PV and poly(I:C) (Fig. 3, Table I).

Indirect role for IFN-αβ

As a result of the ability of LCMV, PV, and poly(I:C), all good IFN-αβ inducers, to sensitize naive CD8 T cells and the inability of VV, a poor inducer of IFN-αβ, to do so, we questioned whether IFN-αβ levels correlated with the sensitization process. Thus, mice were inoculated with 10-fold dilutions of poly(I:C) from 0.01–100 μg/mouse. Decreasing the dose of poly(I:C) resulted in lower induction of total functional IFN, as assessed by a VSV-mediated cytopathic effect-inhibition bioassay (Fig. 4*A*), and there was a linear correlation between the amount of functional IFN induced and the ability of P14 CD8 T cells to rapidly express IFN-γ in response to $GP_{33–41}$ stimulation (r^2 = 0.560) (Fig. 4*B*). Likewise, we found correlations between the induction of functional IFN and the upregulation of GrzB (r^2 = 0.563) and the ability of T cells to undergo enhanced Ag-driven degranulation (r^2 = 0.389) (Fig. 4*C*, 4*D*).

We next asked whether IFN-αβ was required for the sensitization of naive P14 CD8 T cells by using IFN-αβR KO mice, which synthesize less total functional IFN than wild-type (WT) mice (Fig. 4*E*), likely due to the inhibition of a positive feedback loop initiated by IFN-α4 and IFN-β signaling (29, 30). Additionally, to address whether direct IFN-αβ signals were required for sensitization, we crossed the P14 transgenic mice to the IFN-αβR KO mice to generate P14 CD8 T cells that did not express the IFN-αβR and, thus, could not respond directly to IFN-αβ signals. WT or IFN-αβR KO P14 CD8 T cells were adoptively transferred into congenic WT or IFN-αβR KO recipients, which were then inoculated with poly(I:C). WT or IFN-αβR KO P14 CD8 T cells in the IFN-αβR KO host mice (WT→KO and KO→KO) were sensitized much less efficiently by poly(I:C) than by either type of P14 cells in WT host mice (WT→WT and KO→WT) (Fig. 4*F*). The most pronounced defect in IFN-γ production was the KO→KO group, but the ratio of IFN-γ^+ P14 CD8 T cells for the poly(I:C)-treated mice over the untreated mice in this group was similar to the WT→KO group. These data indicated that IFN-αβ is required for sensitization, but direct IFN-αβ signals on the P14 CD8 T cells are not.

Influence of other cytokines and cytokine-producing cells on sensitization

Because of the indirect requirement for IFN-αβ in the sensitization of naive P14 CD8 T cells, we questioned whether IFN-αβ was

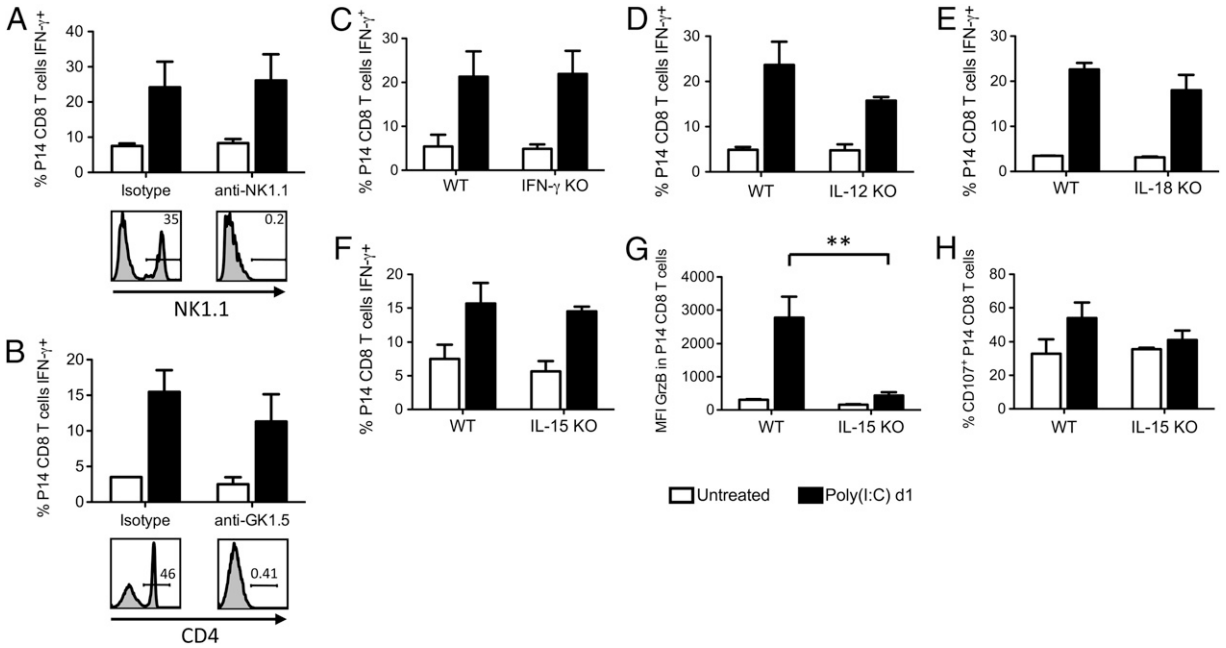

FIGURE 5. NK cells, CD4 T cells, IFN-γ, IL-12, IL-18, and IL-15 are not required for the sensitization to rapid IFN-γ production after poly(I:C) treatment. *A*, *B*, P14 CD8 T cells were adoptively transferred into WT congenic recipients, followed by i.v. inoculation with anti-NK1.1 or IgG2a (*A*) or anti-GK1.5 or IgG2b (*B*) to deplete NK cells or CD4 T cells, respectively. One day post-Ab treatment, mice were inoculated i.p. with 200 μg poly(I:C). At days 0 (untreated) and 1 post-poly(I:C) inoculation, splenocytes were stimulated with $GP_{33–41}$ peptide ex vivo, and the intracellular accumulation of IFN-γ was assessed. Splenocytes were also stained with anti-NK1.1 clone PK136 (gated on $CD3^-$ $DX5^+$) and anti-CD4 clone RM4-4 (gated on $CD3^+$) of different clones than the depletion Abs to assess depletion. *C–H*, P14 CD8 T cells were adoptively transferred into WT, IFN-γ KO (*C*), IL-12 KO (*D*), IL-18 KO (*E*), or IL-15 KO (*F–H*) congenic recipients. One day postadoptive transfer, mice were inoculated i.p. with 200 μg poly(I:C). At days 0 (untreated) and 1 post-poly(I:C) inoculation, splenocytes were stimulated with $GP_{33–41}$ peptide ex vivo, and the intracellular accumulation of IFN-γ (*C–F*) and GrzB (*G*) or degranulation (*H*) was assessed.

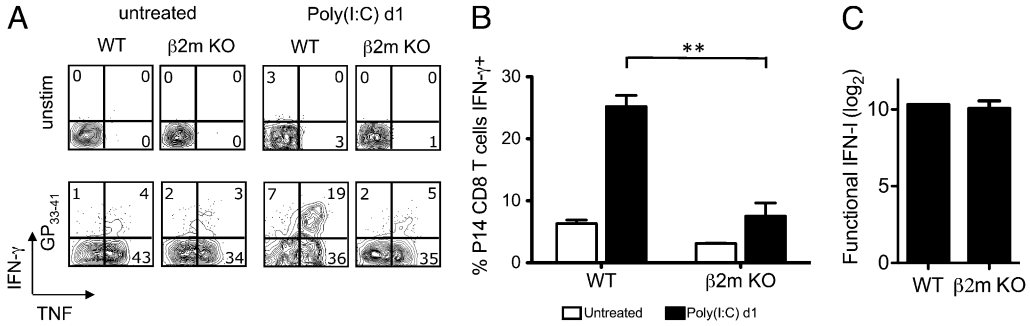

FIGURE 6. MHC I is required for the sensitization of P14 CD8 T cells after poly(I:C) treatment. P14 transgenic CD8 T cells were adoptively transferred into naive WT or β2m KO congenic recipients followed by i.p. inoculation with 200 μg poly(I:C). At days 0 (untreated) and 1 post-poly(I:C), splenocytes were stimulated with $GP_{33–41}$ peptide + exogenous WT splenocytes ex vivo. *A*, P14 CD8 T cells were gated, and intracellular accumulation of IFN-γ and TNF was assessed. *B*, The frequency of IFN-γ–producing P14 cells was graphed (n = 4/group). *C*, Functional IFN production was graphed. Data are representative of four independent experiments. $^{**}p < 0.005$.

inducing sensitization via another mediator. NK cells are activated by IFN-αβ (31), and CD4 T cells can be Th1-skewed by IFN-αβ (32), so it was possible that NK cells or CD4 T cells, perhaps by secreting IFN-γ, were mediating the sensitization of the P14 CD8 T cells. However, depletion of NK cells or CD4 T cells did not affect the sensitization of P14 CD8 T cells after poly(I:C) treatment (Fig. 5*A*, 5*B*). Additionally, P14 CD8 T cells were sensitized by poly(I:C) in IFN-γ KO mice (Fig. 5*C*).

We next questioned whether another cytokine induced by viral infections and poly(I:C) could be mediating sensitization. Because IL-12, IL-18, and IL-15 were shown to induce IFN-γ expression by effector and memory CD8 T cells (33–36), we tested the requirements for these cytokines by using cytokine KO mice. We found that none of these cytokines, at least individually, was required for the sensitization of naive P14 CD8 T cells to rapidly express IFN-γ in response to peptide stimulation after poly(I:C) treatment (Fig. 5*D*–*F*). Each KO mouse group induced a slightly lower frequency of IFN-γ–producing P14 CD8 T cells than did WT counterparts, but these differences were not statistically significant. Interestingly, IL-15 was required for upregulation of GrzB (Fig. 5*G*) and may also play a role in the enhanced degranulation in response to cognate peptide stimulation. Ag-driven degranulation of P14 CD8 T cells was reduced in the poly(I:C)-treated IL-15 KO mice in two independent experiments, but these results did not reach statistical significance (Fig. 5*H*, data not shown).

MHC I is required for the sensitization of naive bystander CD8 T cells

The fact that CD8 T cells of different specificities would be sensitized differently by poly(I:C) suggested that their TCR may play a role in sensitization. T cells are selected in the thymus for their low avidity to self Ags, and naive T cells require MHC-presented self Ags to undergo homeostatic proliferation (37–39). Notably, female HY transgenic CD8 T cells, which recognize a male-encoded Ag, are known to be poor at homeostatic proliferation in female mice (38, 40) and could not be sensitized in

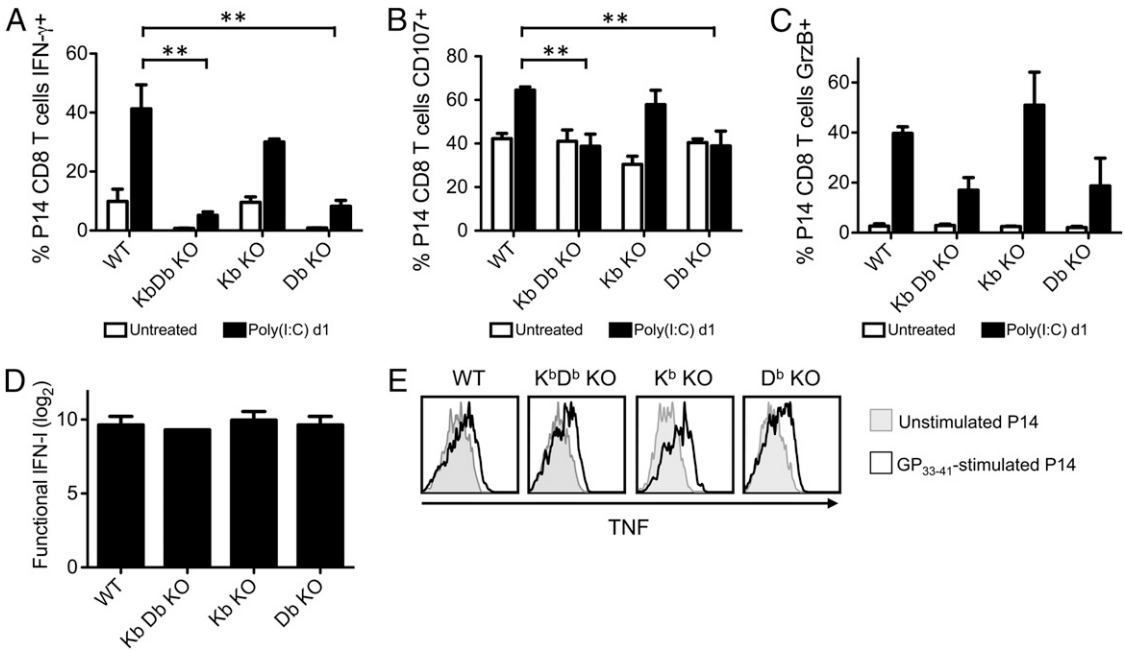

FIGURE 7. $H2D^b$ is required for the sensitization of P14 CD8 T cells after poly(I:C) treatment. P14 transgenic CD8 T cells were adoptively transferred into naive WT, K^bD^b KO, K^b KO, or D^b KO congenic recipients followed by i.p. inoculation with 200 μg poly(I:C). At days 0 (untreated) and 1 post-poly(I:C), splenocytes were stimulated with $GP_{33–41}$ peptide + exogenous WT splenocytes ex vivo. The frequency of IFN-γ–producing (*A*), $CD107^+$ (*B*), and $GrzB^+$ (*C*) P14 CD8 T cells was graphed. *D*, Functional IFN production was graphed. *E*, TNF production by unstimulated and $GP_{33–41}$-stimulated P14 CD8 T cells was assessed. Data depict three or four mice/group and are representative of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.005$; $^{***}p < 0.0005$.

our systems. This suggested that low-affinity cryptically cross-reactive self-Ag, in the presence of proinflammatory signals, may sensitize P14 and OT-I CD8 T cells, which readily undergo homeostatic proliferation. If this were the case, CD8 T cell sensitization would require TCR signaling by class I MHC. To test this, we first sought to determine whether P14 CD8 T cells could be sensitized by poly(I:C) in β2m KO mice, which express class I MHC poorly (41, 42). We compensated for the lack of MHC I Ag presentation during the ex vivo T cell stimulation by providing congenic naive splenocytes to efficiently present the $GP_{33–41}$ peptide to the P14 CD8 T cells. Naive T cells from untreated mice make TNF but not IFN-γ on exposure to their MHC-displayed ligand (43); this allowed us to control for TCR stimulation in vitro, because, under these conditions, P14 cells taken from β2m KO mice produced TNF in response to $GP_{33–41}$ (Fig. 6*A*). However, these P14 CD8 T cells were unable to rapidly express IFN-γ in response to peptide stimulation (Fig. 6*A*, 6*B*), suggesting that they required class I Ag presentation in vivo for sensitization. This difference between β2m KO and WT mice was not due to a defect in IFN-αβ induction, because similar levels of total functional IFN were induced in both strains of mice (Fig. 6*C*).

We also questioned whether cognate MHC ($H2D^b$ for P14) was required for sensitization or whether any MHC I could sensitize the P14 CD8 T cells. To address this, we adoptively transferred P14 CD8 T cells into congenic WT, K^bD^b KO, K^b KO, and D^b KO C57BL/6 mice, followed by inoculation with poly(I:C). As shown in Fig. 7, P14 CD8 T cells were only sensitized in mice expressing $H2D^b$ (WT and K^b KO). Their ability to rapidly express IFN-γ and undergo enhanced degranulation in response to cognate peptide stimulation was abrogated in mice lacking $H2D^b$ but still expressing $H2K^b$ (Fig. 7*A*, 7*B*). The immediate ex vivo expression of GrzB was upregulated in the P14 CD8 T cells after poly(I:C) inoculation in all of the mice, but this upregulation was reduced in mice lacking $H2D^b$ (Fig. 7*C*), suggesting that there was MHC I-dependent and -independent regulation of GrzB expression. Importantly, the inability of P14 CD8 T cells to be sensitized in mice lacking $H2D^b$ was not due to a defect in IFN induction (Fig. 7*D*) or an inability to be activated in vitro, because the P14 CD8 T cells from all recipient mice could synthesize TNF in response to $GP_{33–41}$ stimulation (Fig. 7*E*). These data suggest that cognate MHC ($H2D^b$) displaying cryptic (i.e., unidentified) self-peptides was required for the sensitization of naive P14 CD8 T cells during poly(I:C) treatment.

Induction of Eomes in sensitized P14 CD8 T cells during PV infection and poly(I:C) treatment

IFN-γ gene transcription is regulated by the transcription factors T-bet and Eomes (8–13), and we questioned whether T-bet or Eomes mRNA was induced in bystander-sensitized CD8 T cells. P14 CD8 T cells from naive, LCMV day 6 (effector), LCMV day 40 (memory), poly(I:C) day 1, PV day 5, and PV day 20 infected mice were purified by cell sorting immediately ex vivo and without any exposure to cognate ligand. Their RNA was extracted and used for quantitative real-time PCR to quantify mRNA levels of the transcription factors. As shown in Fig. 8*A* and consistent with published reports, effector CD8 T cells upregulated mRNA for T-bet and Eomes, and memory CD8 T cells further upregulated Eomes but not T-bet mRNA (10, 14). The sensitized bystander P14 CD8 T cells upregulated Eomes, but not T-bet mRNA, after poly(I:C) inoculation and PV infection (Fig. 8*A*). Importantly, the high expression of Eomes mRNA in bystander CD8 T cells was transient; by day 20 after PV infection, at a time when the P14 CD8 T cells could no longer rapidly express IFN-γ (Fig. 2*B*), the level of Eomes mRNA was less than that detected in naive CD8 T cells.

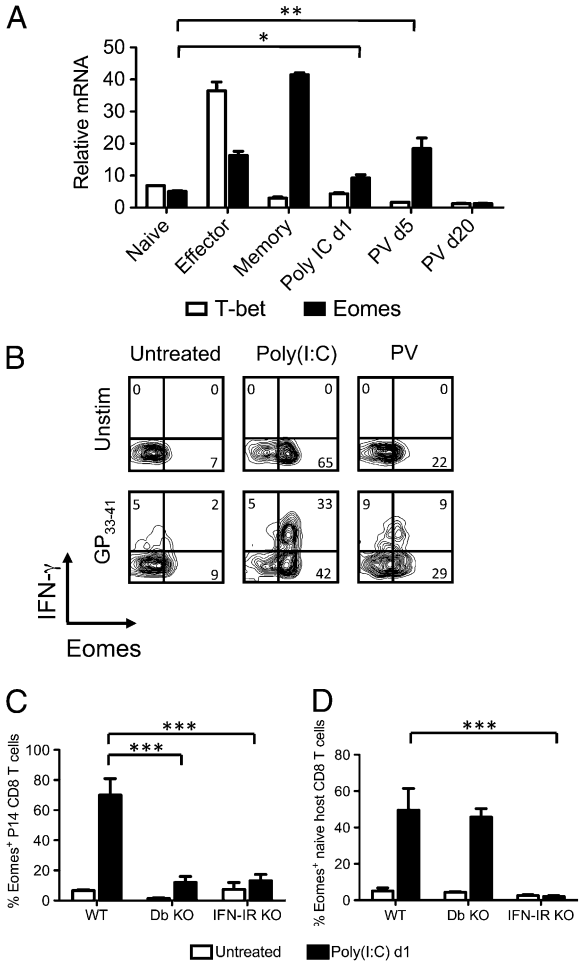

FIGURE 8. Induction of Eomes, but not T-bet, in bystander P14 CD8 T cells during acute PV infection and poly(I:C) treatment. *A*, P14 transgenic CD8 T cells were adoptively transferred into naive congenic recipients followed by i.p. inoculation with 5×10^4 PFU LCMV, 1.5×10^7 PFU PV, or 200 μg poly(I:C). P14 CD8 T cells were sorted from naive P14 mice (Naive), day 6 post-LCMV mice (Effector), day 42 post-LCMV mice (Memory), day 1 post-poly(I:C) mice, day 5 post-PV mice, or day 20 post-PV mice. RNA was extracted and converted to cDNA, followed by real-time PCR using primers for T-bet and Eomes. Standardized mRNA relative to 18S rRNA was calculated from an individual experiment that is representative of four independent experiments. *B–D*, P14 transgenic CD8 T cells were adoptively transferred into naive congenic WT, $H2D^b$ KO, or IFN-αβR KO recipients followed by i.p. inoculation with 1.5×10^7 PFU PV or 200 μg poly(I:C). At day 5 of PV infection or day 1 after poly(I:C) treatment, splenocytes were stimulated with $GP_{33–41}$ peptide ex vivo. *B*, P14 transgenic CD8 T cells were gated, and IFN-γ production and Eomes expression were assessed from representative mice for each group. *C*, The frequency of $Eomes^+$ P14 CD8 T cells in WT, $H2D^b$ KO, and IFN-αβR KO mice was graphed. *D*, The frequency of $Eomes^+$ $CD44^{lo}$ naive host CD8 T cells in WT, $H2D^b$ KO, and IFN-αβR KO mice was graphed. Data in *B–D* are representative of three independent experiments. $*p < 0.05$; $**p < 0.005$; $***p < 0.0005$.

Eomes protein was also induced in sensitized P14 CD8 T cells after PV infection and poly(I:C) treatment and was expressed directly ex vivo, without a requirement for exposure to $GP_{33–41}$ (Fig. 8*B*, 8*C*). Strikingly, most of the P14 CD8 T cells upregulated Eomes protein after poly(I:C) treatment (71 ± 7.7%; $n = 8$), a frequency that was nearly twice that (38 ± 9%; $n = 8$) of the cells that rapidly synthesized IFN-γ in response to $GP_{33–41}$

stimulation ex vivo (Fig. 8*B*). Importantly, about half of the polyclonal naive CD8 T cells in WT B6 mice also upregulated Eomes expression after poly(I:C) treatment (Fig. 8*D*), arguing that this upregulation is a common event and not restricted to only a rare transgenic T cell population.

Because MHC I and indirect effects of IFN-αβ were required for the sensitization of P14 CD8 T cells to rapidly express IFN-γ in response to cognate Ag, we also tested whether these signals were required for the induction of Eomes. To address this, we measured the induction of Eomes protein in P14 and naive polyclonal host CD8 T cells in $H2D^b$ KO and IFN-αβR KO mice. As shown in Fig. 8*C*, P14 CD8 T cells did not upregulate Eomes protein in response to poly(I:C) treatment in $H2D^b$ KO mice, supporting the concept that recognition of MHC was needed for sensitization. In contrast, induction of Eomes protein in naive polyclonal CD8 T cells in the $H2D^b$ KO mice was like that of WT (Fig. 8*D*), indicating that these host CD8 T cells, which had been selected in an MHC $H2K^b$ environment, received sufficient stimulation for sensitization. Neither donor P14 cells nor polyclonal host naive cells were sensitized in mice lacking receptors for IFN-αβ (Fig. 8*C*, 8*D*). Taken together, these data show that IFN-αβ and cognate MHC I are required for the upregulation of Eomes in bystander-sensitized CD8 T cells, and we suggest that the expression of this transcription factor allows for the rapid synthesis of IFN-γ in response to cognate Ag stimulation.

Discussion

We demonstrated in this study that IFN-αβ–inducing acute viral infections and TLR agonists sensitize naive phenotype bystander CD8 T cells, such that they upregulate GrzB prior to cognate Ag stimulation in vivo and prime cells for IFN-γ production and degranulation upon ligand exposure in vitro (Fig. 2). Associated with this acquisition of effector functions was the upregulation of the T-box transcription factor Eomes, known to regulate CTL effector functions (Fig. 8). Hence, these naive bystander T cells were conditioned to behave like memory cells on exposure to high-affinity cognate ligand and, thus, had entered a distinct differentiation pathway when activated by cognate Ag in the presence of this IFN-αβ stimulus.

The sensitization likely required low-affinity MHC–TCR interactions that did not fully activate the T cells, because sensitization did not occur if MHC I was reduced, absent, or of the wrong allotype (Figs. 6, 7). Additionally, if the T cells expressed a TCR with very low self-reactivity, as is the case with the HY TCR transgenic cells (38, 40), they were not sensitized (Fig. 3, Table I). About half of naive host polyclonal CD8 T cells synthesized Eomes protein after poly(I:C) (Fig. 8*D*), suggesting that a substantial proportion of the T cells may become sensitized by virus-induced cytokines and that once sensitized, their response to cognate ligand is altered. Sensitization also required IFN-αβ but not direct IFN-αβ signaling on the CD8 T cells (Fig. 4). IFN-αβ did not sensitize CD8 T cells by way of NK cells or CD4 T cells, nor was IFN-γ, IL-12, IL-15, or IL-18 required, at least individually, for sensitization (Fig. 5). Nevertheless, it is possible that combinations of IFN-γ, IL-12, IL-15, and IL-18 signals sensitize P14 CD8 T cells, because combinations of these cytokines can promote IFN-γ production by effector and memory CD8 T cells better than any of them individually (33, 34). Because of the requirement for MHC I, it is probable that one indirect role of IFN-αβ during PV infection or poly(I:C) treatment is to upregulate expression of MHC I. T cells are positively and then negatively selected in the thymus when IFN is not upregulating MHC, and it has been a mystery why T cells selected at one threshold of MHC do not become autoaggressive during viral infections that induce high levels of MHC expression throughout the host (44). In this study, we showed that these T cells may become sensitized but not fully activated by the enhanced expression of self-MHC during acute viral infections. Full activation only occurs on exposure to their high-affinity ligand and not to cryptically cross-reactive self-ligands present in the host. The simplest explanation of our results is that the IFN-αβ–induced upregulation of class I MHC was all that was needed to sensitize the T cells. However, we cannot rule out that other indirect IFN-induced events modulate this process.

IFN-γ transcription is a tightly controlled process, regulated by chromatin accessibility and the expression of transcription factors. The T-box transcription factors T-bet and Eomes play essential roles in the induction of IFN-γ transcription in virus-specific CD8 T cells (9, 11–13). We showed in this study that Eomes is transiently induced in bystander-sensitized CD8 T cells (Fig. 8). This probably imparts the ability to rapidly express IFN-γ in response to high-affinity cognate Ag. Additionally, another known target of Eomes is the β-chain of the IL-2R and IL-15R CD122 (11), which was not upregulated to the level on virus-specific CD8 T cells but was moderately induced on bystander P14 and naive polyclonal CD8 T cells during acute viral infection and after poly (I:C) treatment (Fig. 1*C*), suggesting that Eomes expression in bystander-sensitized CD8 T cells may also induce IL-15 responsiveness. This is pertinent, given the result that IL-15 was required for the upregulation of GrzB and possibly for enhanced degranulation of bystander-sensitized P14 CD8 T cells after poly(I:C) treatment (Fig. 5*G*, 5*H*).

Taken together, we propose the model shown in Fig. 9, whereby the arenaviruses LCMV and PV and the TLR agonist poly(I:C) induce IFN-αβ (31, 45). IFN-αβ has pleiotropic effects on many

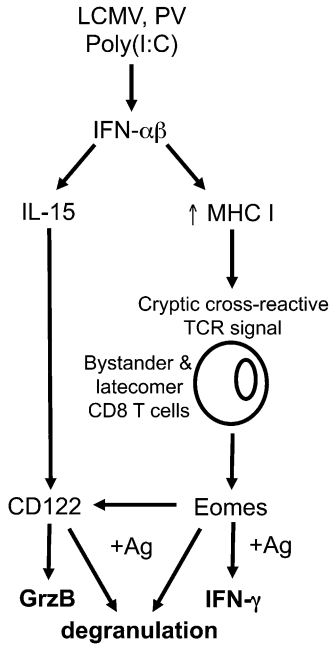

FIGURE 9. Model of the mechanisms that sensitize bystander CD8 T cells during acute viral infections. IFN-αβ induced by viral infection or TLR agonist poly(I:C) induces expression of IL-15 and upregulation of MHC I. Upregulation of MHC I enhances presentation of self- and virus-encoded peptides, which signal through the TCR of bystander CD8 T cells, inducing Eomes expression. Eomes induces expression of CD122, which confers IL-15 responsiveness and induces GrzB expression. In response to cognate Ag, the expression of Eomes allows for the rapid synthesis of IFN-γ. IL-15 expression is also required for the enhanced cognate Ag-driven degranulation.

cell types, including the upregulation of MHC I and the induction of cytokines, including IL-15 (46–48). We propose that the enhanced expression of MHC I during these inflammatory conditions results in TCR signaling by low-affinity cryptically cross-reactive self-peptide–MHC. These signals upregulate the T-box transcription factor Eomes, which allows for the rapid expression of IFN-γ upon cognate Ag stimulation. Eomes-induced expression of CD122 and concomitant IL-15 responsiveness also regulate the expression of GrzB and perhaps the ability of bystander or latecomer CD8 T cells to undergo enhanced Ag-driven degranulation. Therefore, inflammatory signals during acute viral infections initiate the sensitization of naive bystander and latecomer CD8 T cells, such that they rapidly exert effector functions upon cognate Ag stimulation.

These biochemical changes in sensitized T cells that enable them to become immediate effector cells might also affect their proliferation potential. It is difficult to initiate new immune responses during viral infections; in a separate study using the same transgenic models described in this article, we found that these sensitized bystander cells proliferate poorly in vivo in response to their cognate Ag (H.D. Marshall and R.M. Welsh, manuscript in preparation). It was suggested that high-inflammatory environments occurring during viral infection may favor the expansion of short-term effector cells that poorly develop into memory cells (49, 50) and that could be reflecting the IFN-αβ–induced sensitization observed in the current study. It is also intriguing to speculate that chronic viral infections and inflammatory diseases may impact naive CD8 T cell responses, but it is unknown whether sensitization could be maintained under such conditions.

The rules for T cell differentiation events determined by studying naive T cells from unstimulated mice would be different for T cells derived from already inflamed environments. It could be predicted that latecomer T cells recruited at later stages of an immune response would behave differently than those stimulated at the beginning of a response. In this regard, latecomer T cells may be immediately able to produce effector cytokines and to lyse virus-infected cells, without having to proliferate, and may quickly assist in clearing the pathogen. Further, because different responses were seen in transgenic T cells of different specificities, it could be argued that this bystander sensitization may provide a spectrum of T cells receiving a variety of signal strengths from TCR and cytokine receptors and, perhaps, ultimately inducing different fates.

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Disclosures

The authors have no financial conflicts of interest.

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