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In Vivo Generation of Pathogen-Specific Th1 Cells in the Absence of the IFN-γ Receptor[1]

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The precise mechanisms that govern the commitment of CD4 T cells to become Th1 or Th2 cells in vivo are incompletely understood. Recent experiments demonstrate colocalization of the IFN-γR chains with the TCR during activation of naive CD4 T cells, suggesting that association of these molecules may be involved in determining lineage commitment. To test the role of IFN-γ and its receptor in the generation of Th1 Ag-specific CD4 T cells, we analyzed mice after infection with *Listeria monocytogenes* or lymphocytic choriomeningitis virus. In the absence of IFN-γ, Ag-specific CD4 T cells were generated in response to both these infections. In addition, IFN-γ-producing (Th1) Ag-specific CD4 T cells were generated in mice lacking the ligand-binding chain of the IFN-γR (IFN-γR1$^{-/-}$) or the signaling chain (IFN-γR2$^{-/-}$). There was no increase in the number of IL-4-producing Ag-specific CD4 T cells, nor was there a decrease in the expression of T-bet in the absence of functional IFN-γ signaling, indicating that the cells were committed Th1 cells. Thus, both chains of the IFN-γR are dispensable for the generation of Th1 Ag-specific CD4 T cells after infection in vivo. ***The Journal of Immunology,*** **2005, 175: 3117–3122.**

Early during an immune response naive CD4 T cells commit themselves to either a Th1 or a Th2 developmental pathway. This decision is strongly influenced by the cytokines present in the localized environment during activation of the CD4 T cells. In particular, IL-12 and IFN-γ have been identified as key regulators of Th1 development, and IL-4 is considered the key regulator of Th2 development (1). The lack of proper lineage development can adversely affect the outcome of infection, which is most clearly observed in C57BL/6 (B6) and BALB/c mice infected with the parasite *Leishmania major*. B6 mice develop a protective Th1 response and clear the infection, whereas BALB/c mice mount a nonprotective Th2 response, fail to control the infection, and develop progressive disease (2).

Most of the data addressing the mechanisms underlying Th1-Th2 lineage commitment by CD4 T cells have been acquired using in vitro experiments that involve culturing of cells in specific cytokine environments. It is has been recently demonstrated that CD4 T cells cultured under Th1 conditions express the transcription factor T-bet (3, 4), whereas CD4 T cells cultured under Th2 conditions express the transcription factor GATA-3 (5, 6). The expression of these transcription factors is extremely specific for each lineage. Forced expression of T-bet can make Th2 CD4 T cells produce IFN-γ, a Th1-specific cytokine (3).

It has long been observed that the cytokines that foster the development of Th1 cells inhibit the development of Th2 cells and vice versa (7, 8); however, the exact mechanism(s) by which this is accomplished is currently unknown. A recent report (9) demonstrated that both chains of the IFN-γR colocalized with the TCR during activation of naive CD4 T cells in vitro with either Ag-expressing APCs or TCR cross-linking with Ab. In addition, the authors demonstrated that the TCR-IFN-γR colocalization did not occur in the presence of IL-4. These data suggest the intriguing hypotheses that TCR-IFN-γR colocalization could be important for the generation of Th1 CD4 T cells and that inhibition of this process could be the mechanism by which the Th2-inducing cytokine IL-4 inhibited the development of Th1 CD4 T cells.

To address the potential requirement for TCR-IFN-γR colocalization in Th1 CD4 T cell generation in vivo, we infected wild-type (wt),[3] IFN-γ$^{-/-}$, IFN-γR1$^{-/-}$, and IFN-γR2$^{-/-}$ mice with *Listeria monocytogenes* (LM) or lymphocytic choriomeningitis virus (LCMV), both of which induce vigorous CD4 T cell responses to well-characterized Ags (10–12). Our data demonstrate that Th1 Ag-specific CD4 T cells can be generated in the absence of either chain of the IFN-γR, indicating that colocalization of the TCR with an intact IFN-γR is not required for the generation of Th1-committed Ag-specific CD4 T cells.

Materials and Methods

Mice

C57BL/6 mice (National Cancer Institute), 129SVE mice (Taconic Farms), and IFN-γ$^{-/-}$ and IFN-γR1$^{-/-}$ mice (B6 background; The Jackson Laboratory) were purchased for use in these experiments. IFN-γR2$^{-/-}$ mice (129SVE background) (13) were generously provided by Dr. P. Rothman (University of Iowa, Iowa City, IA). All animal experiments followed approved institutional animal care and use committee protocols.

Bacteria and viruses

LM expressing OVA was a gift from Dr. L. Lefrancois (University of Connecticut, Farmington, CT) (14). An attenuated version of this strain was created by introducing an in-frame deletion in the *actA* gene as previously described (15). Bacteria were grown and quantified as previously described (16, 17). All bacterial infections were via i.v. injection. The Armstrong strain of LCMV was propagated and titrated as previously described (18). Approximately 2×10^5 PFU was given to each mouse i.p.

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[3] Abbreviations used in this paper: wt, wild type; ICS, intracellular cytokine staining; LCMV, lymphocytic choriomeningitis virus; LLO, listeriolysin O; LM, *Listeria monocytogenes*; NP, nuclear protein; p.i., postinfection.

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Intracellular cytokine staining (ICS) and quantification of Ag-specific CD4 T cells

Surface staining for CD4 (L3T4; BD Pharmingen) and Thy1.2 (53-2.1; BD Pharmingen) and intracellular staining for IFN-γ (XMG1.2; BD Pharmingen), TNF (MP6-XT22; e-Bioscience), IL-4 (11B11; BD Pharmingen), or IL-5 (TRFK5; BD Pharmingen) were performed as previously described after incubation with listeriolysin O $(LLO)_{190-201}$, gp61–80, nuclear protein $(NP)_{309-328}$, or no peptide (19). The total number of Ag-specific CD4 T cells per spleen was calculated by multiplying the frequency of $CD4^+$/$Thy1.2^+$/IFN-γ^+ or TNF^+ cells after stimulation with specific peptide by the total number of splenocytes.

ELISPOT

The ELISPOT assay was performed as previously described (20). Briefly, microtiter plates were coated with anti-IL-4 (clone 11B11; eBioscience). Whole splenocytes (5×10^6 to 2.5×10^5) from wt 129SVE and IFN-$\gamma R2^{-/-}$ mice on day 7 postinfection (p.i.) were plated in triplicate together with 1×10^6 irradiated splenocytes pulsed with 1 mM $LLO_{190-201}$ or nonpulsed splenocytes. Cells were cultured for 41 h before the biotinylated anti-IL-4 capture Ab (clone BVD6-24G2; eBioscience) was added. The assay was developed using the substrate 3-amino-9-ethylcarbazole. The number of spots per 1×10^6 CD4 T cells was calculated using the frequency of $CD4^+$ cells determined by FACS. The plates were analyzed using an immunospot analyzer (Cellular Technology) according to the manufacturer's instructions.

CD4 T cell purification and RT-PCR

Splenocytes were stained with PE-conjugated CD4 (clone L3T4), then labeled with anti-PE-coated magnetic beads according to manufacturer's instructions (Miltenyi Biotec). Labeled CD4 T cells were then recovered by AutoMACS separation (Posseld program). Purity was assessed by FACS analysis before RNA isolation. All cell samples were purified to >90% $CD4^+$. RNA was isolated from purified T cells using the RNeasy Mini Kit with additional on-column DNase treatment according to the manufacturer's instructions (Qiagen). cDNA was synthesized using a reaction mix including random hexamers and Moloney murine leukemia virus reverse transcriptase.

Amplification of cDNA for T-bet and GATA-3 from purified CD4 T cells was performed using RT-PCR as previously described (21). GAPDH was amplified together with each target gene and was used to normalize expression levels. Appropriate bands on ethidium bromide-stained gels were quantitated using ImageQuant 3.3. Oligos used in these experiments were (5′-3′): T-bet forward, GCGCCAGGAAGTTTCATTTGGGAA; T-bet reverse, ACAGCTCGGAACTCCGCTTCATAA; GATA-3 forward, AGAAAGAAGGCATCCAGACCCGAA; and GATA-3 reverse, AG GACCTCTTCGCACACTTGGAGA. GAPDH primers were part of the TaqMan Rodent GAPDH Control Reagents kit (Applied Biosystems).

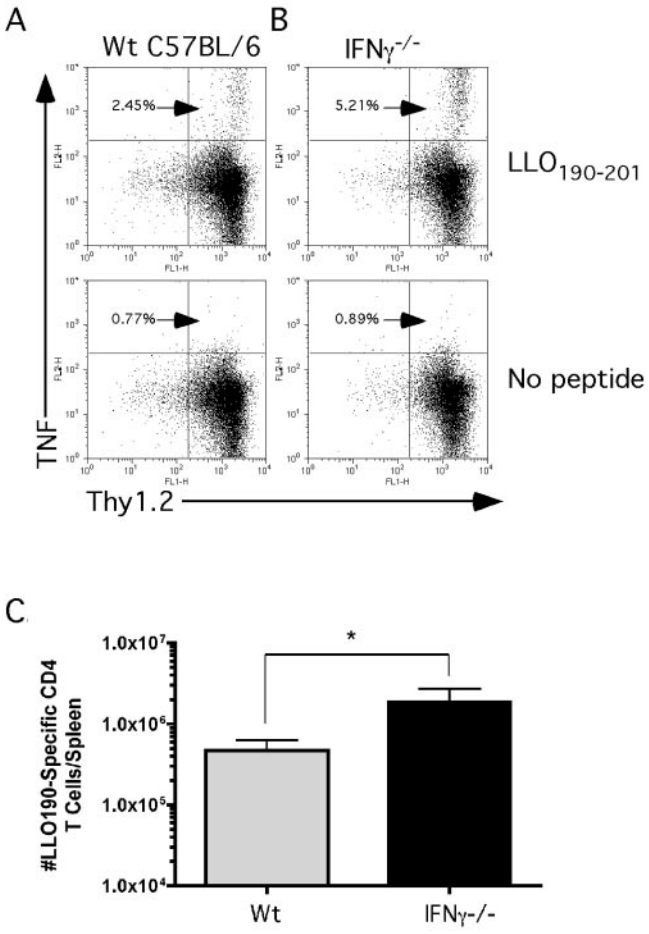
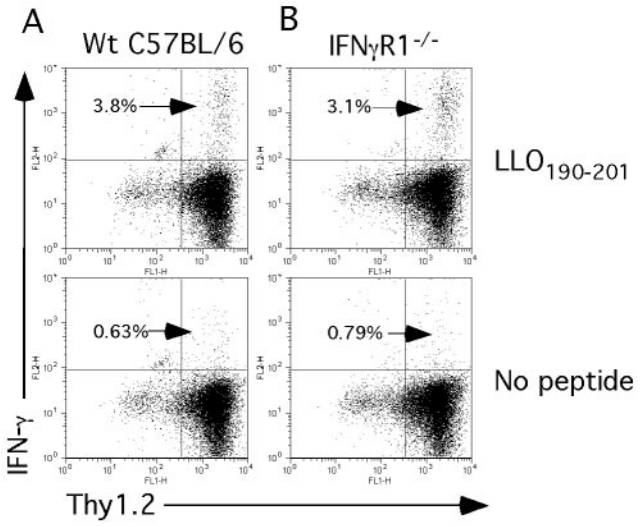


FIGURE 1. Ag-specific CD4 T cell generation in the absence of IFN-γ. IFN-$\gamma^{-/-}$ (B6 background) and wt B6 mice were infected with LM. *A* and *B*, ICS for TNF was performed on splenocytes harvested from wt B6 (*A*) and IFN-$\gamma^{-/-}$ (*B*) mice on day 7 p.i. after a 6-h in vitro stimulation with $LLO_{190-201}$ (*top panels*) or no peptide (*bottom panels*). FACS plots were first gated on $CD4^+$ cells and are representative of three experiments. Numbers indicate the frequency of $Thy1.2^+$/TNF^+ cells within the $CD4^+$ population. *C*, Total number of Ag-specific CD4 T cells per spleen. Statistical analysis was performed using Student's *t* test. ∗, $p < 0.05$.

Results

IFN-γ can up-regulate the autocrine production of IFN-γ by DCs (22) and is considered a major effector cytokine of Th1 CD4 T cells (23). To determine the requirement for IFN-γ in the generation of Ag-specific CD4 T cells, wt B6 and IFN-$\gamma^{-/-}$ B6 mice were infected with ~0.1 LD_{50} of an attenuated *actA*-deficient strain of LM that is cleared from both strains by day 5 p.i. (data not shown) (16). CD4 T cells specific for $LLO_{190-201}$, an I-A^b-restricted CD4 T cell epitope, were detected by peptide stimulation and ICS for TNF. As shown in Fig. 1, TNF-producing $LLO_{190-201}$-specific CD4 T cells were detected in both wt B6 mice (Fig. 1*A*) and IFN-$\gamma^{-/-}$ B6 mice (Fig. 1*B*) on day 7 p.i. When total numbers of $LLO_{190-201}$-specific CD4 T cells per spleen were calculated (Fig. 1*C*), it was determined that the IFN-$\gamma^{-/-}$ B6 mice were capable of generating a slightly larger Ag-specific CD4 T cell response than the wt B6 mice. Total numbers of splenocytes were not different between the two groups of mice (wt B6, $1.45 \times 10^8 \pm 2.85 \times 10^7$; IFN-$\gamma^{-/-}$, $1.42 \times 10^8 \pm 3.40 \times 10^7$; $p = 0.86$). These data demonstrate that IFN-γ is not required for the generation of Ag-specific CD4 T cells after infection with LM.

The IFN-γR is composed of two unique chains, IFN-γR1 and IFN-γR2 (24, 25). Both chains are required for responsiveness to IFN-γ (26). IFN-γR1 is the ligand-binding portion of the receptor and is constitutively expressed on most cells (27), including naive T cells (28). A recent report demonstrated that IFN-γR1 colocalized with the TCR during, but not before, naive CD4 T cell activation in vitro (9). Colocalization of IFN-γR1 with the TCR was abrogated in the presence of IL-4 via a process dependent on STAT6, which is a major signaling component directly downstream of the IL-4R. Thus, IFN-γR recruitment to the TCR could potentially serve an important function in Th1 lineage commitment by CD4 T cells (9). To test the requirement for IFN-γR1 colocalization with the TCR for the generation of Th1 Ag-specific CD4 T cells in vivo, we infected wt and IFN-$\gamma R1^{-/-}$ mice with $actA^-$ LM and analyzed splenocytes from these mice on day 7 p.i. for the presence of IFN-γ-producing (Th1) $LLO_{190-201}$-specific CD4 T cells. IFN-$\gamma R1^{-/-}$ mice cleared the $actA^-$ LM infection by day 5 p.i. (data not shown). As shown in Fig. 2, substantial expansion of IFN-γ-producing $LLO_{190-201}$-specific CD4 T cells was detected on day 7 p.i. in both wt B6 and IFN-$\gamma R1^{-/-}$ mice, which resulted in similar frequencies (Fig. 2, *A* and *B*) and a <2-fold difference in total numbers of Ag-specific CD4 T cells (Fig. 2*C*). Total numbers of splenocytes were not different between the two groups of mice



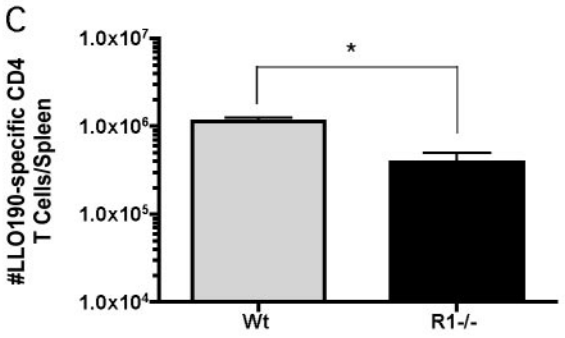


FIGURE 2. IFN-γR1 is not required for the generation of Th1 Ag-specific CD4 T cells after infection. Wt B6 and IFN-γR1$^{-/-}$ mice were infected with LM. *A* and *B*, ICS for IFN-γ was performed on splenocytes harvested from infected wt (*A*) and IFN-γR1$^{-/-}$ mice (*B*) on day 7 p.i. after incubation with $LLO_{190-201}$ (*top panels*) or no peptide (*bottom panels*). The FACS plots presented were first gated on CD4$^+$ cells and are representative of three mice per group. Numbers indicate the frequency of Thy1.2$^+$/IFN-γ$^+$ cells within the CD4$^+$ population. *C*, Total number of Th1 Ag-specific CD4 T cells per spleen. Statistical analysis was performed using Student's *t* test. *, $p < 0.05$.

(wt B6, $1.45 \times 10^8 \pm 2.85 \times 10^7$; IFN-γR1$^{-/-}$ mice, $1.40 \times 10^8 \pm 2.56 \times 10^7$; $p = 0.72$). These data indicate that in the absence of IFN-γR1$^{-/-}$, and therefore colocalization of this cytokine receptor component with the TCR during T cell activation, Ag-specific CD4 T cells capable of producing IFN-γ (probably Th1 cells) are generated after infection with LM.

IFN-γR2 has a very short extracellular domain and does not bind IFN-γ directly. It is not normally preassociated with IFN-γR1, but instead complexes with IFN-γR1 upon ligand binding and is required for subsequent signaling (24, 25). IFN-γR2 was also shown to colocalize with the TCR during naive CD4 T cell activation (9). To directly test a role for IFN-γR2 in the generation of Th1 Ag-specific CD4 T cells in vivo, we infected wt 129SVE and IFN-γR2$^{-/-}$ mice (129SVE background) (13) with LM and measured the $LLO_{190-201}$-specific CD4 T cell response on day 7 p.i. by ICS for IFN-γ. IFN-γR2$^{-/-}$ and wt 129 SVE mice cleared the *actA*$^-$ LM infection by day 5 p.i. (data not shown). The ICS data indicate that IFN-γ-producing (Th1) $LLO_{190-201}$-specific CD4 T cells were generated with similar frequencies in wt and IFN-γR2$^{-/-}$ mice (Fig. 3, *A* and *B*). When total numbers of $LLO_{190-201}$-specific CD4 T cells per spleen were calculated, the data showed that IFN-γR2$^{-/-}$ mice had approximately twice as many Th1 Ag-specific CD4 T cells on day 7 p.i. compared with wt 129 mice. The total numbers of splenocytes were not different between the two groups of mice (wt 129 mice, $1.15 \times 10^8 \pm 1.84 \times 10^7$; IFN-γR2$^{-/-}$ mice, $1.30 \times 10^8 \pm 2.66 \times 10^7$; $p = 0.17$). In combination, the data presented above indicate that neither IFN-γR1 nor IFN-γR2 is required for generation of Ag-specific CD4 T cells capable of making IFN-γ, and, therefore, probably Th1 cells, after infection with LM.

To ensure that these results were not peculiar to LM infection or the $LLO_{190-201}$ epitope, we infected wt B6, IFN-γR1$^{-/-}$, and IFN-γR2$^{-/-}$ mice with the arenavirus LCMV Armstrong strain and measured IFN-γ-producing Th1 CD4 T cells specific for gp61–80 and $NP_{309-328}$ on day 8 p.i. (Fig. 4*A*). IFN-γ-producing CD4 T cells specific for each Ag were generated with similar frequencies (Fig. 4*A*) and total numbers (Fig. 4, *B* and *C*) in the

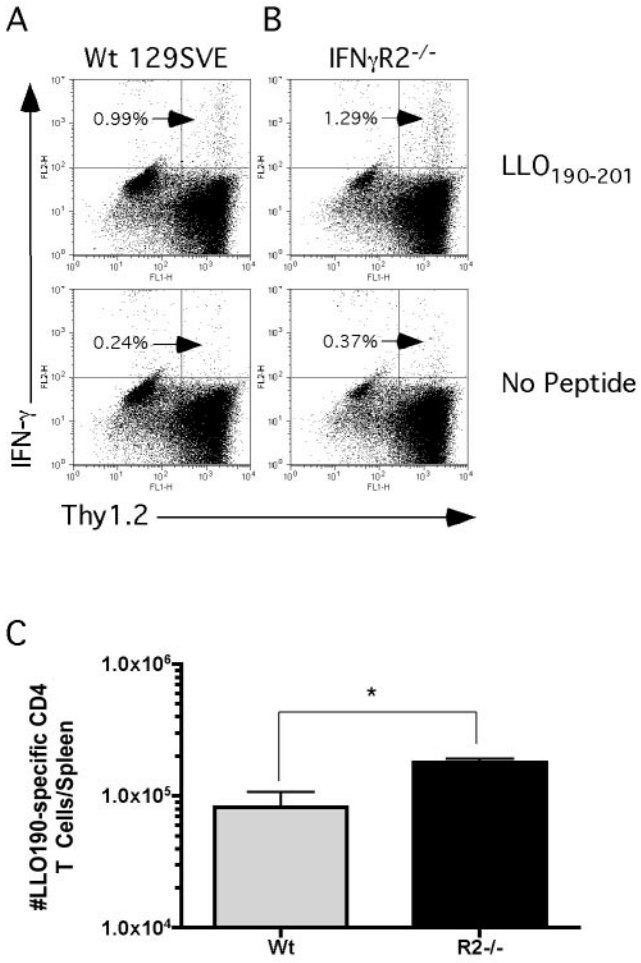


FIGURE 3. IFN-γR2 is not required for the generation of Th1 Ag-specific CD4 T cells after infection. Wt 129SVE and IFN-γR2$^{-/-}$ mice were infected with LM. *A* and *B*, ICS for IFN-γ was performed on splenocytes harvested from infected wt (*A*) and IFN-γR2$^{-/-}$ (*B*) mice on day 7 p.i. after incubation with $LLO_{190-201}$ (*top panels*) or no peptide (*bottom panels*). FACS plots presented were all first gated on CD4$^+$ cells and are representative of at least three mice per group. Numbers indicate the frequency of Thy1.2$^+$/IFN-γ$^+$ cells within the CD4$^+$ population. *C*, Total number of Th1 Ag-specific CD4 T cells per spleen. Statistical analysis was performed using Student's *t* test. *, $p < 0.05$.

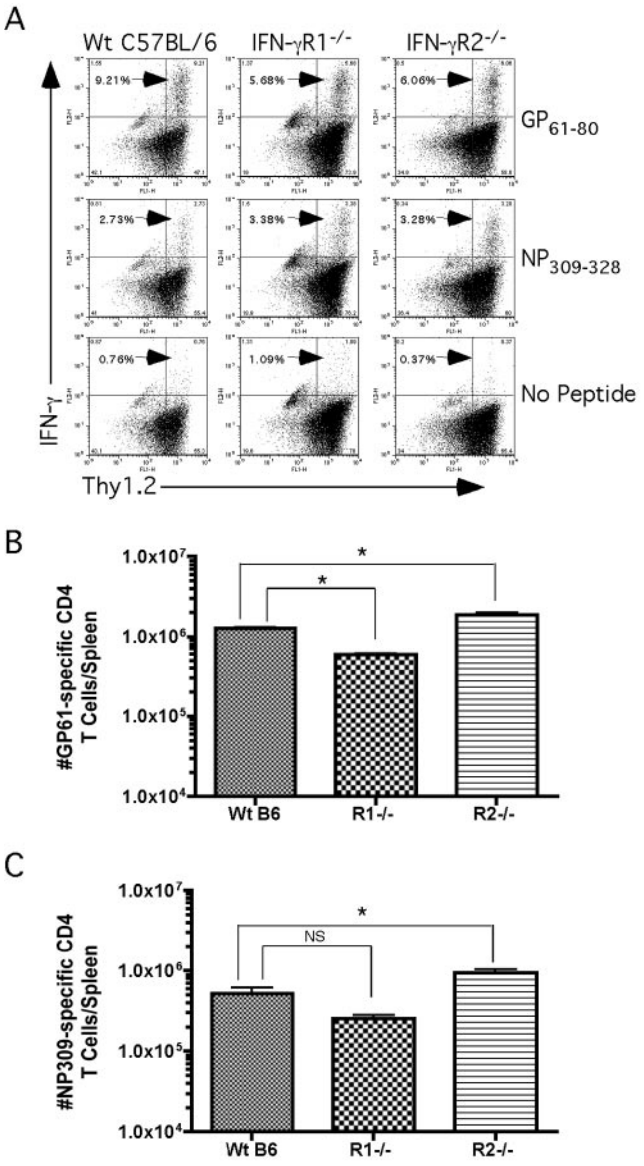


FIGURE 4. Th1 Ag-specific CD4 T cells are generated in the absence of IFN-γR1 or IFN-γR2 after viral infection. Wt B6, IFN-γR1$^{-/-}$, and IFN-γR2$^{-/-}$ mice were infected with LCMV. *A*, ICS for IFN-γ was performed on splenocytes harvested on day 8 p.i. after incubation with gp61–80 (*top panels*), $NP_{309-328}$ (*middle panels*), or no peptide (*bottom panels*). The FACS plots presented were first gated on CD4$^+$ cells and are representative of at least three mice per group. Numbers indicate frequencies of Thy1.2$^+$/IFN-γ$^+$ within the CD4$^+$ population. *B*, Total number of Th1 gp61–80-specific CD4 T cells per spleen. *C*, Total number of Th1 $NP_{309-328}$-specific CD4 T cells per spleen. Statistical analysis was performed using Student's *t* test. *, $p < 0.05$.

different groups of mice. It has been previously reported that wt 129 mice clear LCMV Armstrong infection on approximately day 8 p.i. In contrast, there was prolonged infection in IFN-γR2$^{-/-}$ mice, with clearance reported to be between days 10 and 15 p.i., which resulted in increased total numbers of CD8 T cells at these time points (29). Consistent with these results and as was observed after LM infection, IFN-γR2$^{-/-}$ mice consistently exhibited higher numbers of Th1 Ag-specific CD4 T cells compared with wt 129 mice (Figs. 3, *B* and *C*, and 4). We also infected IFN-γ$^{-/-}$ mice with LCMV and used ICS for TNF to detect gp61–80- and $NP_{309-328}$-specific CD4 T cells. We were able to detect CD4 T cells specific for each of these epitopes on day 8 p.i. (data not shown), indicating that IFN-γ is also not required for the generation of Ag-specific CD4 T cells after infection with LCMV.

Although the ability to produce IFN-γ is a hallmark characteristic of Th1 CD4 T cells, it remained possible that Ag-specific CD4 T cells generated after LM or LCMV infection in the absence of the IFN-γR were uncommitted to either a Th1 or Th2 lineage. To formally show that these cells were Th1 Ag-specific CD4 T cells after infection and did not also possess characteristics of Th2 cells, we stimulated splenocytes from IFN-γR2$^{-/-}$ and wt 129SVE mice on day 7 p.i. with $LLO_{190-201}$ and costained for IFN-γ and either IL-4 or IL-5. As shown in Fig. 5, *A* and *B*, we were unable to detect any IFN-γ-producing $LLO_{190-201}$-specific CD4 T cells that also produced IL-4 or IL-5 in either wt 129SVE or IFN-γR2$^{-/-}$ mice. In addition, using a more sensitive ELISPOT assay, we determined that the number of IL-4-secreting $LLO_{190-201}$-specific CD4 T cells was not statistically different between wt 129SVE mice and IFN-γR2$^{-/-}$ mice (Fig. 5*C*). In summary, we did not detect uncommitted Ag-specific CD4 T cells in IFN-γR2$^{-/-}$ mice, nor did we document any increase in the number of Ag-specific Th2 CD4 T cells.

To further investigate Th1 and Th2 characteristics of CD4 T cells after LM infection, we purified CD4 T cells from wt 129SVE and IFN-γR2$^{-/-}$ mice on day 7 p.i. and performed RT-PCR for the transcription factors T-bet and GATA-3 (Fig. 5, *D* and *E*). Compared with CD4 T cells isolated from wt mice, IFN-γR2$^{-/-}$ CD4 T cells did not express statistically less T-bet or more GATA-3, indicating that they were not more Th2-like than wt CD4 T cells. Taken together, these data strongly support the conclusion that Th1-committed Ag-specific CD4 T cells are generated in the absence of the IFN-γR.

Discussion

Using two different infectious model systems, we have shown that the development of Th1 Ag-specific CD4 T cells does not require either of the IFN-γR chains. It is a strong likelihood that during the process of Th1-Th2 lineage commitment, when multiple signals are being received by naive CD4 T cells in a short period of time, ligation of one receptor may influence how signals received through other receptors are translated into cellular responses. These interactions may be amplified under in vitro stimulation conditions, when the full spectrum of inflammatory signals may not be present.

There are statistical differences in the magnitudes of the Th1 Ag-specific CD4 T cell responses on day 7 (LM) and day 8 (LCMV) p.i. between wt and the various knockout mice. However, without thorough kinetic experiments and determination of the peak of the CD4 T cell response in IFN-γ and receptor-deficient mice, statistical differences in the numbers of cells at this one time point may be misleading. We sought to determine whether Th1 Ag-specific CD4 T cells could be primed in the absence of IFN-γ or its receptor. Our results clearly show this to be true, although the number of IFN-γ-producing CD4 T cells was not always equivalent. As we have previously documented, CD8 T cell responses in the absence of IFN-γ exhibit different kinetics than those in wt mice (18). We have generated preliminary data to suggest that Ag-specific CD4 T cell response kinetics are also aberrant in IFN-γ-deficient mice. Studies are currently underway to carefully explore interesting differences in the magnitude and kinetics of Ag-specific CD4 T cell responses in the absence of IFN-γ or its receptor.

It remains a possibility that the IFN-γR participates together with TCR signaling in Th1 lineage commitment of CD4 T cells

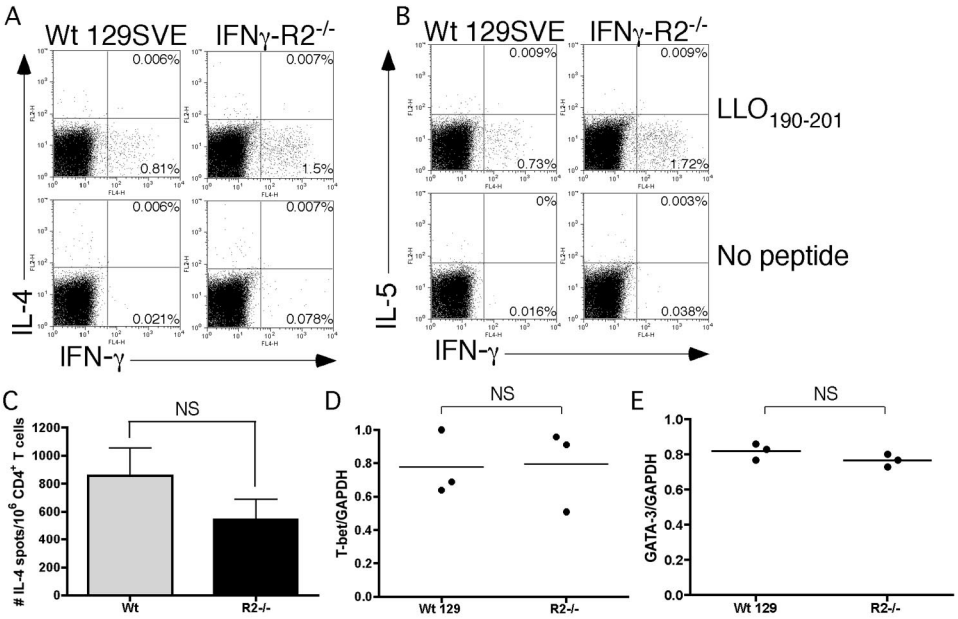


FIGURE 5. Ag-specific CD4 T cells generated after LM infection are committed Th1 cells. CD4 cells from wt 129SVE and IFN-γR2$^{-/-}$ mice on day 7 p.i. with LM were analyzed for their ability to produce Th2 cytokines and expression of Th1- or Th2-specific transcription factors. ICS for IFN-γ and IL-4 (*A*) or IL-5 (*B*) was performed after incubation with $LLO_{190-201}$ (*top panels*) or no peptide (*bottom panels*). Numbers indicate the frequency of IFN-γ$^+$/IL-4 or IL5$^+$ or IFN-γ$^+$/IL-4 or IL-5$^-$ cells within the Thy1.2$^+$/CD4$^+$ population. Data are representative of three mice per group. *C*, Results of an IL-4 ELISPOT assay reported as the number of IL-4-producing spots per 1×10^6 CD4 cells. Cells were either stimulated for 41 h with $LLO_{190-201}$ or left unstimulated. The number of spots detected in the unstimulated wells was subtracted from the number detected in the peptide-stimulated wells. Data are the mean ± SD of three mice per group. Statistical analysis was performed using Student's *t* test. *D* and *E*, Total CD4 T cells were purified from the indicated mice on day 7 p.i. RT-PCR was performed for T-bet (*D*) and GATA-3 (*E*). Data are reported as a ratio of T-bet or GATA-3 expression to GAPDH expression. Each symbol represents one mouse. Lines indicate averages per group.

under some conditions. Perhaps during infections where IL-4 is produced in much higher quantities than after LM or LCMV infections, the inhibition of IFN-γR colocalization with the TCR may negatively influence Th1 polarization of CD4 T cells, resulting in the development of Th2 Ag-specific CD4 T cells as is seen in *L. major* infection of BALB/c mice (30, 31). However, our experiments clearly demonstrate that after infection with LM or LCMV, signaling through the IFN-γR is not a requirement for the development of Th1 CD4 T cells in vivo.

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Disclosures

The authors have no financial conflict of interest.

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