A Role for CD4 in Peripheral T Cell Differentiation

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Summary

Naive CD4 T helper cells (Th) differentiate into one of two well-defined cell types during immune responses. Mature Th1 and Th2 cells regulate the type of response as a consequence of the unique cytokines that they secrete. CD4 serves a prominent role in potentiating antigen recognition by helper T cells. We have examined the role of CD4 in peripheral T cell differentiation by studying helper T cells from mice with a congenital defect in CD4 expression. After protein immunization or infection with Leishmania major, CD4-deficient mice were incapable of mounting antigen-specific Th2 responses, but retained their Th1 potency. CD4-deficient, T cell receptor transgenic T cells were also incapable of Th2 differentiation after in vitro activation. Expression of a wild-type CD4 transgene corrected the Th2 defect of CD4-deficient mice in all immune responses tested. To investigate the role of the cytoplasmic domain, mice reconstituted with a truncated CD4 molecule were also studied. Expression of the tailless CD4 transgene could not rescue the Th2 defect of CD4-deficient mice immunized with protein or CD4-deficient transgenic T cells activated in vitro, raising the possibility that the cytoplasmic domain of CD4 may influence Th2 generation. Expression of the tailless transgene was, however, capable of restoring Th2 development in CD4-deficient mice infected with L. major or CD4-deficient transgenic T cells activated in the presence of recombinant IL-4, demonstrating that the cytoplasmic domain is not absolutely required for Th2 development. Together, these results demonstrate a previously undescribed role of the CD4 molecule. The requirement for CD4 in Th2 maturation reflects the importance of molecules other than cytokines in the control of helper T cell differentiation.

A key functional characteristic of CD4 T cells is their capacity to secrete cytokines when stimulated with peptides presented by MHC class II molecules. During an immune response, a differentiation process gives rise to one of two types of mature helper T cells (see reviews in references 1 and 2). Th1 cells produce IFN-γ and provide help for cell-mediated immunity, whereas Th2 cells produce IL-4 and support the generation of humoral immunity. These two types of helper cells derive from a common naive precursor that has a fate determined in large part by exposure to cytokines. In particular, IL-12 is a potent inducer of the Th1 fate while IL-4 drives cells toward the Th2 fate (1, 2).

Although cytokines have a dominant role in helper T cell differentiation, there is mounting evidence that TCR signaling and costimulation can also influence helper T cell fate (2-4). Of the many cell surface molecules involved in T cell activation, CD4 has a unique capacity to enhance TCR signaling through direct interactions with MHC class II molecules on antigen presenting cells (5) and through its intracellular association with the src-related tyrosine kinase p56
t (6-10). The possible involvement of CD4 in regulating the differentiation of helper T cells has been suggested by several studies (11-13), but remains unclear.

We have studied the functional properties of a unique population of helper T cells that develops in CD4-deficient (CD4
) mice. Earlier studies have demonstrated the capacity of these cells to become Th1 cells (14). To examine the role of CD4 in the full spectrum of peripheral differentiation, we have generated CD4 mice on the Th2-prone BALB/c background and examined their response in several well-characterized models of helper T cell maturation. Under all circumstances tested, we found that helper T cells lacking CD4 are defective in their capacity to adopt the Th2 fate. CD4 mice were bred with mice expressing a

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Abbreviations used in this paper: β2m, β2-microglobulin; CD4, CD4-deficient; CD4
t, CD4 mutant lacking the cytoplasmic tail; CD4wt, wild-type CD4; mRN A, messenger RNA.
transgene encoding either wild-type CD4 or a mutant form of the molecule lacking its cytoplasmic portion (15). While the full-length molecule was capable of reconstituting Th2 development, the tailless molecule was only partially capable of rescuing Th2 maturation. Thus, our results define a novel function for CD4 in the determination of peripheral T cell fate through its selective potentiation of Th2 maturation.

Materials and Methods

CD4-deficient Mice. CD4-/- mice used in these experiments were from the fifth backcross to C57BL (C57BL/6 or B10.D2) or fifth backcross to BALB/c. Heterozygote and wild-type littersmates were used as controls. Mice expressing transgenes encoding a wild-type or truncated CD4 molecule have been previously described (15). CD4+ mice and CD4 transgenic mice were also bred to mice expressing a transgenic TCR specific for a peptide from chicken ovalbumin (OVA) presented by I-A^d (16; provided by Dennis Loh, Washington University, St. Louis, MO). All TCR transgenic experiments were performed using cells from mice of the BALB/c background. Mice were housed in a specific pathogen-free environment. Genotypes were determined by flow cytometry using antibodies for commercially conjugated fluorescent mAbs against CD4 and CD8 (CALTAG Labs., South San Francisco, CA). All work was performed in accordance with University of Chicago (Chicago, IL) guidelines for animal use and care.

KLH Immunization. BALB/c or C57BL mice plus normal littermate controls were immunized in the hind footpads with 150 μg KLH (Calbiochem Corp., San Diego, CA) emulsified in complete Freund's adjuvant (Sigma Chemical Co., St. Louis, MO). To induce T helper subset differentiation in vivo, CD4+ T cells were isolated from spleens and lymph nodes of fifth BALB/c CD4+/+ or CD4-/- or CD4+ T cell receptor transgenic mice (16) by depletion of B220+ and CD8+ cells using mAbs and magnetic beads (PerSeptive Biosystems, Cambridge, MA). Where indicated, CD4-/- mice also expressed a transgene encoding either a full-length or truncated CD4 molecule. An aliquot was stained with mAb KJ1-26 to calculate the percentage of cells expressing clonotypic antigen receptors. 2.5 × 10^5 clonotypic cells were cultured in 1 ml with 2.5 × 10^5 irradiated (2,500 rads) T-deficient splenocytes and 0.3 μg/ml OVA peptide in the presence or absence of IL-4 (20,000 U/ml). After 5 d, cells were washed extensively and 2 × 10^5 cells were restimulated with 8 × 10^6 irradiated splenocytes and 7.5 μg/ml OVA peptide in 200 μl. Supernatants were harvested at 48 h for quantitation of IL-4 and IFN-γ by ELISA.

A nti-CD3 Stimulation of T Cells. CD8-/- cells (>95% pure) were isolated from lymph nodes of B10.D2 CD4+/+ or CD4-/- mice by depletion of B220+, CD8+, NK 1.1+, and MHC class II+ cells using mAbs and magnetic beads. 5 × 10^6 cells were cultured in wells precoated with anti-CD3 (2C11; 10 μg/ml; a gift of Dr. Jeff Bluestone, University of Chicago, Chicago, IL) plus rIL-4 (10,000 U/ml) in 1 ml. After 5 d, cells were washed extensively and 10^6 cells were restimulated in anti-CD3-coated 96-well flat-bottom plates in triplicate for an additional 48 h. Cell number and anti-CD3 concentrations vary where indicated. IL-4 and IFN-γ were quantitated from supernatants by ELISA. For determination of thymidine incorporation, 1 μCi of methyl-[3H]thymidine (American Life Science, Arlington Heights, IL) was added to wells in 25 μl after 48 h. Cultures were harvested 12–14 h later onto filters, and radioactivity was measured (Betaplate 1205 counter; Wallac, Turku, Finland).

Competitive PCR Analysis. 10^6 purified CD8-/- lymph node cells were cultured in the presence of rIL-4 (10,000 U/ml) on plates precoated with anti-CD3 (10 μg/ml). After 48 h, the cells were pelleted and RNA was extracted with Trizol Reagent (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's instructions. RNA was reverse transcribed using random hexamer primers (Pharmacia, Piscataway, NJ). Semi-quantitative PCR was performed as described (14). In brief, CDNA was amplified in the presence of a polycoteterminator construct that contains addition mutations of the authentic cDNA. Resolved on an agarose gel, the larger molecular weight bands provide an internal standard for the relative amounts of the lower molecular weight experimental cDNA. Concentrations of cDNA were adjusted using the housekeeping gene hypoxanthine phosphoribosyltransferase before assay of lymphokine gene transcription. Results were verified by repetition of both individual samples and whole experiments.

Results

Impaired In Vivo Th2 Responses in the Absence of CD4. To induce T helper subset differentiation in vivo, CD4+ mice were immunized with the protein antigen, KLH. Draining lymph node cells were restimulated 7 d later with antigen in vitro, and the cytokine profile was assessed. Cultures from CD4+ animals produced abundant IL-4, as well as moderate amounts of IFN-γ, whereas the cells from CD4-/- mice made the opposite response, producing very little IL-4 and accentuated levels of IFN-γ (Fig. 1 A). The addition of an anti-MHC class II mAb to the cultures blocked all antigen-driven cytokine production, confirming that the responses were indeed mediated by MHC class II-restricted helper T cells (data not shown). Moreover, the CD4-deficient helper T cells manifested the same Th2 defect in the absence of MHC class I expression in doubly deficient CD4(-/-)B220-microglobulin (μ2m)-/- mice (Fig. 1 B). Thus,
Figure 1. The absence of CD4 impairs Th2 differentiation during immunization. (A) BALB/c CD4+ or CD4− mice were immunized with KLH, and draining lymph node cells were harvested 7–10 d later for a re-stimulation assay as described in Materials and Methods. Supernatants were harvested at 48 h for quantitation of IL-4 (filled bars) and IFN-γ (open bars) by ELISA. The results shown are the mean of triplicate determinations from two CD4+ and three CD4− mice, and are representative of 16 separate experiments involving 6 BALB/c CD4+, 9 BALB/c CD4−, 25 C57BL CD4+, and 24 C57BL CD4− mice. Cells cultured without the addition of KLH produced <25 U/ml of IL-4 and 0.5 U/ml of IFN-γ. Standard deviations are expressed as y-axis error bars throughout the figures. (B) β2m-deficient mice (β2m−/−) and double mutant (β2m−/−) were immunized with KLH and restimulated as described above. Results are the mean cytokine measurements of triplicate determinations using three animals in each group.

in vivo Th2 differentiation in response to KLH immunization is dependent on CD4 expression.

To examine T helper cell differentiation in response to chronic infection with a parasite, we challenged mice with L. major. Infected BALB/c CD4+ mice developed progressively, enlarging footpad lesions. In striking contrast, BALB/c CD4− mice developed minimal footpad swelling, which resolved over time and was indistinguishable from genetically resistant controls (Fig. 2 A). Parasite cultures from the feet and spleens of infected mice confirmed that BALB/c CD4+ mice had resolved infection (data not shown). When lymph node cells from infected animals were restimulated in vitro with parasite antigens, the cells from CD4+ mice produced high levels of IL-4 and low levels of IFN-γ, whereas the cells from CD4− mice produced high levels of IFN-γ and minimal IL-4 (Fig. 2 B). The addition of an anti-MHC class II antibody to the cultures blocked all cytokine production, again confirming that class II–restricted helper T cells mediated these effects (Fig. 2 B). Thus, like the response to KLH, L. major infection in BALB/c CD4+ mice is characterized by defective Th2 differentiation.

Impaired In Vitro Th2 Differentiation in the Absence of CD4. The absence of CD4 decreases positive selection in CD4+ mice resulting in a reduced number of helper T cells (14, 17). To correct for this deficiency in cell number, the capacity of CD4-deficient helper T cells to adopt the Th2 fate was examined using in vitro differentiation assays. CD4+ mice were bred to mice expressing a transgenic TCR specific for a peptide from chicken OVA presented by I-A(d) (16). The clonotypic TCR formed by pairing of the transgenic α and β chains is recognized by the mAb KJ1-26 (16). Equal numbers of CD4 lineage (CD8-negative) KJ1-26+ helper T cells were stimulated in vitro with OVA peptide and APCs. After 5 d, cells were washed extensively and restimulated to assess their differentiation state. CD4+ T cells produced high levels of IL-4 and low levels of IFN-γ, whereas CD4− T cells produced greatly reduced levels of IL-4 and accented levels of IFN-γ (Fig. 3). The addition of rIL-4 to primary cultures strongly biases toward Th2 differentiation (1). CD4+ T cells activated in the presence of rIL-4 still yielded only minimal IL-4 production upon restimulation (Fig. 3). Thus, even when responding helper T cell numbers were equalized, the CD4+ cells were still incapable of antigen-driven Th2 differentiation.

As a further test of the intrinsic differentiation capacity of CD4-deficient helper T cells, we established an antigen-MHC–independent system of T cell activation thereby eliminating differences in T cell–APC adhesion of CD4+ and CD4− T cells. Highly purified CD8− helper T cells were stimulated with plate-bound anti-CD3 mAb plus rIL-4 for 5 d. Cells were then washed and restimulated with plate-bound anti-CD3 to assess their maturational state. CD4+ T cells were capable of significant IL-4 and modest IFN-γ production, whereas the T cells from CD4− mice produced high amounts of IFN-γ with negligible IL-4 (Fig. 4 A). We also attempted to augment the efficiency of Th2 maturation or IL-4 secretion by increasing the density of T cells in the primary or secondary cultures, respectively. For CD4+ T cells, increasing cell number in the primary cul-

Figure 2. BALB/c CD4+ mice are resistant to L. major. (A) BALB/c CD4+ (+/+), CD4− (−/−), and C57BL/6 littermates, and genetically resistant C57BL/6 mice were injected with 5 × 106 metacyclic L. major promastigotes in each hind footpad. Disease progression was assessed by measuring footpad thickness with a metric caliper. The results shown are the mean footpad measurements of individual BALB/c mice and are representative of 10 separate experiments using 24 BALB/c CD4+, and 20 BALB/c CD4− mice. (B) Infected BALB/c CD4+/+, CD4+/−, or CD4− mice were killed after 7 wk, and popliteal lymph node cells were cultured with either no addition (M ) or with the addition of 100 μg/ml of soluble parasite antigens in triplicate (A nigen). Wells receiving antigen were cultured without antibody (−) or with either anti-MHC class II (w11) or a control antibody (iso). After 48 h, supernatants were harvested for quantitation of IL-4 (filled bars) and IFN-γ (open bars) by ELISA. The results shown are the mean of triplicate wells from individual mice and are representative of eight experiments using 19 CD4+ and 14 CD4− mice.
ture was sufficient to promote Th2 maturation, even in the absence of rIL-4 (Fig. 4 B). For CD4\(^+\) T cells, even the highest cell number plus the addition of rIL-4 failed to elicit IL-4 production (Fig. 4 B). Moreover, neither the addition of rIL-2 nor co-culture of CD4\(^+\) with CD4\(^-\) T cells could rescue Th2 differentiation in the CD4\(^-\) population (data not shown).

Despite the inability to generate a Th2 response, there was no deficiency in the proliferative response of the CD4\(^-\) T cells (Fig. 4 C). Thus, an inability to proliferate normally does not appear to account for the Th2 defect. Helper T cells initially transcribe multiple lymphokine genes in response to an activation stimulus (18). The addition of rIL-4 to cultures enhances IL-4 messenger RNA (mRNA) and suppresses IFN-\(\gamma\) mRNA within 48 h (18, 19). Competitive reverse transcriptase PCR was performed on highly purified helper T cells that had been stimulated for 48 h with anti-CD3 plus rIL-4. Although the levels of IFN-\(\gamma\) mRNA were higher in the CD4\(^+\) T cells, both populations made equivalent levels of IL-4 mRNA at this early time point (Fig. 5). Furthermore, the viability of unstimulated CD4\(^-\) helper T cells was substantially augmented by the addition of rIL-4 as a survival factor (data not shown). Taken together, these results suggest that the absence of CD4 impairs the intrinsic ability of T cells to commit to the Th2 fate, but does not impede their ability to proliferate, receive signals through the IL-4 receptor, or transcribe the IL-4 gene.

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**Figure 3.** The absence of CD4 impairs antigen-specific Th2 responses in vitro. CD8\(^-\) T cells were isolated from spleens and lymph nodes of CD4\(^+\) or CD4\(^-\) BALB/c D011.10 TCR transgenic mice. An equivalent number of clonotypic cells were cultured in 1 ml with irradiated T-deficient splenocytes and OVA peptide without (left) or with (right) the addition of rIL-4. After 5 d, cells were washed and restimulated with irradiated splenocytes and OVA peptide. Supernatants were harvested at 48 h for quantitation of IL-4 (filled bars) and IFN-\(\gamma\) (open bars) by ELISA. The results shown are the mean of triplicate determinations and are representative of two separate experiments.

**Figure 4.** The absence of CD4 impairs Th2 differentiation when T cells are primed without APCs. (A) Purified CD8\(^-\) T cells were isolated from lymph nodes of CD4\(^+\) or CD4\(^-\) mice, and 5 \(\times\) 10\(^5\) cells were plated in wells precoated with anti-CD3 (10 \(\mu\)g/ml) plus rIL-4. After 5 d, cells were washed extensively and 10\(^5\) cells were restimulated on anti-CD3-coated plates in triplicate for an additional 48 h. IL-4 (filled bars) and IFN-\(\gamma\) (open bars) were quantitated from supernatants by ELISA. The results represent the mean of triplicate determinations and are representative of six separate experiments. (B) Purified helper lineage T cells were primed as described in A, using varying numbers of cells in the primary stimulation (x-axis) and 2 \(\times\) 10\(^5\) cells/ml in the secondary stimulation (B, top), or 2.5 \(\times\) 10\(^6\) cells/ml in the primary stimulation and varying numbers of cells in the secondary stimulation (x-axis; B, bottom). rIL-4 was withheld (B, left) or added (B, right) to the primary cultures where indicated. (C) CD8\(^-\) T cells isolated from lymph nodes of CD4\(^+\) or CD4\(^-\) mice were cultured on plates precoated with the indicated concentration of anti-CD3. After 48 h, \[^3\text{H}^\]thymidine was added, and plates were harvested 12-14 h later. The mean of triplicate wells was used to determine the percent maximum thymidine incorporation for each group.
Effect of CD4 Transgenes on Th2 Development. The association of CD4 with p56\textsuperscript{ck} has been implicated in signaling and in the recruitment of CD4 to the TCR complex (20, 21). To study the role of this association in the potentiation of the Th2 fate, we used transgenic mice expressing wild-type CD4 (CD4\textsuperscript{WT}) or a mutant lacking the cytoplasmic tail (CD4\textsuperscript{Δcyt}). Both of these transgenes are capable of rescuing helper T cell development in CD4\textsuperscript{−/−} mice, although overexpression of the tailless version is required for this effect (15). CD4\textsuperscript{WT} and CD4\textsuperscript{Δcyt} transgenic mice were backcrossed onto the BALB/c background and then intercrossed with CD4\textsuperscript{−/−} mice expressing the OVA-specific transgenic TCR. The CD4WT transgene corrected the Th2 defect when expressed in CD4\textsuperscript{−/−} OVA-specific T cells, but the CD4Δcyt transgene was ineffective in promoting the Th2 fate (Fig. 6). When exogenous IL-4 was added to the primary culture, however, either transgene was capable of reconstituting Th2 induction (Fig. 6). Similar results were observed in vivo. The CD4\textsuperscript{WT} transgene rescued the Th2 response to KLH immunization, whereas the CD4\textsuperscript{Δcyt} transgene did not (Fig. 7). Both types of transgenes, however, restored susceptibility (Fig. 8 A) and Th2 responses (Fig. 8 B) to leishmaniasis in BALB/c CD4\textsuperscript{−/−} mice. Thus, the Th2 defect caused by a null mutation in the CD4 gene can be fully corrected by ectopic expression of wild-type CD4, and partially corrected by a mutant form that lacks the cytoplasmic domain.

Discussion

The results presented in this paper suggest that the function of CD4 influences the outcome of helper T cell differentiation. CD4\textsuperscript{−/−} helper T cells exhibited a consistent bias toward the Th1 fate in several types of immune responses, even under optimal in vitro conditions of cell number and in the presence of exogenous IL-4. Furthermore, the defect in Th2 differentiation was observed for CD4\textsuperscript{−/−} mice on both the Th2-prone BALB/c background and for mice on the C57BL/6 background. We cannot fully exclude the possibility that CD4\textsuperscript{−/−} T cells exit the thymus with an incomplete helper potential. However, CD4\textsuperscript{−/−} T cells were capable of proliferating and generating IL-4 mRNA early in the response to TCR ligation, showing that the absence of CD4 does not impair these common aspects of T helper cell activation. Rather, the results of in vitro assays suggest that the lack of CD4 results in an intrinsic defect in the capacity of helper T cells to generate signals for proper Th2 differentiation. Thus, the results support a novel role for CD4 in the potentiation of the Th2 fate during immune responses.

In an effort to explore the mechanism by which CD4 influences the fate of T helper cells, we used two previously described lines of transgenic mice that express either CD4\textsuperscript{WT}, or CD4\textsuperscript{Δcyt} (15). Th2 differentiation was fully restored by expression of the former molecule, but only partially by the latter. For example, Th2 responses to KLH required the presence of the cytoplasmic domain of CD4, but Th2-dependent susceptibility to Leishmania did not. The partial rescue of Th2 differentiation mediated by the tailless molecule indicates that the cytoplasmic domain of CD4 can be dispensable for Th2 differentiation under certain circumstances. This result implies that Th2 cells can be formed in the absence of any unique signaling pathway that initiates exclusively at the CD4 cytoplasmic domain. Thus, it is possible that the primary Th2-promoting effect of the tailless molecule is to stabilize the TCR engagement of pep-
The cytoplasmic domain of CD4 is not essential for susceptibility or Th2 responses after L. major infection. (A) BALB/c CD4 WT and CD4 Δ4 transgene-reconstituted mice (CD4 Δ4 WT, CD4 Δ4 WT Δ4 WT) were infected with L. major as described in Materials and Methods. The results shown are the mean footpad measurements of two animals each and are representative of five separate experiments using 9 CD4 Δ4 WT and 11 CD4 Δ4 Δ4 WT mice. (B) Poptidal lymph node cells from infected BALB/c CD4 WT and CD4 Δ4 transgene-reconstituted mice (WT, Δ4 WT) were analyzed for parasite-specific cytokine secretion after restimulation in vitro. The results shown are the mean of triplicate determinations from individual mice, and are representative of four separate experiments using eight CD4 WT and nine CD4 Δ4 WT mice.

Figure 8. The cytoplasmic domain of CD4 is not essential for susceptibility or Th2 responses after L. major infection. (A) BALB/c CD4 WT and CD4 Δ4 transgene-reconstituted mice (CD4 Δ4 WT, CD4 Δ4 WT Δ4 WT) were infected with L. major as described in Materials and Methods. The results shown are the mean footpad measurements of groups of two animals each and are representative of five separate experiments using 9 CD4 Δ4 WT and 11 CD4 Δ4 Δ4 WT mice. (B) Poptidal lymph node cells from infected BALB/c CD4 WT and CD4 Δ4 transgene-reconstituted mice (WT, Δ4 WT) were analyzed for parasite-specific cytokine secretion after restimulation in vitro. The results shown are the mean of triplicate determinations from individual mice, and are representative of four separate experiments using eight CD4 WT and nine CD4 Δ4 WT mice.
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