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CD28, Ox-40, LFA-1, and CD4 Modulation of Th1/Th2 Differentiation Is Directly Dependent on the Dose of Antigen

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The involvement of specific accessory/costimulatory molecules in differentiation to Th1 and Th2 phenotypes is controversial. Reports suggest that molecules such as CD4, CD28, and Ox-40 support Th2 differentiation and suppress Th1 differentiation, whereas others such as LFA-1 support Th1 responses and suppress Th2 responses. We have previously defined an in vitro model of differentiation that is absolutely dependent on the initial dose and affinity of peptide presented to a naive CD4 cell. The dose and affinity of Ag regulate autocrine production of IL-2, IL-4, and IFN-γ, which in turn govern differentiation to Th1 and Th2 phenotypes. We have used this system to confirm that CD4, CD28, and Ox-40 interactions can promote, and LFA-1 interactions can suppress, differentiation of cells secreting the Th2 cytokines IL-5 and IL-13. However, for CD4 and LFA-1, this is only seen over a certain range of peptide doses. In addition, CD28 and Ox-40 interactions also promote Th1 differentiation. In general, agonist Abs to accessory molecules shifted the response curves for IFN-γ over a certain range of peptide doses. It also influenced by how Ag is seen in the context of accessory molecule help. Studies of the initial interaction of peptide/MHC complexes with individual TCRs have supported a kinetic model of activation involving serial triggering of many TCRs over several hours. The number of peptide/MHC complexes, and the half-life of their interaction with a TCR, appears to determine the overall level of intracellular signals achieved and consequently the extent of T cell activation. Within this model can be incorporated the actions of costimulatory/accessory molecule receptors such as CD4, CD28, and LFA-1, which may promote T cell activation by both allowing peptide to be seen over a longer period of time (adhesion) and supplying additional intracellular signals which feed into the TCR pathway (costimulation). In general this “strength of signaling” model has suggested that “more is better” for initiating T cell activation. This integration of peptide dose/affinity and accessory molecules has been visualized both molecularly at the cell membrane and functionally for IL-2 production (8). Data from other laboratories have directly or indirectly supported such a model (11–16). We also hypothesized that accessory molecules would integrate with the dose and affinity of Ag to regulate differentiation by effectively increasing or decreasing the strength of signal depending on their presence or absence on the T cell and APC. This scenario would only apply if one accessory molecule did not have a distinct action from another molecule. In contrast to this hypothesis, a number of reports over the past few years have suggested that individual molecules selectively regulate differentiation into Th1 or Th2 phenotypes. For example, interaction of CD28 with B7-2 has been suggested to promote Th2 differentiation but not Th1 differentiation (17–20), as has the interactions of CD4 with class II MHC (21, 22), and Ox-40 with Ox-40 ligand (23, 24). In contrast, LFA-1 interacting with ICAM-1 or -2 (25, 26), and CD28 interacting with B7-1 (18, 27), have been proposed to be inhibitory for Th2 differentiation and therefore integral to Th1 differentiation. One caveat to these studies is that they were largely performed in situations where the TCR signals were not varied, i.e., either one concentration of mitogen or one concentration of Ag was used.

In this report, we have revisited the question of whether Ag dose integrates with accessory molecule signaling by using agonist Abs.

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to CD28 and Ox-40 or reagents that block CD4, LFA-1, and CD28 in a defined model of differentiation that uses naive TCR transgenic T cells responding to a peptide of moth cytochrome c (MCC). We confirm that CD28, Ox-40, and CD28 ligation can suppress Th2 cytokines. However, we demonstrate that multiple effector cytokine phenotypes are generated depending on the initial dose of peptide and the efficiency of stimulation or inhibition provided by the accessory molecule Abs. In general, agonist Abs shifted the cytokine phenotypes are generated depending on the initial dose of peptide or peptides of low affinity (7). To determine the effect of accessory molecules on T cell proliferation, we used a single dose of Ag and assayed for the ability to secrete cytokines. An equivalent number of live T cells were restimulated with a single dose of Ag and assayed for the ability to secrete cytokines. Peak IFN-γ secretion was seen from effectors generated with high doses of peptide (10–100 μM), whereas peak IL-5 and IL-13 secretion were observed from effectors elicited at low/intermediate doses (0.1–1 μM). At the early time point of 4 days, IL-13 was produced in much higher amounts than IL-5 and was the best indicator of Th2-type cells. Very little IL-5 was detected from cells generated after 4 days in culture (Fig. 1, upper panel). In contrast, IL-5 levels were greatly increased with differentiation over time (12 days; Fig. 1, lower panel), showing the typical Th2 cytokine bell-shaped profile, whereas IL-13 levels were down-regulated, although still produced with a similar dose-response curve. IL-5 production was also more variable than IL-13, and in some experiments, very little IL-5 (or IL-4; data not shown) was detected. IFN-γ was similar regardless of the length of differentiation, although on average produced at slightly lower levels over time.

Accessory molecules can modulate naive T cell IL-2 secretion and T cell recovery

In a previous report we showed that expression of CD80 (B7-1) and CD54 (ICAM-1) on APCs allowed naive CD4 cells to upregulate activation Ags (CD25 and CD69), secrete IL-2, and proliferate more efficiently to low doses of peptide or peptides of low affinity (7). To determine the effect of accessory molecules on T

Materials and Methods

Mice

AND TCR transgenic mice bearing T cells reactive with peptides of MCC or pigeon cytochrome c (PCC), and expressing the Vβ3/Vα11 TCR, were bred on a B10.BR background as previously described (28). B10.BR mice were bred at the La Jolla Institute for Allergy and Immunology (San Diego, CA) and used at 8–12 wk of age.

Peptides

T102S, a variant peptide of MCC, bearing a single amino acid substitution of S for T at position 102 (aa 88–103, ANERADLIAYKQA K), was synthesized in the peptide facility at the La Jolla Institute for Allergy and Immunology. This peptide has helicotropic (super agonist) activity, being 5- to 10-fold more stimulatory for a naive CD4 cell than MCC, as described previously (10).

Antibodies

Abs (or Fc fusion protein) to the following Ags were added to cultures at 0.1–20 μg/ml as described in the figure legends. Control Ig (rat IgG1, rat IgG2a, rat IgG2b, and hamster IgG) and anti-LFA-1 (M1/74, rat IgG2a) were purchased from Pharmingen (San Diego, CA). Anti-CD28 (37.51, hamster IgG) was purified from hybridoma supernatant and was a kind gift of Dr. J. Allison (University of California, Berkeley, CA). Anti-Ox-40 (CD134) (Ox-86, rat IgG1) was a gift from Dr. A. Weinberg (Earle Chiles Institute, Portland, OR). Anti-CD4 (GK1.5, rat IgG2b) was a gift from Dr. S. Schoenberger (La Jolla Institute for Allergy and Immunology). Fusion protein CTLA4-Ig, which blocks CD28 and CTLA4 interaction with CD80/CD86, was a gift from Dr. P. Linsley (Rosetta Inpharmatics, Kirkland, WA) and was purified from a hybridoma supernatant by passage over a Gammabind column (Pharmacia Biotech, Piscataway, NJ).

T cells

CD4+ T cells were purified from spleen and lymph nodes of TCR transgenic mice (28) by nylon wool depletion, followed by Ab and complement treatment. T cells were incubated with Abs to CD8 (3.155), heat-stable Aggenic mice (28) by nylon wool depletion, followed by Ab and complement treatment. T cells were incubated with Abs to CD8 (3.155), heat-stable Ags (or Fc fusion protein) to the following Ags were added to cultures at 0.1–20 μg/ml as described in the figure legends. Control Ig (rat IgG1, rat IgG2a, rat IgG2b, and hamster IgG) and anti-LFA-1 (M1/74, rat IgG2a) were purchased from Pharmingen (San Diego, CA). Anti-CD28 (37.51, hamster IgG) was purified from hybridoma supernatant and was a kind gift of Dr. J. Allison (University of California, Berkeley, CA). Anti-Ox-40 (CD134) (Ox-86, rat IgG1) was a gift from Dr. A. Weinberg (Earle Chiles Institute, Portland, OR). Anti-CD4 (GK1.5, rat IgG2b) was a gift from Dr. S. Schoenberger (La Jolla Institute for Allergy and Immunology). Fusion protein CTLA4-Ig, which blocks CD28 and CTLA4 interaction with CD80/CD86, was a gift from Dr. P. Linsley (Rosetta Inpharmatics, Kirkland, WA) and was purified from a hybridoma supernatant by passage over a Gammabind column (Pharmacia Biotech, Piscataway, NJ).

CD4+ T cells were incubated with Abs to CD8 (3.155), fibroblast cells (DCEK-ICAM) transfected with I-Ek and ICAM-1 (CD54), and constitutively expressing B7-1 (CD80), were used for restimulation of T cells. APC populations were treated with mitomycin C (50–100 μg/ml) for 30 min at 37°C before use.

Cell cultures

 Cultures were set up in 1 ml of 10% FCS-RPMI in 48-well plates (Sarstedt, Newton, NC). Naive CD4 T cells were plated at 5 × 10^4/ml with 4 times as many T-depleted spleen APCs and various concentrations of T102S peptide. Abs to cell surface molecules were added on day 0 of culture. Anti-Ox-40 and CTLA4-Ig were added again on day 2. Stimulation was conducted for 4 or 12 days, after which viable cells were recovered and counted by trypan blue exclusion. T cells (3 × 10^5/ml) were replaced in 0.25-ml volumes in 96-well plates in quadruplicate with half as many DCEK-ICAM fibroblast APC, prepulsed for 2–4 h with 20 μM PCC peptide as described previously (10). Supernatants were collected for cytokine analyses 24–48 h later. SDs between replicates were <15% of the means.

Cytokine secretion

Duplicate supernatants were recovered from quadruplicate cultures at 24 h (IL-2) and 48 h (IFN-γ, IL-5, and IL-13) after T cell stimulation to assess cytokine content. Cytokines were measured by ELISA as in previous studies (10) using the Abs JES6-1A12 (PharMingen) and biotin-JES6-5H4 (PharMingen) for IL-2, R4-62 and biotin-XMG1.2 (PharMingen) for IFN-γ, and TRFK5 and biotin-TRFK4 for IL-5 detection. Standard curves were constructed with purified IL-2, IL-5, and IFN-γ (supernatants from the respective X63Ag cell lines). Anti-IL-13 (clone 38213.11, MAB413), biotin-anti-IL-13 (BAF413), and recombinant human IL-13 were purchased from R&D Systems (Minneapolis, MN). The sensitivity of each assay was similar, with levels of detection being 50–100 pg/ml for IL-2, IL-5, and IFN-γ and 200 pg/ml for IL-13.

Results

Th1/Th2 differentiation determined by varying doses of agonist peptide

We have previously shown that differentiation of effectors secreting Th1 (IFN-γ) or Th2 (IL-4, IL-5) cytokines can be controlled by the dose or affinity of peptide initially presented to the naive CD4 cell (10). This is again illustrated in Fig. 1, and we now show that IL-13 is also regulated by Ag dose in a manner similar to IL-5. Naive CD4 cells from AND TCR transgenic mice were stimulated with varying doses of the MCC analogue, T102S, presented by syngeneic T-depleted splenic APCs. Cultures were left for 4 or 12 days (Fig. 1, upper panel and lower panel, respectively), and then an equivalent number of live T cells were restimulated with a single dose of Ag and assayed for the ability to secrete cytokines. Peak IFN-γ secretion was seen from effectors generated with high doses of peptide (10–100 μM), whereas peak IL-5 and IL-13 secretion were observed from effectors elicited at low/intermediate doses (0.1–1 μM). At the early time point of 4 days, IL-13 was produced in much higher amounts than IL-5 and was the best indicator of Th2-type cells. Very little IL-5 was detected from cells generated after 4 days in culture (Fig. 1, upper panel). In contrast, IL-5 levels were greatly increased with differentiation over time (12 days; Fig. 1, lower panel), showing the typical Th2 cytokine bell-shaped profile, whereas IL-13 levels were down-regulated, although still produced with a similar dose-response curve. IL-5 production was also more variable than IL-13, and in some experiments, very little IL-5 (or IL-4; data not shown) was detected. IFN-γ was similar regardless of the length of differentiation, although on average produced at slightly lower levels over time.

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Abbreviations used in this paper: MCC, moth cytochrome c; PCC, pigeon cytochrome c; PI-3-kinase, phosphatidylinositol-3-kinase; PLCγ1, phospholipase Cγ1; MAPK, mitogen-activating protein kinase.
cell differentiation and how they integrate with Ag dose, we utilized several agonist Abs (anti-CD28, anti-Ox-40) and antagonist Abs (anti-LFA-1, anti-CD4) or Ig fusion protein (CTLA4-Ig) to various molecules. To confirm their stimulatory or inhibitory capacities, and thus agonist or antagonist properties, we initially assessed naive T cell IL-2 production, shown in Fig. 2, and the effects on T cell survival over time (Table I).

As shown in Fig. 2, addition of anti-CD28 induced higher IL-2 secretion at all peptide doses, whereas anti-Ox-40 only slightly increased IL-2 in this system. Anti-CD4 resulted in a 10- to 20-fold shift in IL-2 secretion to the higher peptide concentrations, whereas anti-LFA-1 and CTLA4-Ig resulted in more profound inhibition (100- to 1000-fold shift). These results agree with many others who have used these particular Abs. We have previously shown that Ox-40 stimulation alone at the naive T cell stage has only slight effects on IL-2, whereas more profound effects are seen at late time points and for promoting clonal expansion (8). In addition, we have confirmed that this Ab is stimulatory in vivo (unpublished data).

Agonist and antagonist reagents induced a corresponding increase or decrease in T cell recovery after a 4-day culture (Table I). Anti-CD28 and anti-Ox-40 enhanced cell recovery, especially with low doses of peptide, whereas anti-CD4, anti-LFA-1, and CTLA4-Ig reduced cell recovery at these doses. We previously demonstrated that over time, cell survival is optimal when naive T cells are stimulated with intermediate doses (0.1–1 μM) of the high affinity peptide with reduced recovery seen in cultures that initially received high doses of peptide (10). This is illustrated in Table I for day 12 cultures. Anti-CD28 and anti-Ox-40-treated cells showed higher cell recoveries at all Ag doses (except 0.001 μM with anti-Ox-40); however, optimal recovery was still seen with 0.1–1 μM. In contrast, anti-LFA-1-, anti-CD4-, and CTLA4-Ig-treated cells did not survive well at low doses of peptide, and reduced recoveries were seen at other doses. The exception was with anti-LFA-1 where cell survival was greatly enhanced with high dose peptide (1–100 μM). Therefore, blocking or stimulating accessory molecule interactions can reduce or enhance cell survival depending on the Ag dose. There was some correlation, although not strict, with initial IL-2 production (Fig. 2), and overall, it could be argued that provision of costimulation allowed naive T cells to respond and survive when less Ag was presented, whereas

![Figure 1](image1.png)

**FIGURE 1.** Th1/Th2 cytokine production is directed by peptide dose and duration of culture. Naive T cells from TCR transgenic mice were stimulated at 5 × 10^9/ml in 1-ml cultures with four times as many T-depleted spleen APC and varying doses of T102S peptide. After 4 or 12 days, equivalent numbers of viable T cells were recultured at 3 × 10^6/ml in 0.25-ml cultures in quadruplicate with half as many DCEK-ICAM fibroblast APC prepuled with 20 μM PCC peptide. Supernatants were recovered 24–48 h later. Duplicate wells were assayed for cytokine content by ELISA. Results for each dose are the average with SD from at least four experiments. In cultures receiving no peptide (0), no IL-5 or IL-13 was detected above the background level of the assay. Not assayed.

![Figure 2](image2.png)

**FIGURE 2.** Abs or fusion proteins to T cell surface proteins can modulate naive T cell IL-2 secretion. Naive T cells were stimulated as in Fig. 1 with different doses of peptide presented on T-depleted APC, in the presence or absence of Abfusion protein given at the start of culture. Data are shown for 1 μg/ml anti-CD28, 10 μg/ml anti-Ox-40, 3 μg/ml anti-CD4, anti-LFA-1, and 20 μg/ml CTLA4-Ig. Control Ig cultures received 10 μg/ml rat IgG and hamster IgG. Supernatants were collected at 40 h and assayed for IL-2 by ELISA. Similar results were seen in at least three repeated experiments. Errors for individual points were less than 15% of the means.

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* T cells were stimulated as in Fig. 1 with T102S, and cell recoveries were measured by trypan blue exclusion on day 4 and day 12 of culture. Abs were added to cultures as described in Materials and Methods. Results are the average of at least three experiments from each Ag dose. Recovery in the absence of Ag was 52% after 4 days and <10% after 12 days.
inhibition of costimulation suppressed response and survival when Ag dose was suboptimal.

Accessory molecule interactions can promote or suppress Th1/Th2 effector development but in a strictly Ag dose-dependent fashion

The ability of accessory molecules to modulate Th1 and Th2 development was initially assessed with three doses of Ag. Fig. 3 shows cytokine secretion in a single representative experiment from equivalent numbers of cells restimulated after 12 days in culture. In all cases, the cells recovered were CD4 positive and all expressed the transgenic TCR, Vβ3/Vα11.

T cells initially cultured with 0.01 μM peptide developed a Th2 phenotype with secretion of predominantly IL-5, some IL-13, and little IFN-γ (Fig. 3, left). None of the Abs significantly affected IFN-γ at this dose. Anti-CD28 and anti-Ox-40 enhanced IL-5, to a limited extent, and IL-13, to a great extent. In contrast, Abs to CD4, LFA-1, and fusion protein CTLA4-Ig almost completely inhibited development of both IL-5- and IL-13-secreting cells. With an intermediate dose of Ag (1 μM; Fig. 3, center), some similar

and some distinct changes were seen. Anti-CD28 and anti-Ox-40 again resulted in enhanced levels of IL-5 and IL-13, but additionally promoted more IFN-γ secretion. Anti-CD4, CTLA4-Ig, and anti-LFA-1 inhibited cells secreting IFN-γ, but had differing effects on IL-5 and IL-13. CTLA4-Ig had little effect on either cytokine, whereas increases were seen with anti-CD4, and particularly with anti-LFA-1. The trend was similar with 100 μM compared with 1 μM, although again differences were noted (Fig. 3, right). Both anti-CD28 and anti-Ox-40 resulted in enhanced IFN-γ, although a much higher level was now seen with anti-CD28. Again, anti-CD4, CTLA4-Ig, and anti-LFA-1 inhibited cells secreting IFN-γ. In contrast, anti-LFA-1 to a great extent, anti-Ox-40 less, and anti-CD28 and anti-CD4 even less, augmented IL-5 and IL-13 production.

In summary, anti-CD28 and anti-Ox-40 could promote differentiation of T cells secreting both Th1 and Th2 cytokines. Anti-CD4 and anti-LFA-1 could suppress development of T cells secreting IFN-γ; however, they had differential effects on Th2 cytokines (IL-5 and IL-13). CTLA4-Ig could suppress secretion of both Th1 and Th2 cytokines. Thus, agonist Abs could enhance both Th1 and Th2 cytokines, whereas antagonist reagents showed differential effects on Th1 and Th2 cytokines. The most important finding was that all responses were dependent on the initial dose of peptide.

These data suggested that enhancing or suppressing the actions of accessory molecules was primarily affecting the Ag dose-response patterns. We therefore set up full dose-response titrations in the presence or absence of the various Abs added at the initiation of culture. Figs. 4 and 5 show the mean cytokine secretion from effector T cells restimulated after 4 and 12 days from at least three experiments.

In Fig. 4, addition of anti-CD28 and anti-Ox-40 enhanced development of T cells secreting both Th1 (IFN-γ) and Th2 (IL-5, IL-13) cytokines over a wide range of peptide concentrations. For both Abs, at day 4 (upper panel), the dose-response curve for IFN-γ and IL-13 was shifted ~10- to 100-fold to the lower Ag concentrations. Anti-CD28 was more potent than anti-Ox-40 in shifting the cytokine dose response. Minor differences were observed for the Th2 cytokines in that anti-CD28 had greater effects at very low doses (0.001 μM) and little/no effect at very high doses (10–100 μM). However, the quantity of cytokine and shape of the curve were similar for both anti-CD28 and anti-Ox-40-treated T cells.

The enhancement of IFN-γ and IL-13 secretion, by anti-CD28 and anti-Ox-40 was retained when T cells were restimulated at 12
FIGURE 5. Anti-CD4, anti-LFA-1, and CTLA4-Ig alteration of Th1/Th2 differentiation is Ag dose-dependent. Naive T cells were stimulated as in Fig. 1 for 4 or 12 days in the presence of anti-CD4 (3 μg/ml), anti-LFA-1 (3 μg/ml), CTLA4-Ig (20 μg/ml), or equal amounts of isotype-matched Abs rat IgG2a and IgG2b (control Ig) given at the initiation of culture. Equal numbers of T cells were then restimulated with PCC-pulsed DCEK-ICAM cells, and supernatants were collected at 24 and 48 h.

Data are the average cytokine secretion from three or more experiments.

days (Fig. 4, lower panel) although the overall levels of each cytokine was lower than at day 4 (upper panel). Both anti-CD28 and anti-Ox-40 also enhanced IL-γ secretion, especially in cultures that initially received low doses of peptide (0.001–0.01 μM) with peak production occurring at 10-fold lower doses vs control cultures (0.01 v. 0.1 μM). Therefore, both anti-CD28 and anti-Ox-40 could enhance the ability to produce Th1 and Th2 cytokines in addition to altering the dose-response curves, with peak production of cytokine occurring in cultures that received lower doses of priming Ag.

The effect of antagonist reagents (anti-CD4, anti-LFA-1, and CTLA4-Ig) on Th1/Th2 development is shown in Fig. 5. Cytokine secretion is shown from T cells restimulated after 4 (top panel) and 12 days (middle and bottom panels). There was little effect on IL-2 secretion; however, CTLA4-Ig could inhibit IL-2 secretion from cells that initially received low doses of Ag (0.001–0.1 μM), in effect, shifting the dose response ~100-fold. After short-term culture (top panel), both anti-CD4 and anti-LFA-1 shifted the IL-13 response to higher Ag doses, whereas CTLA4-Ig greatly inhibited IL-13 secretion. Anti-LFA-1 and CTLA4-Ig also inhibited IFN-γ secretion, whereas anti-CD4 had little effect, although in two of three experiments anti-CD4 actually shifted the IFN-γ dose response to higher Ag concentrations. Restimulation of cells after 12 days showed that anti-CD4, anti-LFA-1, and CTLA4-Ig inhibited development of IFN-γ-secreting cells at all Ag concentrations, effectively shifting the dose-response curves to the higher concentrations. These Abs similarly inhibited IL-5 production when low doses of peptides were used (0.01–0.1 μM) and either inhibited IL-13 or had no effect. At high doses of priming Ag (1–100 μM), both anti-CD4 and anti-LFA-1 treatments resulted in augmented IL-5 and IL-13 vs control cultures. This increase was particularly striking with anti-LFA-1. In contrast, CTLA4-Ig inhibited or had no effect on subsequent IL-5 and IL-13 secretion. Although the absolute amounts of cytokines varied, these data suggest that anti-CD4 and anti-LFA-1 can alter Th2 dose response while inhibiting and/or shifting the dose response for Th1 (IFN-γ-secreting) cells. CTLA4-Ig profoundly inhibited Th2 development and partially Th1 development though lower doses of CTLA4-Ig appeared to shift the Th1/Th2 Ag dose-response curves (data not shown).

Therefore, although modulation of individual accessory molecules can influence overall levels of cytokine secreted and thus Th1 and Th2 development, there does not seem to be a link between a single ligand/receptor interaction and a particular phenotype. For example, anti-CD28 and anti-Ox-40 augmented both Th1 and Th2 cytokines, whereas anti-CD4, anti-LFA-1, and CTLA4-Ig inhibited or shifted the Th1/Th2 cytokine dose response to higher Ag concentrations.

Multiple cytokine phenotypes result depending on the amount of Ab reagent used to modulate accessory molecules

In initial experiments to assess the effects of blocking various accessory molecules, we had noted that differing cytokine profiles result depending on the amount of Ab used, presumably related to the extent of blocking achieved, and the expression and use of the molecules in this particular system. This is illustrated in Fig. 6 for anti-LFA-1. With lower amounts of anti-LFA-1 (0.1 μg/ml), which partially blocked naive IL-2 secretion (data not shown), a more subtle phenotype was seen than previously shown in Figs. 3 and 5. IFN-γ was blocked at the lower concentrations of peptide, but not significantly at very high concentrations. Similarly, IL-13 and IL-5 were inhibited at very low peptide doses (0.01–0.1 μM) but not at high doses, and IL-5 was increased although only modestly. With increasing amounts of anti-LFA-1, it was obvious that a shift in the peptide dose response was occurring with all three cytokines, peaking at higher peptide concentrations. This effectively resulted in lower levels of all cytokines at the low Ag doses and higher levels of IL-5 and IL-13 at the high Ag doses. IFN-γ was blocked at 50 μM (and 100 μM, Fig. 5) with 10 μg/ml anti-LFA-1; however, it could be argued that increased levels may have been seen if we could have used sufficient amount of peptide (100–200 μM is the maximum that is feasible in these cultures).

In addition to anti-LFA-1, titration of anti-CD28 also shifted the cytokine dose-response curves in an Ag dose-dependent manner (data not shown). Although the amounts of cytokines secreted did
not change with addition of more anti-CD28, the dose responses for IFN-γ, IL-5, and IL-13 were shifted ~10-fold to lower Ag doses when 10 times more anti-CD28 was used (1 μg/ml vs 0.1 μg/ml anti-CD28). Overall, it can therefore be seen that multiple phenotypes result depending on the amount of agonist/antagonist Ab used, and that the overall conclusion as to the role of various accessory molecules can be heavily biased depending on the extent of intervention. Again, most importantly, modulation of the cytokine phenotype was strictly correlated with the dose of Ag.

The kinetics of development of IL-5- and IL-13-secreting cells can be altered by suppressing or promoting costimulation

Our previous studies showed that IL-5 was only produced at high levels following a long differentiation period. More recently, we also noted that the kinetics of IL-13 were opposite to IL-5 in that higher levels of IL-13 were seen earlier (on day 4) rather than later (on day 12) (see Fig. 1). We therefore assessed whether modulating costimulation would affect the kinetics of these responses. Fig. 7 shows IL-5 production at day 4 vs day 12 when costimulation from anti-CD28 was provided, and IL-13 production when anti-LFA-1 was used to inhibit costimulation. Again, the conclusions were dependent on the dose of Ag used initially, with multiple effects on cytokine phenotypes being seen depending on the amount of peptide presented. However, significantly, provision of CD28 costimulation resulted in enhanced or rapid differentiation to IL-5 production. Cells treated with anti-CD28 and restimulated after 4 days showed and IL-5 response pattern similar to that in control cultures at 12 days (compare anti-CD28, day 4 to control, day 12). In addition to IL-5, the kinetics of IL-13 secretion which is opposite to that of IL-5 (see Fig. 1), was also altered. Instead of peak production on day 4, peak production of IL-13 was seen on day 12 from T cells initially cultured with anti-LFA-1. Therefore, inhibiting LFA-1 interaction prevented the down-regulation of IL-13 at late times (day 12) or shifted the kinetics of peak IL-13 secretion. The overall conclusions reached, regarding the role of each molecule in regulating individual cytokines, therefore depend on the time of analysis, the concentration of reagents used, and most importantly, the initial dose of Ag presented to the responding T cell.

Discussion

In this report, we have shown that differentiation of naive CD4 T cells into divergent cytokine-secreting effector cells can be affected by the participation of accessory molecule interactions. As opposed to several studies which have implied that individual molecules control distinct cytokines, our data show that both positive and negative effects on multiple cytokines can be seen when promoting or suppressing costimulation. Most importantly, the ultimate phenotype achieved is intimately linked to the dose of peptide that is available for presentation, showing that the integration of signals produced from TCR/peptide/MHC ligation and those from accessory molecules is as important for T cell differentiation as it is for the initial phases of T cell activation.

Although we did see some results that do not exactly fit our model when manipulating certain accessory molecules, including differences in total cytokine levels, it is clear that in many cases the effect of providing or taking away costimulation was to alter the dose-response curve to peptide. Thus, in general, agonist Abs produced a similar dose-response curve, but skewed toward the lower peptide concentrations, and antagonist Abs resulted in a similar dose-response pattern shifted toward the higher peptide concentrations. Variations were seen between anti-CD4, CTLA4-Ig, and anti-LFA-1 for example, but it could be argued that this may have been determined by the expression levels and use of each molecule in the normal response, or proficiency of blocking of the reagents, rather than any intrinsic differences between the actions of the molecules. Therefore, rather than an individual accessory receptor/ligand interaction positively regulating one cytokine and negatively regulating another, our data favor a model depicted in Fig. 8 where the overall strength of stimulation governs T cell differentiation. This is dictated by the balance between the extent of TCR signaling (dose and/or affinity of peptide) and the extent of
accessory molecule involvement at the time of T cell/APC interaction. In such a model, differentiation to a Th2 phenotype (IL-5, IL-13) will be induced by a low/moderate dose of Ag presented in the context of many accessory interactions, or alternatively by a high dose of Ag presented with little accessory molecule help. Reducing both sets of signals sufficiently will result in largely uncommitted effector T cells being generated over time (IL-2 primarily), whereas maximizing both sets of signals will favor predominantly IFN-γ-secreting cells.

These results raise a number of questions. First, is it valid to refer to two distinct sets of signals, brought about by peptide/MHC or accessory molecule interactions, or should they be viewed more as a continuum of events funneling into one single pathway? Originally, costimulation was defined as a separate signaling pathway employing molecules distinct from those used by the TCR. This was based on the fact that T cell clones would become anergic, and primary T cells did not proliferate appreciably or secrete IL-2 when stimulated with Abs to the TCR/CD3 complex, or with peptide presented on APCs with disrupted membranes, or on APCs artificially made with only MHC on their surface. The initial description of CD28, preventing anergy and inducing proliferation and IL-2, and the finding that it utilized phosphatidylinositol-3-kinase (PI-3-kinase), a molecule not thought to be part of the TCR signaling pathway, fulfilled that original definition of costimulation. However, more recent data from multiple sources suggest that the issue is more complex. It is now clear that TCR signals alone are sufficient to activate a T cell (applicable to both naive CD4 and CD8 cells), resulting in blastogenesis, entry into cell cycle, expression or modulation of several cell surface proteins (CD69, IL-2R, Ox-40), secretion of a low level of IL-2, and transient proliferation (7, 30–32). However, these activation events only occur in the absence of accessory molecules when high dose and high affinity peptide are presented. With low dose/affinity peptide, T cells remain quiescent. The ligation of accessory/costimulatory receptors enhances the ability of peptide to initiate early events of T cell activation and, importantly for a naive T cell, to promote high level IL-2 production, proliferation over time, and cell survival. This has been demonstrated in one form or another for multiple molecules including those studied here (CD28, LFA-1, Ox-40, CD4) and others such as 4-1BB, CD27, and SLAM (reviewed in Ref. 33). Although differences have been noted between individual receptors (8, 34), it can be argued that these are related to the system studied, the affinity of interaction between ligand and receptor, and more relevant, their expression on the T cell membrane over time, and the expression of their ligands on the APCs over time. Thus, it may be valid in one sense to talk about distinct signals from the TCR vs accessory molecules, but not necessarily from one accessory molecule vs another.

On the other hand, are there really distinct intracellular events that occur when the TCR or accessory receptors are engaged? Studies from Lanzavecchia and colleagues (2, 5) showed that CD28 ligation reduced the number of TCRs that needed to be engaged, and the length of the engagement period required for T cell triggering. However, a similar level of activation could be achieved in the absence of CD28 if the length of stimulation and number of engaged TCRs were increased (2, 5). Similarly, the presence of CD4 has been shown to be crucial for efficient T cell activation, but only when weak peptide agonists are presented (35, 36). In the absence of CD4, a strong agonist produces signaling patterns identical to those seen with a weak agonist (37). We have seen similar results with ICAM/LFA interactions and weak agonists (7). Although much is unknown, studies of intracellular signals from accessory receptors now suggest that pathways converge, in many cases before the level of the nucleus. CD28 can affect inositol phospholipid hydrolysis, phospholipase Cγ1 (PLCγ1) phosphorylation, and activation of p21Ras and the mitogen-activated protein and stress-activated protein kinases (reviewed in Refs. 38 and 39). It is now clear that all of these are also recruited to some extent by TCR/CD3 ligation, including PI-3-kinase. CD4 and LFA-1, which in many cases have been considered as adhesion molecules, are also obviously capable of enhancing the intracellular signals induced in a T cell. CD4 through its association with p56Lck can feed into the PLCγ1 pathway, and LFA-1 binding also results in PLCγ1 phosphorylation, leading to inositol phospholipid hydrolysis and increases in intracellular calcium (40, 41). The TNFR family of costimulatory molecules initially could have been viewed as the exception to these common signaling pathways, each acting through one or more of the TNFR-associated factor (TRAF) molecules, such as TRAF2 and -3 in the case of Ox-40 (42). However, it is now clear that one of the results of TRAF binding is activation of NF-κB, via NF-κB-inducing kinase (NIK), and its translocation into the nucleus. The recent demonstration that CD28 can also activate the NIK/NF-κB axis through a kinase termed Cot (43), again implies that all signals (TCR and accessory receptor) converge before or at the level of the nucleus. Whether truly separate pathways are initiated from individual receptors may therefore be unlikely, although many more studies are obviously needed.

In light of the above arguments, it then makes sense that a single accessory ligand/receptor interaction will not control one function (e.g., Th2 cytokines) as suggested by a number of reports (17, 18, 21–26). Rather, as implied by our data, the integration of signals from the TCR and multiple accessory receptors will determine the overall response pattern of the T cell, with the dominant response being determined by the dose and affinity of Ag and the expression characteristics of the various accessory molecules on the T cell and APC. How then is Th1/Th2 differentiation controlled by strength of signaling? At present we do not know, but think that it will be determined in the initial days after activation by the balance of signals received by the T cell. IL-2R signaling plays some role in this process as exogenous IL-2 can shift the Ag dose-response patterns for effector cytokines to the lower concentrations (10), and blocking IL-2 can mimic to a certain extent the effects seen here when blocking with anti-CD4 and CTLA4-Ig (unpublished observations). This is not surprising as a major role of TCR and accessory signals is to regulate IL-2 production, and signaling through the IL-2R also involves some of the same intracellular substrates such as Lck, Fyn, Ras, mitogen-activating protein kinase (MAPK), and PI-3-kinase. We also know that in our system the balance of IFN-γ and IL-4 initially produced by the naive T cell is critical to
subsequent differentiation into Th1 and Th2 phenotypes (10, 44, 45), and this has also been seen in several other systems (16, 46, 47). Thus, IFN-γR signaling controls the ability to subsequently make high levels of IFN-γ, and IL-4R signaling controls the ability to make high levels of IL-5 and IL-13, as demonstrated using blocking Abs to these cytokines (Ref. 10, and data not shown). IL-12, which potentially could override the action of IL-4, was found not to be a major factor in these studies (10).

In summary, we therefore believe that the signals that control the balance of autocrine IL-4 and IFN-γ made by the responding naive T cell will provide the answer to how the strength of signaling regulates Th1/Th2 differentiation. Although great strides have been made in understanding the signaling events initiated through the IL-4R and the IFN-γR, there is no information on what regulates the first burst of IL-4 and IFN-γ. As both cytokines are produced relatively late after naive CD4 activation, with mRNA first seen around 24 h compared with several h for IL-2 (44–46, 48), the amount of IL-2 and hence also the level of signaling through the IL-2R may be critical to the level of transcription of IL-4 vs IFN-γ. Data from Yamashita et al. (49, 50), using dominant negative transgenic mice, recently showed that the Lck/Ras/MAPK pathway may be critical to Th2 differentiation. Thus, an intriguing possibility, that could involve both TCR, accessory receptor, and IL-2R signaling, is that the ability to preferentially activate this pathway may favor IL-4 over IFN-γ, and therefore Th2 differentiation over Th1 differentiation. Obviously, studies of this nature are still in their infancy and many more will be needed before a clearer picture emerges.

References
3. Lanzavecchia, A. 1996. IL-12, which potentially could override the action of IL-4, was blocking Abs to these cytokines (Ref. 10, and data not shown).