Brief Definitive Report

Extent of T Cell Receptor Ligation Can Determine the Functional Differentiation of Naive CD4$^+$ T Cells

By Stephanie Constant, Christiane Pfeiffer, Ann Woodard, Theresa Pasqualini, and Kim Bottomly

From the Section of Immunobiology, Yale University School of Medicine, and The Howard Hughes Medical Institute, New Haven, Connecticut 06510

Summary

Naive CD4$^+$ T cells can differentiate into cells predominantly involved in humoral immunity, known as T helper type 2 cells (Th2), or cells involved in cell-mediated immunity, known as Th1 cells. In this report, we show that priming of CD4$^+$ T cells bearing a transgene-encoded T cell receptor can lead to differentiation into Th1-like cells producing abundant interferon γ when the cells are exposed to high antigen doses, while low doses of the same peptide induce cells with the same T cell receptor to differentiate into Th2-like cells producing abundant interleukin 4. Thus antigen dose is one factor that can control the differentiation fate of a naive CD4$^+$ T cell.

Adaptive immune responses are largely dependent on the induction of effector CD4$^+$ T cell function during the initial exposure to antigen. After antigenic stimulation, CD4$^+$ T lymphocytes can differentiate into helper T cells (Th2) capable of initiating humoral responses or into cells mediating cell-mediated responses (Th1). This distinction, which was initially reported using cloned T cell lines in the mouse (1–3), has now been shown using normal CD4$^+$ T cells (4–6) and cells derived from humans (7, 8). The distinction is important in a variety of infectious diseases (6, 8–10), including AIDS (9, 11). Thus, it is crucial to understand the factors that control the differentiation of naive, resting CD4$^+$ T cells into armed effector cells upon encounter with antigen. Dominance of one or the other effector cell type has been largely attributed to cytokines present in the priming environment; thus, the addition of high doses of exogenous cytokines in vivo and in vitro can direct the differentiation of CD4$^+$ T cells, with IFN-γ (9, 12) and IL-12 (13, 14) leading to Th1-like cells, and IL-4 (15–18) leading to Th2-like cells. These experiments leave open the mechanism by which these cytokine environments may be generated in the in vivo setting. It has been proposed that innate immune responses of natural killer cells and mast cells might provide the requisite priming environments.

In addition, in some cases where Th1 or Th2 responses are dominant, cytokine environments do not appear to be distinct, suggesting that mechanisms other than cytokine environments must be able to determine the fate of naive CD4$^+$ T cells encountering antigen for the first time. We have previously hypothesized that antigen dose might be one critical parameter in determining the differential activation of naive CD4$^+$ T cells (19). In particular, we have analyzed the response to a single peptide of inbred strains of mice that differ only in MHC class II genotype, and found that mice of one genotype respond by making Th1-like CD4$^+$ T cells, while mice of all other genotypes produce Th2-like responses to the same peptide (20). One explanation put forward for these results is that the density of peptide–MHC class II complexes presented to undifferentiated CD4$^+$ T cells differs in the two genotypes. Thus, a high ligand density leads to efficient priming of one Th subset, whereas low ligand provides optimal priming for the other subset. Although the same peptide was used to prime both Th1 and Th2 cells in this system, the MHC molecule with which the peptide combines will differ according to genotype, and therefore the ligands cannot be compared directly. Moreover, the T cells in the starting populations are heterogeneous in their specificity and may bind to the ligand with varying affinity. Finally, antigen dose cannot be rigorously controlled in this in vivo system. Other interpretations of these results are therefore possible.

In this report, the effect of the priming antigen dose on the generation of Th1 or Th2 cells was examined under rigidly defined conditions. To eliminate the affinity of the TCR as a potential variable, and to gain greater control over the antigen dose, we investigated the priming by peptide antigen in vitro of purified CD4$^+$ T cells from mice transgenic for a TCR of known specificity (21). Our results show that priming antigen dose can indeed control the differentiation fate of naive CD4$^+$ T cells such that low doses favor development of Th2-like cells and high doses Th1-like cells.

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Materials and Methods

Mice. B10.A (5R) (5R) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The αβ TCR-transgenic mice (TCR specific for the carboxy terminus of pigeon cytochrome c; 21) were derived from a heterozygous mouse, which was kindly provided to us by J. Kaye (The Scripps Research Institute, La Jolla, CA). This founder mouse was mated to 5R mice to generate transgenic animals with an $H\text{-}2^b$–restricted TCR. Subsequent generations were then maintained as transgene heterozygotes on a 5R background by back-crossing to 5R breeders.

Antigen. The antigen used throughout these studies was a synthetic peptide coding for residues 81–103 of tobacco hornworm moth cytochrome c (pMCC), and it was prepared as described previously (22).

Preparation of Accessory and $CD4^+$ T Cells. T cell–depleted APC were prepared by antibody-mediated complement lysis of 5R splenocytes using anti-Thy1, -CD4, and -CD8 mAb (22). Purity of the resulting APC was >95% as determined by staining with anti–MHC class II mAb. The APC were treated with 50 μg/ml mitomycin c (Boehringer Mannheim Biochemicals, Indianapolis, IN) before culture. $CD4^+$ T cells were isolated from the combined lymph nodes and spleens of TCR-transgenic mice by immunomagnetic negative selection (22) using mAbs to CD8 and MHC class II followed by incubation with anti–mouse and –rat Ig–coated magnetic beads (Collaborative Research Inc., Bedford, MA). Purity of the recovered $CD4^+$ T cells was >85%, as determined by staining with an anti-CD4 mAb (the remaining 15% were usually a mixture of granulocytes and class II^+ cells) with 95–99% expressing the transgenic α and β chains. For some experiments, populations of purified $CD4^+$ T cells were depleted of previously stimulated cells (cells having low/negative expression of the CD45RB molecule; 23) by MACS® (Miltenyi Biotec, Sunnyvale, CA) separation (24), using biotinylated anti-CD45RB (clone 16A; 23) and MACS streptavidin-conjugated beads. The resulting $CD45RB^{hi}$ ($16A^{hi}$) cells were then used for in vitro cultures.

In Vitro Stimulation of TCR-transgenic T Cells. Primary cultures were set up in 25- or 75-cm^2 tissue culture flasks (Corning, Bellingham, MA) using purified (total or $16A^{hi}$) $CD4^+$ T cells (5×10^5/ml) from TCR-transgenic mice and T-depleted APC (5×10^5/ml) from 5R mice plus various concentrations (0.0005–50 μg/ml) of pMCC in EHAA medium (GIBCO BRL, Gaithersburg, MD) supplemented with 5% FCS. In some experiments, murine rIL-2 (Boehringer Mannheim) was added to each culture (50 U/ml) 24 h after setting up primary stimulations. We and others (18) have found that the addition of exogenous IL-2 during priming does not influence the outcome of the secondary responses in these assays, but increases the yield of primed cells significantly. After an additional 3 d of priming, supernatant was collected from each bulk culture for cytokine analysis, and the viable $CD4^+$ T cells isolated from the cultures by gradient centrifugation. The T cells were then resuspended with fresh APC (without antigen) for a 2-d "rest" period. For secondary cultures, rested T cells were restimulated at 5×10^5/ml with 5 μg/ml pMCC plus fresh APC (5×10^5/ml) in 24-well plates. 2 d later, the supernatant was collected for cytokine analysis. In some experiments, anti–IL-4 antibody (at a concentration capable of blocking 225 U/ml rIL-4) was included in primary or secondary cultures.

Measurement of Cytokine Production. The presence of IL-4 was determined by the induction of proliferation of the CT.4S cell line (obtained from W. E. Paul, National Institutes of Health, Bethesda, MD). The release of IFN-γ was detected by the inhibition of proliferation of the WEHI-279 cell line (American Type Culture Collection, Rockville, MD). CT.4S (5×10^3/well) and WEHI-279 (10^4/well) cells were incubated with dilutions of test supernatants from T cell cultures or with control recombinant cytokines for 48 h and then 1 μCi [^{3}H]methyl-thymidine (6.7 Ci/mmol; ICN Biomedicals Inc., Irvine, CA) was added for a further 24 h to measure proliferation. Units of IL-4 and IFN-γ were calculated from the regression analysis of standard curves of proliferation or inhibition of the cell lines with known units of recombinant cytokines. The specificity of the responses in each cytokine assay was confirmed by the addition of anti–IL-4 or anti–IFN-γ antibodies.

Results and Discussion

To determine whether the priming dose of antigen controls the differentiation of a homogeneous population of $CD4^+$ T cells into either Th1 or Th2 cells, $CD4^+$ T cells from naive mice transgenic for a TCR recognizing pMCC bound to $E_\beta{}^b$:$E_\alpha{}^k$ (I-E^b) were used (21). Total $CD4^+$ T

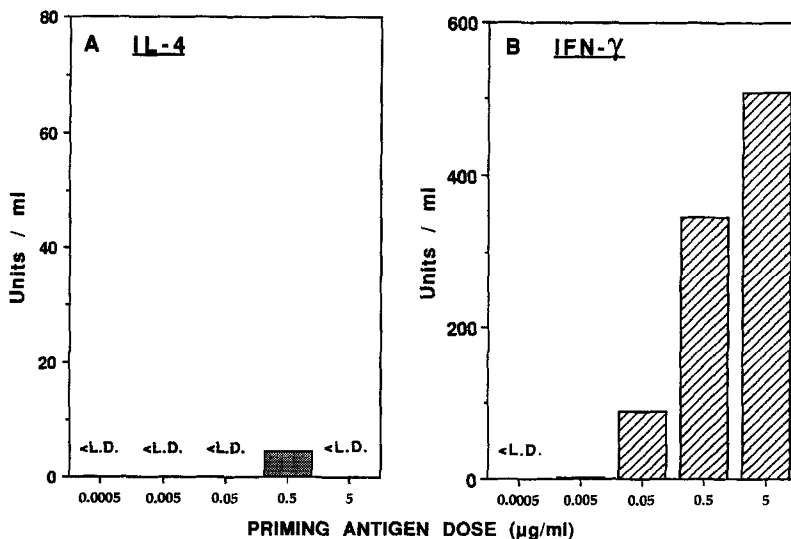

Figure 1. Cytokine production from total $CD4^+$ T cells stimulated with varying doses of antigen. Total $CD4^+$ T cells from TCR transgenic mice (prepared as described in Materials and Methods) were primed in vitro with different doses of pMCC antigen (0.0005, 0.005, 0.05, 0.5, or 5 μg/ml) and APC for 4 d. The viable $CD4^+$ T cells from each group were purified, rested for 2 d in the absence of antigen, and then restimulated for 2 d with 5 μg/ml antigen and fresh APC. The supernatant from these secondary cultures was collected for cytokine analysis. The figures show the units of (*A*) IL-4 and (*B*) IFN-γ (both per 1 ml of culture supernatant) after secondary antigenic stimulation. The lower limit of detection (LD) in these experiments was 0.9 U/ml IL-4 and 1.9 U/ml IFN-γ. These data are representative of three independent experiments.

cells were cultured with T-depleted splenocytes (APC) and doses of pMCC ranging from 0.5 ng/ml to 50 μg/ml (in the absence of any priming antigen, the viable cell recovery after 4 d of culture is $<10^5$ per culture). After 4 d in culture, the primed CD4+ T cells were repurified, rested in the presence of fresh APC without antigen for 2 d, and then restimulated with fresh APC and pMCC at a single peptide dose. As seen in Fig. 1, the priming of CD4+ T cells with doses of 50 ng/ml or greater led to IFN-γ production (Fig. 1 *B*). Lower doses of pMCC in the initial culture, however, did not generate Th2-like cells (Fig. 1 *A*). The failure to produce either IFN-γ or IL-4 after priming with <50 ng/ml of antigen was not the result of the CD4+ T cells having become anergic since they showed levels of secondary proliferation similar to those of T cells primed with higher doses of antigen (data not shown).

We next asked whether the failure of the CD4+ T cells to be primed to produce IL-4 might be due to the presence of IFN-γ in the primary culture. Several studies have shown that the addition of IFN-γ in vivo (9) or in vitro (12) blocks the generation of Th2 cells and IL-4 production. Furthermore, it has been reported that CD4+ T cells from mice transgenic for the TCR recognizing pMCC contain a small population of memory cells (25) that might be capable of producing IFN-γ in vitro. Therefore, to determine whether the presence of IFN-γ might explain our failure to observe Th2 priming in our own experiments, we analyzed supernatants from the primary cultures for the presence of IFN-γ. For comparison, we stimulated a population of TCR-transgenic CD4+ T cells that had been purified for naive cells. These naive CD4+ T cells were prepared by depletion of cells having low/negative expression of the CD45RB isoforms detected by the mAb 16A ($16A^{hi}$), which identifies cells previously stimulated by antigen (23) (Fig. 2 *A*). When we examined IFN-γ release in the cultures of both unseparated and naive CD4+ T cells, IFN-γ was produced in the primary cultures of unseparated CD4+ T cells only (Table 1).

Table 1. *Cytokine Production in Primary Cultures of Unseparated and $16A^{hi}$ CD4+ T Cells*

CD4+ T cells	Cytokines (U/ml) IFN-γ	IL-4
Unseparated	37.1	<LD
Naive ($16A^{hi}$)	<LD	<LD

The CD4+ T cells were stimulated in vitro with APC and 5 μg/ml pMCC for 4 d as described in Figs. 1 and 2, and culture supernatants were then analyzed for the presence of IFN-γ and IL-4. The LD in these experiments was 1.9 U/ml IFN-γ and 2.8 U/ml IL-4.

Since there was no IFN-γ in the primary cultures of naive CD4+ T cells, we asked whether we could induce Th2-like cells from $16A^{hi}$ CD4+ cells by varying the dose of peptide used in the initial culture. The naive cells were cultured with APC and different doses of pMCC, rested, and then restimulated with a single dose of peptide in the secondary culture. As seen in Fig. 2 *B*, IL-4 production was observed at priming doses lower than 50 ng/ml; at 50 ng/ml or above, IL-4 production was undetectable. By contrast, IFN-γ production increased with increasing priming dose (Fig. 2 *C*). Thus, priming with 0.5 or 5 ng/ml leads to dominant IL-4 production in the second culture, whereas priming doses greater than 50 ng/ml leads to dominant IFN-γ production. The same phenomenon was observed when using CD4+ T cells separated by the L-selectin marker (unpublished observations). These data demonstrate that the induction of IL-4–producing CD4+ T cells depends both on the dose of antigen used for priming and on the use of naive T cells as the starting population. Despite the careful isolation of the naive subset of CD4+ T cells for our cultures, in a few experiments, we failed to generate IL-4–producing cells using low doses of priming antigen: in all these experiments, IFN-γ (possibly derived from

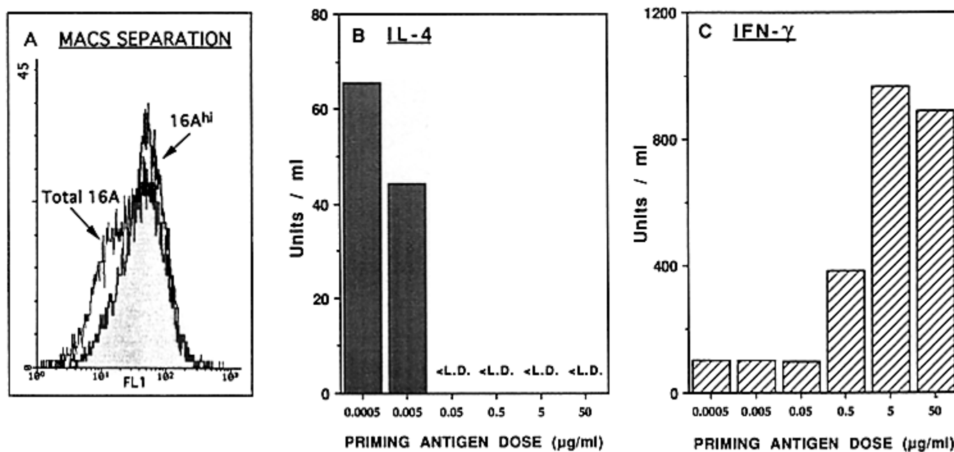

Figure 2. Priming $16A^{hi}$ CD4+ T cells with low doses of antigen generates CD4+ cells producing high levels of IL-4. Purified CD4+ T lymphocytes from TCR-transgenic mice were depleted of previously stimulated cells (cells with a low expression of the CD45RB molecule recognized by mAb 16A) using MACS separation. (*A*) Flow cytometric analysis of CD4+ T cells stained with 16A mAb before (*open*) and after (*shaded*) separation. The purified $16A^{hi}$ cells were then primed in vitro with different doses of pMCC antigen (0.0005–50 μg/ml). Murine rIL-2 was added to each culture (50 U/ml) 24 h after setting up primary stimulations to ensure cell survival, especially at the lower antigen doses. After 4 d of priming and 2 d of rest, the CD4+ T cells from each group were restimulated with 5 μg/ml of antigen for an additional 2 d, and then cytokines were measured in the supernatants sampled from each culture. Units of (*B*) IL-4 and (*C*) IFN-γ (both per 1 ml of culture supernatant) after secondary antigenic stimulation are shown. The LD in these experiments was 1 U/ml IL-4 and 2 U/ml IFN-γ. These data are representative of seven independent experiments.

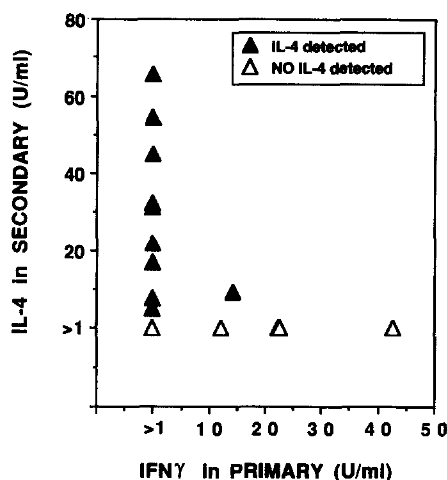

Figure 3. The relationship between IFN-γ in primary cultures and secondary IL-4 production. In a summary of 15 independent experiments examining the role of antigen dose and $16A^{hi}$ $CD4^+$ T cell differentiation, 9 out of 10 experiments in which IL-4 was generated with a low dose of peptide (*closed triangles*), no IFN-γ was detected in primary cultures. In 4 out of 5 experiments where no secondary IL-4 was generated (*open triangles*), significant levels of IFN-γ were detected in primary supernatants.

non-$CD4^+$ T cells) was detected in the primary cultures. Fig. 3 summarizes the results obtained from 15 experiments and illustrates how the presence of very small levels of IFN-γ in primary cultures is sufficient to prevent the generation of Th2-like cells by low doses of antigen.

It might be argued that the Th2-priming effect observed when using low doses of pMCC is caused by the release of small but undetectable quantities of IL-4 during the primary culture that augments Th2 differentiation. To test this possibility, we primed naive $CD4^+$ T cells with either a high (5 μg/ml) or low (0.5 ng/ml) dose of pMCC in the presence of anti–IL-4 antibody. As shown in Table 2, the secondary production of IL-4 after priming with a low dose of antigen was not abrogated by neutralization of endogenous IL-4 during priming. In addition, we observed no significant changes in the levels of IFN-γ production (data not shown). Confirmation that the antibody could deplete IL-4 completely within these in vitro assays was demonstrated by its ability to neutralize IL-4 release in secondary cultures (Table 2). Therefore it seems unlikely that priming for the Th2 cells with low doses of antigen is the result of endogenous IL-4 production. Furthermore, it should be noted that other studies have shown that Th2 priming requires the addition of more than 100 U/ml IL-4 (18).

Thus, these experiments establish that naive $CD4^+$ T cells with a homogeneous receptor respond to different doses of peptide antigen by differentiating into distinct effector cell types. This mechanism may be particularly important in responses to antigens that do not trigger those innate immune responses that are believed to generate the cytokine environment that conditions $CD4^+$ effector T cell differentiation in some infectious diseases. Examples may be inhaled allergens, which arrive via the airways at very low doses; it is estimated that the total dose of ragweed allergen inhaled does not exceed 5 μg per person per year. Our findings that low doses of antigen preferentially activate Th2-like $CD4^+$ T cell responses while avoiding induction of Th1-like cells could explain why such noninfectious, low dose antigens preferentially stimulate IgE antibody responses. By contrast, Th1-like cells are needed predominantly to activate macrophages bearing intracellular pathogens that generally are slow growing and cause little direct pathology. Since these pathogens are concentrated in the intracellular vesicles of infected cells, such macrophages could be expected to develop high levels of peptide from the infecting organism on their surface, stimulating a Th1-like response. Finally, these findings may explain why B cells, which bind and internalize specific antigens to give a very high level of surface peptide–MHC class II complexes, appear to be needed to prime Th1-like responses to soluble protein antigens, but not Th2-like responses to these same antigens (26). While several other studies have shown a role for antigen dose in the type of effector response elicited (27, 28), they concluded that low doses induce delayed-type hypersensitivity and higher doses induce antibody production. These studies, however, involved priming with complex antigens, such that different peptides might be presented at varying densities depending on the antigen dose. Indeed, our findings could explain why mice immunized with complex protein antigens usually generate both Th1- and Th2-like cells specific for the same protein within the same priming environment. Sercarz and co-workers (29) have in fact reported that some peptides of β-galactosidase elicit helper T cells, perhaps Th2 cells, while others elicit proliferating T cells, possibly Th1 cells, as found in the response to human collagen IV (20). Moreover, we have recently reported that the immunodominant peptide of human collagen type IV binds 10,000 times better to the MHC class II molecule, which is associated with priming

Table 2. *Priming of Th2 Cells with a Low Dose of Antigen Is Independent of Endogenous IL-4*

	Secondary IL-4 production (U/ml)			
	Experiment A		Experiment B	
	Anti–IL-4 added in primary		Anti–IL-4 added in secondary	
Priming antigen dose (μg/ml)	No Ab	+ Anti–IL-4	No Ab	+ Anti–IL-4
0.0005	52.8	54.0	65.2	<LD
5	<LD	<LD	<LD	<LD

Naive $16A^{hi}$ $CD4^+$ T cells were stimulated in vitro with either a low (0.0005 μg/ml) or high (5 μg/ml) dose of pMCC for 4 d, rested for 2 d, and then restimulated for an additional 2 d, as described in Fig. 2. Anti–IL-4 antibody was added to cultures during either primary (Experiment A) or secondary (Experiment B) stimulations. The LD for IL-4 in both experiments was 2.8 U/ml. These data are representative of three independent experiments.

Th1-like cells that proliferate and secrete IFN-γ, than to the MHC class II molecules that give rise to T cells that help and produce IL-4 but do not proliferate (30). Thus, antigen dose can play a critical role in determining the differentiation of naive $CD4^+$ T cells, different from and perhaps complimentary to the effects of cytokine environment on this process. It remains to be determined which of these or other effects is critical in various disease states, like AIDS, leishmaniasis, or leprosy, in which one or the other of these subsets dominates the response. How any of these influences produces its effect is likewise uncertain, but the fact that antigen dose can influence $CD4^+$ T cells to differentiate into Th1- or Th2-like cells strongly suggests that differences in signalling from the TCR can affect cytokine gene transcription, offering the potential for pharmacologic manipulation of this process.

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Address correspondence to Kim Bottomly, Section of Immunobiology, Yale University School of Medicine, and Howard Hughes Medical Institute, 310 Cedar Street, LH 408, New Haven, CT 06510.

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