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*J Immunol* 1998; 161:1194-1203;
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CD4 Regulation of TCR Signaling and T Cell Differentiation Following Stimulation with Peptides of Different Affinities for the TCR

David Leitenberg,*† Yvan Boutin,∗ Stephanie Constant* and Kim Bottomly²,∗

To define the role of CD4 in modulating T cell signaling pathways and regulating Th1 and Th2 differentiation, we have examined the activation and differentiation characteristics of naive T cells from CD4 mutant mice. Using peptides with differing affinities for the moth cytochrome c-specific TCR, we test the hypothesis that differences in coreceptor recruitment and signaling explain the qualitatively distinct signaling pathways seen in CD4 T cells following high affinity agonist and low affinity altered peptide ligand (APL) ligation. We find that the absence of CD4 signaling during stimulation with a strong agonist peptide does not qualitatively change the pattern of early TCR-mediated biochemical signaling events into a pattern resembling the response of CD4⁺ T cells to APLs. In contrast, the response to APL stimulation, by T cells bearing the same TCR, does require a component of CD4 signaling. The proliferative response and calcium signals normally seen following APL stimulation are markedly diminished in the absence of CD4. In addition, we find that naive T cell differentiation into Th2 effector cells is impaired in the absence of CD4. These data suggest that the altered pattern of biochemical signals generated by APLs require CD4 coreceptor function and that some of these signals may be required to initiate Th2 differentiation. The Journal of Immunology, 1998, 161: 1194–1203.

Upon recognition of MHC class II-peptide complexes on the surface of APCs, CD4⁺ T cells are induced to proliferate and can differentiate into distinct effector cell subsets. These late activation events are correlated with a cascade of biochemical signaling pathways initiated by TCR ligation (1, 2). The earliest biochemical signaling event after TCR stimulation is tyrosine phosphorylation of the immune receptor tyrosine-based activation motif (ITAM) in the CD3ζ components associated with the TCR, which is thought to be mediated by the src family tyrosine kinases lck and/or fyn. Following tyrosine phosphorylation of the CD3ζ components, a second protein tyrosine kinase associated with the TCR complex, ZAP70, is activated, which in conjunction with a series of adapter proteins can activate the phosphatidylinositol second messenger pathway resulting in protein kinase C activation and the mobilization of intracellular calcium stores. The regulation of these early biochemical signaling events generated following TCR/CD3 stimulation has been characterized in various models of T cell activation using long term T cell cloned lines or T cell leukemic lines primarily in relation to IL-2 gene transcription and proliferation. However, relatively little is known about the regulation of early signaling events generated by peptide stimulation of naive T cells, and the relation of these events to T cell differentiation into Th1 and Th2 effector cell subsets.

Several groups of investigators have shown in T cell cloned lines that the initial TCR-mediated signaling pathways can be modified by stimulating T cells using peptides with low affinity (having high dissociation rates) for a particular TCR (3). These peptides, termed altered peptide ligands (APL),³ can be generated by substituting specific amino acid residues of an agonist peptide at the TCR contact area, and can induce a distinct signaling pattern compared with stimulation with agonist peptides: there is an altered pattern of ζ phosphorylation, absent ZAP70 phosphorylation, and low amplitude, transient calcium mobilization (4–6). These signaling events generally do not result in T cell proliferation and IL-2 production, but correlate with other possible outcomes of T cell activation including antagonist function and IL-4 secretion in T cell cloned lines (7, 8) and, in naive T cells, with differentiation into Th2 effector cells (9). Because these variant peptides induce different outcomes of T cell activation and because the altered pattern of signals cannot be mimicked by low doses of agonist peptide (3–5, 9), it has been proposed that the signaling events associated with variant peptides with a low affinity for the TCR are qualitatively different compared with the events induced by agonist peptides. However, the molecular basis for the different signaling patterns induced by these variant peptides is not well understood. Furthermore, it remains unclear how these different signals are interpreted by the T cell to result in different outcomes of activation. It has been demonstrated in several model systems, in vivo and in vitro, that providing a weak TCR-mediated signal can preferentially generate IL-4-producing cells and Th2 differentiation (10–18). These data suggest that the pattern of signaling events induced by low affinity peptides is sufficient to induce Th2 differentiation; however, it remains uncertain whether these events are necessary or whether the full signaling pattern seen following agonist peptide stimulation simply inhibits Th2 differentiation.

One hypothesis that has been proposed to explain the different signaling patterns seen following APL stimulation is that CD4 and the associated src family tyrosine kinase, lck, are not effectively

²Abbreviations used in this paper: APL, altered peptide ligand; MCC, moth cytochrome c. peptide (e.g., pMCC); CD4⁻/−/CD4 transgene lacking the cytoplasmic tail of CD4.

³This work was supported by National Institutes of Health Grants AI26791 (K.B.) and AB01314 (D.L.).
recruited to the TCR complex, either because of a failure in the formation of TCR/CD4 oligomeric complexes or due to a change in TCR conformation that prevents or disrupts effective TCR/CD4 association (3, 19, 20). Failure to recruit and/or activate lck to the TCR complex could then result in a truncated pattern of TCR ζ-chain phosphorylation and a failure to phosphorylate ZAP70. In support of this hypothesis are recent experiments by Madrenas and coworkers using T cell cloned lines. These experiments indicate that prevention of CD4 interaction with MHC class II by using anti-CD4 mAbs or MHC class II mutants which cannot bind CD4 results in conversion of an agonist signaling phenotype and a proliferative response into an APL signaling phenotype, failure to proliferate, and anergy (21). These data indicate that in fully differentiated T cell cloned lines, the failure to recruit CD4/ltk to the TCR complex can modulate TCR signaling pathways.

However, since CD4 is expressed normally in these experiments, the role of CD4 signaling apart from the TCR complex remains uncertain. Furthermore, although the above-referenced data suggest that in the absence of CD4 recruitment to the TCR, the response to a high affinity peptide can resemble the response to an APL, the role of CD4 during low affinity, APL-induced T cell stimulation was not determined. Indeed, data from several different systems suggest that peptides with a poor affinity for the TCR may be more dependent on the presence of a coreceptor to stabilize TCR/MHC class II interactions and to enhance the efficiency of TCR signaling (22, 23). If this is the case, then APL signaling and subsequent Th2 differentiation may be abrogated in the absence of CD4. Indeed, recent studies using CD4-deficient mice have suggested that CD4 expression on peripheral T cells is required for the generation of a Th2 effector response in vivo and in vitro (24, 25). However, changes in TCR signaling pathways were not examined in these studies. Thus, the mechanism for the CD4 requirement to induce a Th2 effector response remains unclear.

Since we had previously shown that stimulation with APLs is associated with Th2 differentiation (17), we have now examined the requirement for CD4 signal transduction following activation with peptides of different affinity and analyzed the role of CD4 in early signaling events, in T cell proliferation, and in priming of naive T cells to differentiate into Th1 and Th2 subsets. To do this, we bred mice with a transgenic TCR specific for moth cytochrome c onto CD4-deficient mice or CD4-deficient mice reconstituted with a CD4 transgene lacking the cytoplasmic tail of CD4 (CD4αcyt). We then stimulated CD8− TCR transgenic T cells isolated from these mice with peptides of varying affinity and evaluated the effect of CD4 and CD4-associated lck on early signaling events in naive T cells and on subsequent differentiation into Th1 and Th2 effector cell populations. These experiments showed that, although signaling with agonist peptide in the absence of functional CD4 leads to a moderate reduction in responsiveness as measured by proliferation, it does not lead to any qualitatively distinct changes in the biochemical signaling profile (ζ phosphorylation, ZAP70 phosphorylation, and calcium mobilization) similar to that induced by APLs. In contrast, T cell responses to APL stimulation were severely compromised in the absence of CD4 coreceptor function as measured by loss of calcium mobilization and a marked decrease in proliferation. In addition, we found that in the absence of CD4 coreceptor function, differentiation of naive T cells into Th2 cells was severely impaired, while Th1 differentiation remained unchanged. These experiments show that a component of CD4 signaling is required for the differentiation of CD4 T cells into Th2 cells.

Materials and Methods

Mice

B10.A (5R) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The TCR transgenic mice in which CD4+ T cells express a TCR specific for the carboxyl terminus of pigeon cytochrome c have been previously described (26) and are housed in our facilities and maintained as heterozygotes on a B10.A (5R), B10.BR, or C57BL/6 background. The CD4−/− and CD4+/− B6 mice were generously provided by D. Littman (New York University, New York, NY) and were originally on a B6 background (27). These mice were backcrossed four to six times on to a B10.BR background, and then heterozygote CD4−/+ or CD4+/+ B6 mice were crossed to obtain homozygous CD4−/− mice. All mice used in these studies were 6 to 10 wk old.

Pepitides

Moth cytochrome c (MCC; peptide 81–103), pMCC = VFAGLKKANE RADLIALYQATK, K99R = VFAGLKKANERADLIALYLRQATK, and pBSA (peptide 141–154) = GKYLIEARHPYF were synthesized by the W. M. Keck Foundation Biotechnology Resource Laboratory (New Haven, CT). All peptides were purified by HPLC before use.

Preparation of APC and CD4+ T cells

T cell-depleted APC were prepared by Ab-mediated complement lysis of 5R splenocytes as previously described (28). The APC were treated with 50 μg/ml of mitomycin C (Boehringer Mannheim Biochemicals, Indianapolis, IN) before use. CD4+ CD8− T cells from lymph nodes and spleens of transgenic mice were isolated by immunomagnetic negative selection (28) using Abs against CD8, CD32/CD16, B220, and MHC class II, followed by incubation with anti-mouse and anti-rat Ig-coated magnetic beads (PerSeptive Biosystems, Framingham, MA). Purity of the recovered Va11+, CD4+ T cells (or Va11+, CD4+ T cells in CD4−/− mice) was usually 85 to 95% as determined by staining with anti-CD4 and anti-Va11 mAb.

Flow cytometric analysis

Blood leukocytes were labeled with mAbs to Va11 (PharMingen, San Diego, CA), CD8α (Life Technologies, Gaithersburg, MD), and CD4 (Sigma, St. Louis, MO), conjugated with FITC, phycoerythrin, and Red 670, respectively, after water lysis. The cells were labeled with saturating amounts of the indicated Abs diluted in PBS supplemented with 5% FCS/0.02% sodium azide and 1 mg/ml mouse Ig and incubated on ice for 30 min. Fluorescence analysis was done using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) and the data analyzed using the LYSYS 2 software package (Becton Dickinson).

Proliferation assays

Proliferation of Ag-stimulated T cells was determined as previously described (29). Briefly, 105 Va11+, CD8− T cells were cultured in triplicate in 96-well flat-bottom microtiter plates and stimulated with different doses of peptide in the presence of 105 mitomycin C-treated APC. After 3 days, the cells were pulsed with 1 μCi/well of [3H]thymidine, harvested 18 to 24 h later, and the level of incorporation determined by scintillation counting.

In vitro differentiation of naive T cells

Induction of naive T cell differentiation was performed as previously described (17) with slight modifications. Briefly, mitomycin C-treated, T cell-depleted splenocytes (2 × 105 cell/ml) were incubated with 20 μM of agonist peptide or APL for 2 h at 37°C. T cells (5 × 105/ml) from TCR-transgenic mice were then cocultured with peptide-primed APC (1 × 106/ml) and rIL-2 (25 U/ml). After 4 days of priming, T cells were harvested and dead cells were removed by gradient centrifugation. Viable T cells were then incubated for a rest period of 2 days with fresh APC only. For the secondary culture, rested T cells (5 × 105/ml) were restimulated with pMCC (2 μM) and fresh APC (5 × 105/ml) for an additional period of 2 days. The presence of IL-4 and IFN-γ in the supernatants of primary and secondary cultures was determined using ELISA kits from Endogen (Cambridge, MA). The supernatants were diluted serially in duplicate and the concentration of cytokine determined in relation to a reference standard supplied by the manufacturer.

Protein biochemistry and immunoprecipitation

CD4+ T cells (5 × 106) were added to peptide-primed APC (5 × 105) and incubated at 37°C for indicated times. At the indicated time points, cells
Table I. Summary of peptide regulation of naive CD4 T cell activation and differentiation

<table>
<thead>
<tr>
<th></th>
<th>High Affinity Peptide (agonist)</th>
<th>Low Affinity Peptide (APL)</th>
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<tbody>
<tr>
<td>TCR-ζ phosphorylation</td>
<td>Full</td>
<td>Partial (transient)</td>
</tr>
<tr>
<td>ZAP70 phosphorylation</td>
<td>Present</td>
<td>Absent</td>
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<tr>
<td>Calcium mobilization pattern</td>
<td>Sustained</td>
<td>Transient</td>
</tr>
<tr>
<td>Proliferation</td>
<td>Strong</td>
<td>Weak</td>
</tr>
<tr>
<td>Th2 cell differentiation</td>
<td>Absent</td>
<td>Present</td>
</tr>
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</table>

* Data are summarized from Boutin et al. (9).

were harvested and lysed in ice-cold lysis buffer (20 mM Tris, pH 7.2, 1% Nonidet P-40, 150 mM NaCl, 1 mM MgCl₂, 1 mM EGTA) containing protease and phosphatase inhibitors (10 mM NaN₃, 10 mM NaF, 1 mM Na₂VO₃, 50 mM NaF, 1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin), and nuclear material was removed as previously described (30). Cell lysates were incubated for 1.5 h with protein A-Sepharose CL-4B beads (Pharmacia Biotech, Piscataway, NJ) that had been pretreated with anti-ζ-chain or ZAP70 rabbit antisera prepared in our laboratory. Immunoprecipitates were washed four times and analyzed for tyrosine phosphorylation by Western blot. Phosphotyrosine-containing proteins were detected by blotting with anti-phosphotyrosine mAb (4G10; Upstate Biotechnology, Lake Placid, NY) followed by goat anti-mouse IgG horse-radish peroxidase conjugate (Bio-Rad, Hercules, CA) and detected by ECL as described by the manufacturer (Amersham, Buckinghamshire, England). Densitometric analysis was determined using a digital imaging system, model IS-1000 (α Innotech, San Leandro, CA).

Calcium mobilization

Calcium signaling following Ag-specific stimulation was monitored as described previously (31). Briefly, CD4⁺ T cells loaded with 5 μM fluo-3/AM ester (Molecular Probes, Eugene, OR) were plated by centrifugation in 96-well plates at a concentration of 5 × 10⁵ cells/50 μl. The cells were then scanned using the ACAS 570 video laser cytometer (Meridian Instruments, Okemos, IL). After initiation of scanning, 4 × 10⁶ T-depleted splenocytes pulsed with 20 μM of peptide were added to the CD4⁺ T cells. The initial average fluorescence of each cell was digitized and normalized to 1, and the results are expressed as changes in normalized fluorescence intensity of individual cells over time. The percentage of responding cells was determined by dividing the number of cells demonstrating an increase in intracellular calcium of >50% by the total number of scanned cells.

Results

Summary of model system used in these studies

We have previously shown in several systems that varying the signal strength of an antigenic peptide during the initial priming of naive CD4 T cells can regulate the development of Th1 and Th2 effector cell populations (11–13, 17, 32). To investigate the molecular basis for this result, we have recently developed an in vitro model of T cell differentiation in which naive CD4 T cells expressing a transgenic TCR specific for MCC are induced to differentiate into Th1 and/or Th2 cells depending upon the nature of the T cell ligand used during priming. In this system, naive MCC-specific CD4 T cells are primed with peptides that have differing affinities for the TCR while maintaining similar association for MHC class II, and then all groups are restimulated with a high affinity (agonist) peptide and assessed for differences in T cell effector populations induced by the peptide used during priming. Using this system, we can analyze the early signaling events that occur during priming and correlate them with the subsequent differentiation of the T cells into different effector subsets (results summarized in Table I). The low affinity peptide (or APL) used in most of these experiments has a substitution of arginine for lysine at position 99 (K99R) and is a characteristic APL in that it has a diminished affinity for the cytochrome c-specific TCR with a high dissociation rate (33) and, in naive T cells, induces an altered pattern of T cell signaling events similar to that seen following APL signaling in T cell cloned lines (3–5). Most interestingly, we have shown that this peptide can induce the differentiation of naive CD4 T cells into Th2 effector cells as compared with stimulation with the agonist peptide, which can only induce Th1 differentiation (17). In the present experiments, we tested whether CD4 coreceptor signaling was required for these differential effects.

Generation of CD4 mutant TCR transgenic mice

To investigate the role of CD4 in naive T cell signaling and differentiation, we bred mice transgenic for the MCC-specific TCR with CD4-deficient mice and subsequently with mice expressing a transgene for CD4 with a deletion of the cytoplasmic tail (CD4Δcyt), originally described by Killeen and Littman (27). It has previously been shown that the CD4Δcyt transgene encoding the full ectodomain of CD4, but lacking the cytoplasmic domain, can significantly restore selection of functional CD4⁺CD8⁺ MHC class II-restricted T cells in CD4 knock-out mice (27), and it appears to be a useful model for examining the role of CD4-associated signaling molecules during peripheral T cell activation. The
MCC-specific TCR transgene used in these experiments is positively selected into the CD4 lineage in mice that express either I-A<sup>b</sup> and I-E<sup>k</sup> (26, 34). Although initial experiments were performed using C57/BL6 (I-A<sup>b</sup>) mice, most of the following data are derived from I-E<sup>k</sup>-expressing mice, which select the MCC-specific TCR more efficiently in the absence of CD4 signaling (data not shown) (34). The results were similar regardless of the mouse strain, although the yield of purified MCC-specific TCR<sup>+</sup> CD8<sup>-</sup> cells from spleen and lymph nodes is better when B10.BR mice are used. As shown in Figure 1, A and B, selection of “CD4<sup>-</sup> lineage” MCC-specific cells (Vα11<sup>+</sup> CD8<sup>-</sup>) is relatively efficient in the CD4-deficient and CD4<del>cyt</del> mice and is ~30 to 50% of the level seen in CD4 wild-type mice. Furthermore, as shown in Figure 1A, the expression level of the CD4<del>cyt</del> transgene is similar to the expression of wild-type CD4.

These data show that selection of T cells bearing an MHC class II restricted receptor can occur in the absence of functional CD4 and allows the examination of the role of CD4 in regulating peripheral T cell responses. Since we have previously shown that stimulation with APLs is associated with Th2 differentiation, we originally hypothesized that T cells from mice that lacked CD4 or lacked the cytoplasmic domain necessary for CD4 signaling function would receive an impaired signal following Ag stimulation similar to that seen following stimulation with APLs and would preferentially undergo Th2 differentiation. To test this hypothesis and to characterize differences in TCR signaling pathways that were dependent on CD4, we undertook a series of experiments using naive T cells from TCR transgenic mice that lacked either CD4 or the cytoplasmic domain necessary for CD4 signaling function; we examined their activation characteristics in proliferation assays and in biochemical assays designed to measure early signaling events.

**CD4 regulation of T cell proliferation following stimulation with peptides with different affinities for the TCR**

Initial experiments were performed to assess the proliferative capacity of purified CD8<sup>-</sup> Vα11<sup>+</sup> CD8<sup>-</sup> T cells isolated from CD4<del>cyt</del>, CD4-deficient, and wild-type cytochrome c-specific-TCR mice following high (agonist) and low affinity (APL) peptide stimulation. In this way, we could determine the relative dependence of the MCC-specific TCR transgenic T cells on CD4 for a late event of T cell activation and compare two peptides with differing affinities for the TCR, which have known differences in their ability to induce proliferation (17). As expected, in T cells from the two CD4 mutant mice, proliferation to the agonist peptide (pMCC) was diminished compared with T cells from CD4<sup>+</sup> wild-type mice (Fig. 2A), although it remained significantly above the level of proliferation induced by the APL (K99R) in T cells from CD4<sup>+</sup> wild-type mice (Fig. 2B, note different scale). As shown in Figure 2B, when the APL (K99R) was used to stimulate wild-type and CD4 mutant TCR transgenic CD4 T cells, Ag-specific T cell proliferation was completely lost in the absence of CD4 and in the absence of the cytoplasmic portion of CD4 (CD4<del>cyt</del> mutant).

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**FIGURE 2.** Proliferation of CD4 mutant T cells following stimulation with agonist and APL. TCR transgenic Vα11<sup>+</sup> CD8<sup>-</sup> T cells were stimulated in triplicate with APC in the presence of different doses of the indicated peptide for 3 days, then pulsed with [3H]thymidine and harvested; thymidine incorporation was determined by scintillation counting. A and B are representative experiments of agonist (pMCC)- and APL (K99R)-induced proliferation, while C and D represent the same experiments performed in the presence of 25 U/ml IL-2.
The addition of exogenous IL-2 to the cultures revealed further differences in the proliferative characteristics of T cells from wild-type and CD4 mutant mice. When IL-2 was added to the T cells stimulated with agonist peptide, T cell proliferation was similar regardless of CD4 expression (Fig. 2C). However, in contrast to agonist peptide stimulation, proliferation in response to the APL (K99R) remained impaired in the CD4 mutant mice despite the addition of exogenous IL-2 (Fig. 2D). Although proliferation was reproducibly observed in the T cells from the CD4&cyt mice in the presence of IL-2, it remained significantly below the level of proliferation observed in T cells from CD4 wild-type mice. Furthermore, no proliferation in response to K99R was observed in T cells from CD4-deficient mice. In total, these data suggest that the requirement for CD4 is more stringent when a peptide with lower affinity for the TCR is used. This is supported by the observation that the specific induction of CD69 expression was markedly reduced 24 h after stimulation with K99R in the CD4 mutant T cells compared with the CD4 wild-type cells (data not shown).

**CD4 regulation of early signaling events following stimulation with peptides of different affinities for the TCR**

We next examined the role of CD4 coreceptor function on three peptide-induced signaling events (ZAP70 phosphorylation, calcium mobilization, and CD3/ζ phosphorylation) that are known to differ depending on the affinity of the peptide for the TCR and have been shown to correlate with different outcomes of T cell activation. In these experiments, we asked whether the early biochemical signaling events initiated upon CD4 T cell stimulation with high affinity peptides required CD4 signaling function and whether, in the absence of CD4 signaling, these events would resemble stimulation with an APL. Furthermore, these experiments examined changes in the relative dependence of T cell signaling pathways on CD4, based on varying the affinity of the peptide for the TCR.

Since ZAP70 phosphorylation is induced in wild-type CD4 T cells following stimulation with the high affinity peptide (pMCC), but not after stimulation with an APL (K99R), we asked whether CD4-associated signaling function was required for ZAP70 phosphorylation following stimulation with the high affinity peptide. As shown in Figure 3B, agonist peptide-induced ZAP70 phosphorylation is detected in T cells isolated from wild-type mice and mice with the CD4 cytoplasmic deletion mutant. Similar results are seen in T cells from CD4-deficient mice (data not shown; Fig. 5). Although the level of ZAP70 phosphorylation is somewhat reduced in this experiment compared with that seen upon stimulation of T cells from wild-type CD4 mice, it is readily detectable compared with the absence of ZAP70 phosphorylation seen following stimulation with the APL (Fig. 3A). These data suggest that CD4 signaling ability is not required for ZAP70 phosphorylation and that the absence of CD4 recruitment does not necessarily result in the signaling phenotype seen following APL stimulation.

Consistent with the ability to induce ZAP70 phosphorylation, the calcium mobilization pattern of T cells isolated from CD4 mutant mice was also intact following high affinity (agonist) peptide stimulation. Similar numbers of cells were induced to flux calcium regardless of their CD4 phenotype (Fig. 4, A and B). In addition, there was no significant change in the pattern of calcium mobilization: most of the responding cells exhibited a strong, sustained pattern of calcium flux induced with the agonist peptide (Fig. 4B). This is in marked contrast to the pattern of calcium flux seen following stimulation with an APL, which induces a small amplitude, transient calcium flux in the majority of responding cells (9) (Fig. 4, C and D). When T cells from the CD4 mutant mice were stimulated with APC pulsed with the APL, calcium mobilization was reduced to near background levels compared with T cells from normal control mice (Fig. 4, C and D).

In summary, the calcium mobilization data indicate that CD4 signaling is not required to generate the sustained pattern of signaling typical of agonist peptide stimulation, and the absence of CD4 does not result in a calcium pattern similar to that induced by APLs. In contrast to agonist peptide-induced calcium flux, the pattern of calcium flux induced by APLs for the TCR is lost in the absence of CD4. This finding is similar to the data from the proliferation assays and suggests that TCR signaling in response to APLs is more dependent on coreceptor signaling than signaling with high affinity peptides.

However, some signaling events in response to stimulation with APLs did occur in the absence of CD4, as seen by ζ-chain phosphorylation. When T cells from wild-type and CD4 mutant mice were stimulated with either agonist or APL, the pattern of p18 and p21 ζ phosphorylation was essentially unchanged in the T cells from CD4 mutant mice compared with the wild-type mice (Fig. 5, A and B). In cells from all three mouse groups, agonist peptide stimulation induced strong phosphorylation of the p21 phospho-ζ band, with concomitant phosphorylation of ZAP70, while stimulation with the APL characteristically induced less p21 phosphorylation relative to the p18 isof orm and failed to induce association with phosphorylated ZAP70. Densitometric analysis revealed a similar ratio of p18:p21 after stimulation with K99R in both wild-type mice and CD4 mutant mice that was approximately half that induced with the agonist peptide (Fig. 5B). These data indicate that in the absence of CD4 signaling, APLs can stimulate early signaling events such as ζ-chain phosphorylation and that the pattern of
this phosphorylation is not changed. However, the altered pattern of \( \xi \) phosphorylation induced by the APL is not sufficient to initiate the transient calcium pattern characteristic of APL signaling.

In summary, the signaling data indicate that agonist peptide signaling in the MCC-specific TCR transgenic mice is qualitatively unchanged in the presence or absence of CD4 signaling function. Although early proliferation was somewhat diminished in the CD4 mutant mice, early signaling events remained intact and did not resemble the signaling pattern seen following stimulation with APLs. In contrast, signaling events following APL stimulation were qualitatively changed in cells lacking CD4 signaling capacity, as exhibited by the failure to induce significant calcium flux, which correlates with a marked reduction in the ability to proliferate to the APL even in the presence of exogenous IL-2.

**Th1 and Th2 differentiation following agonist and APL stimulation**

Although the signaling phenotype of the CD4 mutant T cells after agonist stimulation was not qualitatively different from wild-type CD4 T cells and did not resemble APL-induced signaling, the decrease in proliferation in both the CD4-deficient and CD4\( \delta\text{-cyt} \) mice suggested that the signal received by the TCR in these mice was quantitatively diminished due to the lack of CD4-associated signaling capacity. To determine whether this would affect differentiation into Th1 and Th2 effector subsets, naïve CD4 T cells were primed with either agonist or altered peptide in the presence of exogenous IL-2, allowed to differentiate for 4 to 6 days, then re-stimulated with agonist peptide and assayed for the ability to produce Th1 or Th2 effector cytokines. As we have previously shown, stimulation with agonist peptide primes the cells to secrete IFN-\( \gamma \), but not IL-4, while stimulation with the APL, K99R (with decreased affinity for the TCR), primes the cells to produce IL-4 in addition to IFN-\( \gamma \) upon secondary stimulation (Fig. 6). However, in contrast to CD4 T cells from wild-type mice, when T cells from the CD4\( \delta\text{-cyt} \) mice were primed with the APL, Th2 differentiation was not induced, although, interestingly, IFN-\( \gamma \) production was not affected and was similar to that seen in T cells from CD4 wild-type mice. No defect in the ability of the agonist or APL to prime for IFN-\( \gamma \) production was observed in the absence of CD4 signaling (Fig. 6A). Furthermore, despite the decrease in T cell proliferation, there was no significant production of IL-4 following priming with the high affinity peptide (Fig. 6B). Since the CD4-deficient cells...
did not significantly proliferate upon stimulation with K99R, we
could not reproducibly examine these mice in the double culture
system because insufficient cells were available after primary
stimulation.

These data suggest that CD4 signaling is required for Th2 dif-
ferentiation and that the altered pattern of early signaling events
seen during APL stimulation, which is disrupted in the CD4 mu-
tant mice, may be important in priming for IL-4 production. Fur-
thermore, although we have previously correlated weak signaling
with the TCR with subsequent Th2 differentiation, these data show
that weak signaling by itself is not sufficient for Th2 differentia-
tion, since APL in the absence of CD4 failed to induce Th2 dif-
ferentiation. Rather, there appears to be distinct signaling require-
ments to initiate Th2 differentiation, which are not generated in
the absence of CD4. Interestingly, Th1 differentiation is maintained
despite the absence of CD4 signaling, even upon stimulation with
APLs, suggesting that the induction of Th1 differentiation does not
require a full TCR-mediated signaling pattern.

Effect of exogenous IL-4 on Th2 differentiation in CD4 mutant
mice

We next determined whether the addition of exogenous IL-4 could
overcome the failure in Th2 differentiation seen in cells defective
in CD4 signaling. In this way, we could determine whether the
inability of CD4 mutant T cells to differentiate into Th2 cells is due
to their failure to produce sufficient IL-4 during the primary culture
or if it is due to a general failure of these cells to respond to IL-4
and undergo Th2 differentiation. As shown in Figure 7, when exo-
genous IL-4 is added to T cells from CD4Δcyt mice primed with
the APL (K99R), Th2 differentiation is significantly restored. This
suggests that the signaling failure seen in CD4 mutant T cells after
stimulation with an APL results in part from a failure to produce
sufficient endogenous IL-4 required for Th2 differentiation nor-
mally seen following APL priming (17).

It should be noted, however, that the T cells from CD4 mutant
mice also have a surprising defect in the ability to differentiate in
response to exogenous IL-4 compared with cells with wild-type
CD4. When CD4 mutant T cells and wild-type controls are primed with either high or low affinity peptides in the presence of exogenous IL-4 and tested for the subsequent ability to differentiate into Th2 effector cells, T cells from CD4 mutant mice produce approximately five- to sixfold less IL-4 than cells with wild-type CD4 (Fig. 8A). These data suggest that signaling through the IL-4R may be modulated by the CD4 coreceptor. It appears that some signaling through the IL-4R is intact, however, since IL-4 is able to suppress Th1 differentiation, as shown by the decrease in IFN-γ production in T cells from the CD4 mutant mice (Fig. 8B).

**Discussion**

**Role of CD4 in regulating agonist and APL signaling**

In this report, we have examined the role of CD4 signaling in naive T cell activation and differentiation following stimulation with specific Ag. Using peptides with different affinities for a TCR specific for a peptide of MCC, we have shown that naive T cell activation with a strong agonist peptide is qualitatively unchanged in the absence of CD4. Although there is a quantitative difference in the proliferative response, no qualitative changes in early signaling events were detected, and there was no alteration in the subsequent differentiation of CD4 T cells. In contrast, the activation characteristics following stimulation with an APL are markedly affected both quantitatively and qualitatively by the absence of CD4. The T cells fail to significantly mobilize calcium, and they have a profound defect in proliferation that cannot be fully recovered by the addition of IL-2. Most interestingly, in the absence of CD4 signaling ability, T cells fail to differentiate into Th2 effector cells, while Th1 differentiation is unaffected. This provides support for the hypothesis that the TCR signaling pathways that lead to Th1 and Th2 differentiation are distinct and can be regulated at the level of early T cell signaling events in the absence of exogenously provided cytokines.

The experiments presented here demonstrate that a failure in CD4 recruitment or signaling during stimulation with an agonist peptide does not necessarily result in an APL signaling phenotype, but rather CD4 signaling appears to be required to generate at least some of the signals characteristic of APLs. The relative CD4 independence of agonist signaling seen in our experiments argues against the model of APL signaling proposed by Madrenas et al. in which the APL-signaling phenotype arises from a failure to induce CD4 recruitment to the TCR complex (21). They showed that exposing T cell clones to anti-CD4 Abs during agonist stimulation, or preventing CD4-MHC class II interaction using MHC class II mutants unable to interact with CD4, resulted in an APL signaling phenotype as demonstrated by the failure to phosphorylate ZAP70, an altered ζ-chain phosphorylation, and anergy induction. One possible explanation for the different results is that the agonist peptide used in our studies may be of higher affinity than the agonist stimuli used in the studies of Madrenas et al., which may have been more dependent on the CD4 coreceptor. In cases of relatively weak agonist signaling, CD4 coreceptor function may be more important for the generation of a full signaling pattern, and in the absence of coreceptor, inefficient signaling may occur that resembles APL signaling. This conclusion is consistent with several independent experiments in which sensitivity to anti-CD4 inhibition was dependent on the strength of the ligand to the TCR: strong agonists are generally resistant to anti-CD4 treatment compared with weaker agonists, which are more sensitive (23, 35, 36). Thus, one mechanism for inducing an altered peptide signaling phenotype may be absence of coreceptor recruitment to the TCR.
following stimulation with a weak agonist; however, our data indicate that the absence of CD4 does not necessarily result in APL signaling if there is a sufficiently strong signal to the TCR. Alternatively, the differences observed between studies may be a reflection of differences in the regulation of signaling in long term T cell clones vs primary CD4 T cells.

The CD4 dependence of APL signaling observed in our experiments is consistent with other correlative data showing that low affinity TCR signals appear to be more dependent on the expression of coreceptors (22, 23, 37) and the increased susceptibility of T cell stimulation to anti-CD4 inhibition with partial agonists compared with agonist peptide (23, 35). The increased requirement of CD4 for APL signaling may be due, in part, to the increased dissociation rate of APLs for the TCR, making TCR/peptide interactions less stable and potentially making CD4 adhesion to MHC class II more important for stabilizing the interaction with specific peptide. In addition to adhesion, CD4 signaling function appears to be important, since CD4 T cells with an intact ectodomain but deleted cytoplasmic domain were also impaired in APL signaling. Although the CD4 cytoplasmic domain may interact with a variety of signaling molecules, its interaction with lck is particularly well defined and disruption of CD4-lck association is a potential mechanism for the effects observed in these experiments. Thus, one can infer that APL signaling may be especially dependent on CD4-associated lck, while other tyrosine kinases or non-CD4-associated lck may be sufficient for agonist peptide signaling. Again, this may simply be due to the APL requirement for increased signaling efficiency, which is generated by CD4-associated lck being more effectively targeted to the TCR complex. However, this does not fully explain the qualitatively distinct signals generated by APLs and their subsequent effects on T cell effector function. An alternative hypothesis is that CD4-associated lck may have access to distinct substrates compared with non-CD4-associated lck, and preferential use of CD4-associated lck during APL signaling may result in the qualitatively distinct pattern of signals generated compared with agonist signaling.

It should be noted, however, that while APL-induced calcium signaling and Th2 differentiation are impaired in CD4 mutant T cells, the altered pattern of TCR ζ-chain phosphorylation is essentially unchanged in the absence of CD4 signaling. This result is consistent with the data of Rabinowitz et al., who proposed a hierarchy of signaling events based on the strength of TCR signals with ζ phosphorylation being the least sensitive to changes in TCR signaling efficiency (35). However, the mechanism behind the qualitatively distinct pattern of ζ phosphorylation seen during APL signaling relative to agonist signaling remains unclear. Our data showing that the APL-induced altered pattern of ζ phosphorylation is largely unchanged in the absence of CD4 suggests that CD4-associated lck is not necessary for the differential regulation of ζ-chain phosphorylation seen in T cells stimulated with APL. It remains possible that non-CD4-associated lck, or another tyrosine kinase such as fyn, is still involved. Alternatively, differential tyrosine phosphorylation patterns may also be regulated by the presence of a tyrosine phosphatase such as CD45. It is possible that tyrosine phosphatase association with the TCR complex may be differentially regulated during agonist and APL stimulation, which may affect ζ-chain phosphorylation directly, or indirectly by regulating src family tyrosine kinase activity.

**Signaling events required for Th2 differentiation**

Perhaps the most striking aspect of our data is the failure to induce Th2 differentiation in the absence of CD4 signaling ability. This is especially interesting in light of the experiments of Stumbles and Mason in which in vitro administration of whole anti-CD4 Ab induced Th2 differentiation of naive T cells in a mixed lymphocyte reaction (38). It was unclear from these studies whether the mechanism of CD4 regulation of T cell differentiation was by blocking MHC class II/CD4 interactions or whether the CD4 Ab was directly signaling the T cell. In support of the latter possibility are other studies by Konig and colleagues in which the in vivo administration of peptides that inhibit CD4/MHC class II interaction promote Th1 development (39). In conjunction with our own results, these data imply that CD4 signaling may be required for the initiation of Th2 differentiation.

The experiments presented in the present report clarify the nature of the signals required to induce Th2 differentiation. We have previously proposed a signal strength model of T cell differentiation in which low avidity signals to the TCR promote Th2 differentiation, while high avidity signals promote Th1 differentiation. This model was based on in vivo and in vitro experiments in which CD4 T cell differentiation was regulated by varying the ligand density on the APC (11–14) or by varying the affinity of the TCR/peptide interactions (16, 17). In both instances, Th2 differentiation was correlated with low avidity signaling to the TCR, while Th1 differentiation was seen during high avidity signaling. In the studies of APL-induced Th2 differentiation, we have shown that this is dependent on the production of IL-4 by primary T cells 2 to 3 days after initial priming. These data suggest that low avidity TCR signaling can induce IL-4-driven differentiation of Th2 cells, which is similar to data derived from studies of T cell clones in which APL signaling was sufficient for the selective production of IL-4, but insufficient to induce proliferation (8). Our current data indicate that, at least in terms of APL with a decreased affinity for the TCR, the specific nature of the low avidity signal is important for the induction of Th2 differentiation and is not simply due to an overall weaker level of signaling.

The specific failure of APL to induce calcium flux in CD4 mutant T cells and their subsequent failure to undergo Th2 differentiation suggest that the altered pattern of calcium mobilization normally seen in wild-type CD4 T cells may be a necessary signal for Th2 differentiation. This is consistent with our observation that blockade of the calcium/calmodulin/NF-AT pathway with cyclosporin A during primary stimulation with APL inhibits Th2 development (9). In addition, we have previously shown that APL-induced Th2 differentiation is inhibited by inducing a sustained increase in intracellular calcium using a calcium ionophore. This indicates that the pattern of Ca\(^{2+}\) mobilization may mediate distinct signaling pathways and affect the differentiation of CD4 T cells.

In addition to changes in calcium signaling, there may be additional alterations in signal transduction that occur in the absence of CD4 which influence T cell differentiation. As shown in Figure 8, and as recently reported by others during the preparation of this report (24, 25), CD4 signaling seems to be important for efficient Th2 generation driven by the addition of exogenous IL-4 even in the presence of agonist peptide signaling. The role that CD4 plays in promoting Th2 differentiation in this situation remains uncertain. Optimal doses of agonist peptide prime MCC-specific transgenic T cells to undergo differentiation into Th1 effector cells only, without any detectable Th2 activity (13, 17). Th2 differentiation can be induced, however, by the addition of large amounts of exogenous IL-4 during primary culture (Fig. 8) (40), and it is this step that appears to be diminished in the absence of CD4, suggesting that CD4 signaling may be required for the cells to be optimally receptive to signaling by IL-4. This may be because IL-4R expression is not induced to the same level on the surface of the T cell in the absence of CD4 signaling; or because IL-4R signaling...
pathways are qualitatively affected by CD4 expression. Since inhibition of Th1 differentiation by the addition of IL-4 is unchanged in the absence of CD4, we currently favor the possibility that IL-4R signaling occurs, but that the downstream effect of inducing Th2 differentiation may be differentially regulated in the absence of CD4.

In conclusion, it appears from our present data that CD4 expression can regulate Th2 differentiation in two possible ways: by modulating TCR signaling pathways and by modulating IL-4R signaling pathways. In our experiments with APL-induced Th2 differentiation (and in the experiments of Fowell et al.) (24), peptide normally induces differentiation of both Th1 and Th2 effector populations, while Th2 differentiation is selectively lost in the absence of CD4 signaling (Fig. 6), but can be substantially recovered upon the addition of exogenous IL-4 (Fig. 7). These data suggest that CD4 primarily affects TCR signaling pathways induced by APL, which are necessary for Th2 differentiation. This is quite different from the experiments using the agonist peptide of MCC, which primes for Th1 differentiation only, regardless of CD4 expression, while the addition of exogenous IL-4 will allow Th2 differentiation to occur (Fig. 8). The absence of CD4 signaling in this case causes a significant decrease in Th2 differentiation, but it is not completely lost as is true with APL stimulation. These experiments suggest that the CD4 coreceptor can regulate Th2 differentiation by affecting the response to IL-4 in addition to affecting TCR signaling pathways. In total, these experiments demonstrate an important role for the CD4 coreceptor in modulating TCR and cytokine receptor signaling pathways that are important for Th2 differentiation.

Acknowledgments
We thank Drs. Dan Littman for generously providing CD4-deficient and CD4-deficient mice, Patty Ranney for excellent technical assistance with animal breeding, and Dr. Charles A. Janeway for helpful comments on the manuscript.

References