Functional Plasticity in Memory T Helper Cell Responses

Connie M. Krawczyk, Hao Shen and Edward J. Pearce

J Immunol 2007: 178:4080-4088; doi: 10.4049/jimmunol.178.7.4080
http://www.jimmunol.org/content/178/7/4080

Why The JI?

- **Rapid Reviews!** 30 days* from submission to initial decision
- **No Triage!** Every submission reviewed by practicing scientists
- **Speedy Publication!** 4 weeks from acceptance to publication

*average

References  This article **cites 33 articles**, 8 of which you can access for free at:
http://www.jimmunol.org/content/178/7/4080.full#ref-list-1

Subscription  Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Effective immunity requires that responses be appropriately tailored to the infective agent. This is accomplished through the differentiation of T lymphocytes into functionally distinct cell lineages in response to environmental cues provided by the innate system. These cell lineages possess unique properties that enable them to control diverse pathogens. Upon activation by APCs, naive CD4⁺ T Th precursors (Thp) can differentiate into distinct cell lineages including regulatory T (Treg), Th1, Th2, and Th17 cells. In general, Th1 cells develop following infection with pathogens that have intracellular life stages such as bacteria, viruses, and certain parasites, whereas Th2 cells develop following infection with extracellular pathogens such as helminth parasites.

The cytokine production profiles of Th cells distinguish their functions and have been extensively used as lineage-specific markers (1). IFN-γ and IL-4 are both expressed at low levels in Thp cells (2). However, Th1 cells are potent producers of IFN-γ, whereas Th2 cells produce high levels of IL-4 and other Th2 cytokines such as IL-5 and IL-13. The ifng and Th2 cytokine loci in Th1 and Th2 cells, respectively, acquire epigenetic modifications that positively influence gene expression and allow for heritable expression of cytokine genes (3, 4). Concurrently, cytokine genes characteristic of the opposing lineage are silenced (2). The epigenetic modifications of cytokine loci are thought to be irreversible and to underlie the maintenance of T cell fates (3). Thus, the plasticity of cytokine gene expression in Th cells is thought to be progressively lost as Thp cells differentiate into effector and then memory Th cells, reflecting the permanent commitment of cells to a specific lineage (5, 6). It is believed that this commitment begins early, within several divisions following the activation of Thp cells in the presence of polarizing environmental conditions (2), and is associated with the lineage-specific expression of the transcription factors such as T-bet (Th1) and Gata-3 (Th2) (7–9).

Murine CD4⁺ T cells are thought to be rigid in their ability to produce lineage-specific cytokines and, once differentiated, lose their potential to produce cytokines of other lineages. However, there is evidence that human T cell clones are able to produce new cytokines when cultured in opposing polarizing conditions, suggesting some flexibility in human CD4⁺ T cell responses (10). Th cell differentiation has largely been studied using long-term cell culture systems and has yet to be examined in an Ag-specific manner in vivo. Therefore, it remains to be determined whether Th1 and Th2 cells reflect fully differentiated cell lineages in vivo.

To interrogate Th cell lineage commitment in vivo we used dendritic cells (DCs) pulsed with Ags from pathogens that inherently induce Th1 or Th2 responses along with the potently antigenic IAβ-binding listeriolisin O (LLO) peptide LLO190 (containing aa 190–201 of the LLO protein of Listeria monocytogenes (Lm)) (11) to establish polarized LLO190-specific Th responses. The LLO190-specific cells were reactivated in vivo in conditions that favor the development of the alternative Th lineage. Our data reveal that, upon reactivation, memory Th cells retain the ability to produce cytokines of their original lineage but additionally possess the capacity to respond to environmental cues to produce pathogen-appropriate cytokines of the opposing lineage. Importantly, these findings demonstrate that Th memory responses can be manipulated in vivo in an Ag-specific manner and therefore raise the possibility that immunotherapy can be used to induce the production of ameliorative cytokines by established pathologic Th cells in chronic infectious, allergic, or autoimmune disease settings.

Materials and Methods

Mice and reagents

B6 and Ly5.1 mice were purchased from The Jackson Laboratory and housed according to animal care guidelines at the University of Pennsylvania. Conjugated Abs used to detect CD4, IFN-γ, IL-2, IL-4, Ly5.1, and Ly5.2 for FACS analysis were purchased from BD Pharmingen. LLO190-201...
(NEKYAQAAPYVNS) and gpeh1-20 (GLNGPDYKVGYQFSVEFD) were purchased from Invitrogen Life Technologies. *Listeria monocytogenes* was grown in brain-heart infusion medium for 15 h at 37°C and then injected into mice (3 × 10⁸ CFU per mouse) unless otherwise indicated.

**DC culture**

Bone marrow-derived DCs were generated as described (12). Briefly, bone marrow DC precursors were differentiated for 8–10 days in the presence of 20 ng/ml GM-CSF in RPMI 1640 containing 10% FCS, 100 U/ml penicillin/streptavidin, 0.05 mM 2-ME, and 2 mM L-glutamine. On days 8–10 of culture DCs were pulsed for 18 h with 10 μg/ml *Propionibacterium acnes* (PA) and 50 μg/ml soluble *Schistosoma mansoni* egg Ag (SEA) with or without 10 μg/ml LLO190 peptide. Following incubation with Ags, cells were washed in PBS and 5 × 10⁴ DCs were injected i.p. per mouse.

**Ex vivo cytokine production analysis**

Splenocytes were harvested and restimulated for 6 h at a concentration of 15 × 10⁶/ml with or without 1 μg/ml LLO190 in the presence of 50 U/ml human IL-2 (Peprotech) and GolgiStop (BD Pharmingen) in IScove’s medium supplemented with 10% FCS or 5% normal mouse serum, 100 U/ml penicillin/streptavidin, 0.05 mM 2-ME, and 2 mM L-glutamine. Cytokine production was determined by using the BD Biosciences Cytofix/Cytoperm kit as per the manufacturer’s protocol. Data were collected using BD FACSCalibur and LSRII cytometers and analyzed using FlowJo software.

**LLO190-SEA conjugate (seallo)**

The LLO180–201 peptide was coupled to SEA proteins using N-hydroxysulfosuccinimide and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) in a cross-linking procedure used to couple carboxyl groups to primary amines per the manufacturer’s protocol (Pierce). SEA proteins were dialyzed into the activation buffer (0.1 M MES and 0.5 M NaCl (pH 6.0)). SEA proteins were subsequently incubated with EDC (~2 mM) and 1.1 mg of N-hydroxysulfosuccinimide (~5 mM) for 15 min at room temperature. 2-ME was added to quench the EDC. A synthesized LLO180–201 peptide (Invitrogen Life Technologies) was used and the numbers represent the percentage of lymphocytes that are CD4⁺ and produce the indicated cytokine. Results are representative of three experiments using three mice per group. b. Mice were immunized with DCs pulsed with PA or SEA with or without LLO190. More than 60 days following DC immunization the mice were each challenged with 3 × 10⁸ CFU of *Lm*. Seven days following infection, splenocytes were harvested from immune mice and restimulated ex vivo with LLO190. IL-2 production was determined by ICS and FACS. The legend on the right indicates primary immunization, and all mice were challenged with *Lm*. Values for individual mice are shown and the bars represent the mean. Data from one of five experiments are shown.

**CD4⁺ T cell transfers**

CD4⁺ T cells were enriched by the depletion of class II⁺, CD8⁺, NK1.1⁺, and CD19⁺ cells from spleens of donor mice using MACS (Miltenyi Biotec) as per the manufacturer’s directions. Cells were transferred, i.v. at a 1:1 donor-to-host ratio into congenic recipients. For the central memory Th cell (Tcm) and effecter memory Th cell (Tem) transfers, CD4⁺ cells were sorted based on CD62L expression using flow cytometry.

**Results**

**Immunization with DCs pulsed with LLO190 and polarizing Ags generates peptide-specific Th1 and Th2 CD4⁺ T cells in vivo**

DCs pulsed with LPS or bacteria such as PA induce the differentiation of Thp cells into Th1 cells, whereas DCs pulsed with helminth Ags such as SEA polarize Th responses to the Th2 lineage (12, 13). Because PA and SEA do not share antigenic epitopes, PA- or SEA-pulsed DCs were copulsed with LLO190 (aa 190–201 of the Lm LLO protein) (11) and used to induce the expansion of populations of LLO190-specific Th cells from within the polyclonal Th populations of naive B6 mice.

To establish that Th1 and Th2-polarized LLO190-specific Th cells develop following immunization with DCs pulsed with PA plus LLO190 (DC/PA/LLO190) or SEA plus LLO190 (DC/SEA/ LLO190), respectively, cytokine production by Th cells from mice injected with DCs and stimulated in vitro with LLO190 was assessed by intracellular cytokine staining (ICS). DC immunization was found to induce the expansion of small but significant populations of LLO190-specific Th cells. As anticipated, in mice immunized with DC/PA/LLO190, the Th cells produced IFN-α and IL-2, whereas in mice immunized with DC/SEA/LLO190 the LLO190-specific Th cells produced IL-4 and IL-2 but not IFN-γ (Fig. 1a, and data not shown). Furthermore, immunization with DC/PA/LLO190 or DC/SEA/LLO190 generates long-lived LLO190-specific Th memory cells that are capable of responding to subsequent antigenic challenge (Fig. 1b).

**FIGURE 1.** DC immunization generates peptide-specific Th1 and Th2 effector cells in vivo. a. Mice were immunized (IMM) with DCs pulsed with 18 h with PA or SEA with or without the LLO190 peptide as indicated. Seven days following immunization splenocytes were harvested from immune mice and restimulated ex vivo for 6 h with LLO190. IFN-γ and IL-4 production were determined by ICS and FACS. A live splenocyte gate was used and the numbers represent the percentage of lymphocytes that are CD4⁺ and produce the indicated cytokine. Results are representative of three experiments using three mice per group. b. Mice were immunized with DCs pulsed with PA or SEA with or without LLO190. More than 60 days following DC immunization the mice were each challenged with 3 × 10⁸ CFU of *Lm*. Seven days following infection, splenocytes were harvested from immune mice and restimulated ex vivo with LLO190. IL-2 production was determined by ICS and FACS. The legend on the right indicates primary immunization, and all mice were challenged with *Lm*. Values for individual mice are shown and the bars represent the mean. Data from one of five experiments are shown.
Ag-specific Th2 cells from immune mice produce IFN-γ and continue to produce IL-4 when challenged in a Th1 environment. a–d, Mice were immunized with DCs pulsed with PA or SEA with or without LLO\textsubscript{190} and challenged 60 days later with Lm. Splenocytes were analyzed for LLO\textsubscript{190}-specific cytokine production as described in Fig. 1. a–c. Cells are gated on live splenocytes except when gated on CD4\textsuperscript{+} as indicated by an asterisk (*). Numbers represent the percentage of gated cells producing the indicated cytokines. d, Total number of CD4\textsuperscript{+} splenocytes producing IFN-γ, IL-4, IL-2, and IFN-γ and IL-4. Each point represents one mouse and bars indicate the means. Results are shown from one of five experiments.

e, Mice were immunized i.p. with DCs pulsed with SEA with or without LLO\textsubscript{190}. More than 60 days following DC immunization the mice were challenged in the footpad with either 1 × 10\textsuperscript{4} CFU of Lm or 20 μg of seallo conjugate (conj.) mixed with 30 μg of SEA. Seven days following challenge, popliteal lymph nodes were harvested from immune mice and lymphocytes were restimulated ex vivo with soluble LLO\textsubscript{190} or with SEA-pulsed DCs. IFN-γ and IL-4 production were determined by ICS and FACS. Cells are gated on CD4\textsuperscript{+} T cells. Numbers represent percent gated cells producing the indicated cytokine. Results are representative of two experiments using three mice per group.

f, Mice were immunized with DCs pulsed with PA or SEA with or without GP61 peptide...
Th2 memory cells produce both IFN-γ and IL-4 when challenged in a Th1 environment

Memory Th cells and long-term cultured Th cells are thought to be fixed with respect to the types of cytokines they are able to produce (5, 6). We examined whether, in vivo, memory Th cells are non-responsive to the environment and continue to produce lineage-specific cytokines in the face of opposing polarizing conditions. Th1 and Th2 memory cells were generated by immunization with DC/SEA/LLO190 and DC/PA/LLO190, respectively, and examined for their cytokine production potential when recalled with Lm infection, a Th1-promoting pathogen.

Mice harboring LLO190-specific memory Th1 cells exhibited strong recall responses to Lm as measured by the ≥10-fold increase in the number of IFN-γ producing LLO190-specific Th1 cells relative to control mice mounting a primary response (Fig. 2, a, b, and d). Greater than 20% of CD4+ T cells from these animals produced IFN-γ in response to LLO190 stimulation and most of the IFN-γ-producing cells also produced IL-2 (Fig. 2b and data not shown).

Moreover, the CD4+ T cells from these mice produced very little IL-4, demonstrating the fidelity of cytokine production by memory Th1 cells recalled in a Th1 response-promoting environment (Fig. 2, b and d).

Mice harboring Th2 memory cells also exhibited recall responses greater than those of controls, indicating that the LLO190-specific Th2 memory cells were responding to Lm infection (Fig. 2). DC/SEA/LLO190-immunized mice that had been challenged with Lm contained large numbers (≥5-fold compared with controls) of CD4+ T cells capable of producing IFN-γ in response to LLO190, suggesting that LLO190-specific Th2 memory cells from these mice were capable of producing Th1 cytokines (Fig. 2, c and d). These mice also contained CD4+ T cells that produced IL-4 in response to Lm infection (Fig. 2, c and d), and double staining revealed that most of the IL-4-producing cells also made IFN-γ, IL-2, and IL-5 (Fig. 2c, and data not shown). The IFN-γ/IL-4 double-positive cells on average represented 20–30% of the cytokine-producing cells. Furthermore, the number of LLO190-specific IL-4-producing cells was 10-fold greater than that generated by primary DC immunization (compare Figs. 1a and 2c), strongly suggesting that the IFN-γ/IL-4 double-positive cells are memory Th2 cells that have retained the ability to produce IL-4 but, as a result of Lm infection, acquired the ability to also produce IFN-γ.

To confirm that Th2 memory cells generated by DC immunization produced Th2 cytokines when recalled by a Th2 stimulus, mice immunized with DC/SEA/LLO190 were challenged with seallo, an LLO190/SEA complex that allows the presentation of LLO190 in the context of a Th2 stimulus and, when used to pulse DCs, induces comparable responses as SEA plus the LLO190 peptide (data not shown). As anticipated, DC/SEA/LLO190-immunized mice challenged with seallo exhibited increased numbers of LLO190-specific Th2 cells capable of making IL-4 compared with controls (Fig. 2e). There were also increased numbers of SEA-specific Th2 cells in these animals (Fig. 2e) and little evidence for LLO- or SEA-specific IFN-γ-producing Th cells. Thus, analogous to memory Th1 cells challenged under Th1 conditions, memory Th2 cells challenged under Th2 conditions respond by producing only lineage-specific cytokines. In contrast, when memory Th2 cells were challenged under Th1 conditions through s.c. infection with Lm, a population of Th2 cells capable of producing IL-4 and IFN-γ or IFN-γ alone emerged.

To determine whether the capability of Th2 memory cells to acquire the ability to produce IFN-γ represents a more general phenomenon or is unique to the LLO190-responding population or Lm infection, mice were immunized with DCs pulsed with SEA and the IAβ-restricted lymphocytic choriomeningitis virus (LCMV) epitope gP61–80 (14). More than 60 days later the immunized mice were challenged with the Armstrong strain of LCMV (Fig. 2f). Consistent with previous results, Th2 memory cells generated during DC immunization produced IFN-γ and IL-4 or IFN-γ alone in response to LCMV infection (Fig. 2f). Taken together, these results suggest functional plasticity in the ability of Th2 memory cells to express Th1 lineage-specific cytokine genes in vivo.

IFN-γ/IL-4 double-positive cells are memory CD4+ cells that retain the ability to produce IL-4 while acquiring the ability to produce IFN-γ

Our results suggested that memory Th2 cells can acquire the ability to produce IFN-γ when reactivated under Th1 conditions. However, it remained a possibility that IFN-γ/IL-4+ CD4+ T cells were newly activated but uncommitted CD4+ T cell effectors. To examine this, CD4+ cells from DC/SEA/LLO190-immunized mice and nonimmune mice were transferred to congenic hosts and challenged with Lm. We reasoned that if IFN-γ/IL-4 double-positive cells represent new effector Th cells they would arise equally from both the host and donor populations, whereas IFN-γ+/IL-4− effector memory Th2 cells would be present only within the donor population.

Comparable numbers of LLO190-specific host CD4+ T cells producing IFN-γ were observed regardless of the origin of donor cells (Fig. 3, a and b). Host-derived, LLO190-specific CD4+ T cells did not produce significant amounts of IL-4, indicating that the effectors derived from host naive CD4+ T cells responding to Lm infection were appropriately skewed to the Th1 lineage (Fig. 3, a and c). In addition, naive cells present within the CD4+ population from control DC/SEA-immunized donor animals also mounted a Th1 response to Lm, producing IFN-γ and IL-2 but not IL-4 (Fig. 3, a and b, and not shown). In contrast, only donor cells from mice immunized with DC/SEA/LLO190 contained CD4+ T cells that produced IL-4 upon restimulation with LLO190 (Fig. 3, a and c). Strikingly, CD4+ T cells derived from DC/SEA/LLO190-immunized mice also contained 10-fold more IFN-γ-producing cells than did donor populations from control DC/SEA-immunized animals despite the fact that the recipient mice were infected equivalently with Lm (Fig. 3, a and b), indicating that the enhanced IFN-γ production was the result of the activation of a population of donor-derived memory Th cells. A significant percentage (>13%) of these memory cells produced both IL-4 and IFN-γ, further establishing that memory Th2 cells can acquire the ability to produce IFN-γ when recalled in a Th1 environment. Therefore, the IFN-γ+/IL-4− double positive and many of the IFN-γ−Th cells in the chimeric mice are derived from the memory Th2 cell compartment.

Th1 memory cells can acquire the ability to produce IL-4

Having shown that memory Th2 cells can acquire the ability to produce IFN-γ when faced with a Th1-polarizing infection, we examined whether Th1 memory cells can acquire the ability to...
produce IL-4. Th1 memory cells were generated using *Lm* infection, which induces a highly polarized Th1 response (Figs. 2 and 4). Memory Th1 cells generated by *Lm* infection were transferred into congenic hosts and challenged under Th1-polarizing conditions with *Lm* or DC/PA/LLO190 or, under Th2 conditions, with DC/SEA/LLO190. LLO190-specific memory Th1 cells were recalled by both *Lm* infection and DC/PA/LLO190 immunization as evident by significantly increased numbers of IFN-γ/H9253-producing cells within the donor populations in challenged mice vs unchallenged controls (Fig. 4, a and b). These LLO190-specific effector memory CD4+ T cells were predominantly Th1 because they were able to make IFN-γ and IL-2 but little IL-4 (Fig. 4a, and data not shown). However, mice that had been challenged with the Th2 stimulus DC/SEA/LLO190 contained significant numbers of donor cells that produced both IFN-γ and IL-4 in response to LLO190 stimulation (Fig. 4, a and b). These cells were present in numbers that were significantly greater than what could be accounted for any primary response to DC immunization as evident by significantly increased numbers of IFN-γ-producing cells within the donor populations in challenged mice vs unchallenged controls (Fig. 4, a and b). These LLO190-specific effector memory CD4+ T cells were predominantly Th1 because they were able to make IFN-γ and IL-2 but little IL-4 (Fig. 4a, and data not shown). However, mice that had been challenged with the Th2 stimulus DC/SEA/LLO190 contained significant numbers of donor cells that produced both IFN-γ and IL-4 in response to LLO190 stimulation (Fig. 4, a and b). These cells were present in numbers that were significantly greater than what could be accounted for any primary response to DC immunization as illustrated by the host response (Fig. 4, a and b), indicating that they are derived from memory Th1 cells generated during the primary *Lm* infection in the donor animal. The IFN-γ/IL-4 double producers represented ~20% of the IFN-γ-producing memory cells (Fig. 4c). The host response to *Lm* and DC/PA/LLO190 was predominantly Th1, characterized by IFN-γ and IL-2 (Fig. 4a, and data not shown). It is interesting to note that challenging *Lm*-primed mice with DC/PA/LLO190 led to the appearance of a small population of Th cells that made IL-4 as well as IFN-γ (Fig. 4). We did not observe these double producers in mice primed and challenged with *Lm* (Fig. 4).

We speculate that this difference reflects the fact that a subpopulation of PA/LLO190-pulsed DCs remain immature (and similar in this regard to SEA/LLO190-pulsed DCs) (12) and therefore capable of driving Th2 responses. The host immune system within mice challenged with DC/SEA/LLO190, exhibited a weak and mixed response with some Th1 cells and some Th2 cells, raising the possibility that the memory responses may influence the primary responses (Fig. 4a). Therefore, memory Th1 cells generated during *Lm* infection can acquire the ability to produce IL-4 when challenged with a Th2-inducing stimulus. In addition, these results reveal that SEA-conditioned DCs are sufficient to induce IL-4 production by memory Th1 cells.

Our data show clearly that by 1 wk following challenge in conditions that favor the development of the alternative Th lineage, the effector memory Th1 and Th2 cells have acquired the ability to produce IL-4 and IFN-γ, respectively, in addition to their lineage-specific cytokines. We wondered whether this dual producer status reflected an intermediate stage on the pathway toward a complete lineage switch and hypothesized that if, for example, a gradual recommitment from Th2 to Th1 was occurring in mice immunized with DC/SEA/LLO190, and challenged with *Lm*, we should see the production of IL-4 diminish following an additional subsequent *Lm* challenge. To address this issue, mice were infected with *Lm*

---

**FIGURE 3.** IFN-γ/IL-4 double-positive cells are memory CD4+ cells that have retained the ability to produce IL-4 and acquired the ability to produce IFN-γ. a. Ly5.1 Mice were immunized (IMM) with DCs pulsed with SEA with or without LLO190. More than 60 days later, CD4+ T cells were purified and transferred to Ly5.2 congenic hosts. The following day, mice were infected (CHG, challenged) with 3 × 10⁴ CFU of *Lm*. Seven days following infection with *Lm*, splenocytes were harvested from infected mice and restimulated ex vivo with LLO190 for 6 h. IFN-γ, IL-4, and IL-2 production was then determined by ICS and FACS. Cytokine responses of donor and host cells are indicated. Results are representative of two experiments using 2–3 mice per group. Cells are gated CD4+ T cells and the numbers indicate the percentage of CD4+ T cells producing the indicated cytokines. b and c, Quantitation of total cytokine-producing cells from one experiment. Each point represents one mouse and the bars represent the mean.
60 days following DC/SEA/LLO190 immunization and then infected again with Lm more than 60 days later. Stimulation of the splenocytes from these mice with LLO190 revealed that effector memory Th cells capable of making IFN-γ and IL-4 had in fact persisted in these animals and that a gradual conversion of all of the Th2 cells to the Th1 lineage had not occurred (Fig. 5). These data suggest that under the appropriate circumstances a subpopulation of effector memory Th cells can commit to producing both IL-4 and IFN-γ.

Tcm (central memory) and Tem (effector memory) cells possess differential capacity for new cytokine production

One study using human T cell clones suggests that Tcm cells are unpolarized and, upon secondary challenge, can produce cytokines typical of the Th1 or Th2 lineage (10). In contrast, human Th1 Tem cells remained committed to producing the cytokines characteristic of their lineage (10). Th1 Tem cells begin to produce IL-4 when cultured in Th2 conditions and retain the ability to produce IFN-γ. Human Tem Th2 cells, however, are more rigid in their commitment than are Th1 cells and cannot readily acquire the ability to produce IFN-γ. These findings indicate that the differential potential varies among T cell subsets. The heterogeneous population that is observed when memory Th cells are challenged under conditions that promote the development of the opposing lineage in this study may reflect different levels of commitment of the various Th memory subsets.

To examine the differentiation potential of murine memory T cell populations, CD4 T cells from either DC/SEA/LLO190-immune mice (Th2) or Listeria-immune mice (Th1) were sorted...
FIGURE 6. Differential potential of Tcm and Tem cells. Mice were either immunized with DC/SEA/LLO190 (Th2) or infected with \(3 \times 10^7\) CFU of Ln (Th1). More than 40 days following infection CD4+ T cells were sorted using flow cytometry based on CD62L expression and transferred to congenic hosts. The following day mice were immunized with DCs pulsed with SEA/LLO190 or challenged with \(3 \times 10^7\) CFU of Ln. Seven days after challenge IFN-\(\gamma\) and IL-4 production was determined as in Fig. 1. Cells are gated on donor CD4+ T cells and the values indicate percentage of donor CD4 T cells producing the indicated cytokine.

![Diagram](image)

**Discussion**

The polarization of Th responses is directed by signals delivered from cells of the innate system that use pattern recognition receptors to identify and respond to specific pathogen threats by producing a series of positive and negative signals that favor the differentiation of Thp cells into either Th1 or Th2 cells (13). Differentiation is thought to be a gradual process in which, over the course of several cell divisions, lineage-specific cytokine genes are epigenetically modified to favor continued expression, and genes encoding cytokines produced by alternative Th lineages are epigenetically silenced (3). During the primary response this process leads to the development of increased numbers of similarly polarized effector Th cells and, subsequently, as the Ag diminishes memory is established through the persistence of long-lived Ag-specific cells. There are believed to be two major subsets of memory cells, effector and central (15). The latter are thought to produce only IL-2 in quantity and their commitment to lineage is unclear. Effector memory cells, however, are believed to be lineage-committed and produce lineage-specific cytokines. In this study, by using an in vivo immunization system that allows us to couple polyclonal Th response priming to an individual peptide Ag with polarizing signals delivered by pathogen-derived molecules we show that memory Th cells are committed to producing lineage-specific cytokines but also retain the ability to respond to environmental cues and express situationally relevant, lineage-atypical cytokine genes. These findings support the view put forward by Sallusto and colleagues (10) that differentiated Th cells exhibit flexibility of cytokine production and further question the notion that Th1 and Th2 cells reflect fully differentiated cell lineages. In addition, these results reveal that CD4 T cell responses can be manipulated in vivo and that the manipulation of existing Ag-specific populations could be of therapeutic value because inappropriate cytokine production can cause or exacerbate pathological conditions.

In vitro modeling of memory Th cell development has indicated that long-term culture under polarizing conditions leads to the development of committed Th cells that cannot express the cytokine genes that define alternative lineages (2, 5). Additionally, cytokine production profiles of memory cells grown in vivo following the transfer of in vitro polarized effector cells resemble those of the original effectors, suggesting again that murine memory Th cells remain committed to the same lineage as the effector Th cells from which they were derived (6). However, simple in vitro Th-polarizing conditions use rIL-12 to promote Th1 differentiation and rIL-4 to promote Th2 differentiation, and while IL-12 and IL-4 are clearly important for lineage commitment of Th cells, increasing evidence suggests there are additional factors that affect Th cell fate. For instance, molecules such as Notch-ligands, SLAM, Tim-2, Tim-3, IL-25, IL-27, IL-6, and TGF-\(\beta\) can influence Th response polarization (7, 16 – 27), and Th differentiation is also affected by Ag dose (28, 29). Therefore, in vivo modeling of Th cell polarization is necessary to determine the rigidity of Th cell fate decisions and the potential for manipulation of responses in vivo.

Our results demonstrate that both central and effector memory CD4 T cells can acquire the ability to produce new cytokines in response to environmental cues. We observe a heterogeneous response with respect to the cytokine production profiles of the memory Th population, particularly in cases where mice are primed for Th2 memory and then challenged in Th1 conditions. The results of our adoptive transfer studies coupled with the much larger numbers of the Th1-like cells present after challenge vs primary infection indicate that these cells have arisen from the established memory Th2 pool. Part of the heterogeneity is based on subpopulations within the Th2 memory T cell pool. Tem cells produce cells in which IFN-\(\gamma\)-producing and IL-4 producing is either maintained or silenced, indicating that some Tem cells or daughter cells derived from Tem cells have completely converted to the Th1 lineage and have lost the ability to produce cytokines of the Th2 lineage. Central memory cells generated under Th2 conditions become Th1 cells, producing IFN-\(\gamma\) and not IL-4. Because it is unclear whether Tem cells generated under Th2 conditions retain the ability to produce IL-4, these Th1 cells are either Th2 cells that have lost the ability to produce IL-4 or are Th1 cells newly differentiated from previously uncommitted central memory cells. Central memory cells generated under Th1 conditions are also capable of producing IL-4 when challenged under Th2 conditions. Taken together, these results suggest that central memory cells are largely an unpolarized population that can respond accordingly and produce cytokines of other lineages.

An interpretation of our findings is that priming with apparently polarizing immunizations leads to the development of a small number of cells that have not committed to effector cytokine production or that produce cytokines characteristic of either the opposing lineage or both lineages and it is these cells that are selectively expanded during challenge. Arguing against this, we did not detect any IL-4-producing cells following Ln priming (Fig. 2) or...
in Lm-immune mice that were challenged with Lm (Fig. 5). Nevertheless, a clear population of IL-4-producing cells was evident in Lm-primed mice that were challenged with SEA-conditioned LLO-pulsed DCs (Fig. 5). The fact that all of the IL-4-producing Th cells in the Lm-primed, SEA-conditioned LLO-pulsed DC-challenged mice were also making IFN-γ suggests that they were derived from preexisting Th1 memory cells rather than uncommitted memory cells, because the latter would presumably be expected to give rise to effector cells that produce only IL-4 under these conditions. However, following priming with SEA-conditioned LLO-pulsed DCs and challenge with Lm, it is clear that the population of cells producing IFN-γ and not IL-4 emerges (Fig. 3) and is presumably derived from uncommitted memory cells. Consistent with many reports that Tcm cells are uncommitted, we found that these IFN-γ-producing cells that emerge in Lm-challenged, SEA-conditioned, LLO-pulsed, DC-primer mice can be derived from Tcm cells (Fig. 6). We believe that our data support the view that memory Th responses established under polarizing conditions contain populations of Ag-specific cells that have yet to commit to making particular cytokines (central memory cells) or that did commit to the Th1 or Th2 lineages and that all of these populations contain within them cells that are capable of responding to new situations that require them to make cytokines that they previously have not made.

Many studies of CD4 Th cell lineage commitment show that epigenetic modifications of cytokine gene loci permit stable cytokine gene expression profiles to be passed on to daughter cells as the population expands (3, 4). Our results are in agreement with this view, demonstrating the existence of large numbers of Tcm cells exhibiting the cytokine gene expression patterns established during the primary response. However, findings reported in the present study indicate that Th cells are capable of producing cytokines of distinct lineages, both IFN-γ and IL-4, simultaneously. Production of IFN-γ and IL-4 by CD4 T cells is atypical and is generally considered to be mutually exclusive. These double-positive cells may represent cells that are transitioning from one lineage to another. However, Th2 cells that have also acquired IFN-γ and produce both IFN-γ and IL-4 are able to persist through an additional Lm challenge, suggesting that they may be effector memory cells that have modified both the Ifng and il4 loci for heritable expression. As a Thp cell differentiates, interplay between the pathways that influence Th1 and Th2 development occurs to ensure the adoption of one cell fate. Differential expression of the transcription factors T-bet and Gata-3 underlies the polarization of Th1 and Th2 subsets, respectively. T-bet is considered to prevent the development of Th2 responses through phosphorylation-dependent interaction with Gata-3 (30). Interestingly, T-bet phosphorylation is not detected upon a secondary stimulation of Th1 cells, suggesting that its role in limiting Th2 cell development is restricted to the early stage following Thp cell activation (30). Although Gata-3 expression is important for Th2 cell development, its continued production is not required for established Th2 cells to continue producing IL-4 and it is likely that Th2 memory cells no longer need Gata-3 for IL-4 expression (31). However, forced expression of Gata-3 in Th1 cells has been shown to induce the production of Th2 cytokines and down-regulate IFN-γ production (32, 33) and a report has demonstrated that cultured Th1 cells can produce IL-4 when further primed in Th2 conditions (31), an ability that was dependent on the presence of Gata-3, suggesting that Gata-3 can be induced in Th1 effector cells. Thus, our data raise the possibility that if T-bet and Gata-3 are expressed in memory Th cells they may be capable of positively activating cytokine gene expression but incapable of silencing genes of the opposing lineage, leading to cells that produce cytokines typical of both Th1 and Th2 lineages. The induction of Gata-3 and T-bet has not previously been shown to occur naturally in mature Th1 and Th2 cells, respectively, and whether they can be induced in IFN-γ/IL-4 double-positive cells in our system remains to be determined. A deeper molecular analysis of the IFN-γ/IL-4 double positive cells identified here will shed light not only on Th differentiation, but also on cell fate decisions in general.

In summary, in vivo modeling of Th differentiation potential has revealed unexpected plasticity in the ability of murine Th cells to produce cytokines of opposing lineages. Our data support the studies of human Th cells and suggest that Th cells in general possess the potential to further differentiate in response to environmental cues. We believe that this reflects both changes in individual cells that allow them to begin making cytokines they had previously not made and changes at the population level such that the secondary response is dominated by cells that make cytokines appropriate to the nature of the challenge. Importantly, our discovery reveals that CD4 responses can be manipulated in vivo in an Ag-specific manner, suggesting that it will be possible to treat chronic autoimmune, allergic, or infectious diseases by inducing existing pathological or ineffectual Th cells to produce beneficial cytokines.

Acknowledgments
We thank J. Taylor, R. G. Jones, and E. L. Pearce for critical comments and E. Jung for excellent technical assistance. We thank B. Murphy, A. Bantly, and C. H. Fletcher of the Flow Cytometry and Cell Sorting Resource Laboratory for invaluable assistance.

Disclosures
The authors have no financial conflict of interest.

References


