Cutting Edge: LFA-1 Interaction with ICAM-1 and ICAM-2 Regulates Th2 Cytokine Production[1]

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The role of CD28/B7 and LFA-1/ICAM costimulation in proliferation and Th1/Th2 differentiation of naive CD4$^+$ T cells was addressed using T cells from DO11.10 TCR transgenic mice stimulated by dendritic cells. The blockade of either CD28/B7 or LFA-1/ICAM interactions partially inhibited T cell proliferation. By comparison, blocking CD28/B7 costimulation inhibited IL-4 and IL-5 (Th2 cytokine) production, whereas blocking LFA-1/ICAM-1 or LFA-1/ICAM-2 led to a significant increase (15- to 40-fold) of Th2 cytokines. The combination of anti-ICAM-1 and anti-ICAM-2 mAbs had a synergistic effect with a 100- to 1000-fold increase of Th2 cytokine production. Thus, these two costimulatory pathways have opposing roles in the regulation of Th2 development. *The Journal of Immunology*, 1998, 161: 5138–5142.

T helper type 1 cells, which secrete IL-2, IFN-γ, and lymphotoxin, have a major proinflammatory role in cellular immunity to intracellular pathogens, whereas Th2 cells, which secrete IL-4, IL-5, and IL-10, have a predominant role in humoral immunity against extracellular parasites (1, 2). Many factors regulate Th1/Th2 differentiation. Cytokines such as IFN-γ, IL-12, and IL-4 have been shown to play a key role in skewing the Th cell repertoire (2). In addition, the strength of TCR-mediated signals (3, 4) and costimulatory molecules such as CD30 (5), 4–1BB (6), or CD28 (7) have been reported to regulate Th cell differentiation. The nature of the APCs can also alter this differentiation. Several groups have shown that B cells promote Th2 differentiation (8), while dendritic cells (DCs),[3] the major APCs in initiation of primary immune responses (9), promote Th1 differentiation (10–12). This property of DC is due, at least in part, to the production of IL-12 by the DC, but could also reflect differences in the level of MHC expression or in the nature of signals delivered through certain costimulatory/adhesion molecules.

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[3] Abbreviations used in this paper: DC, dendritic cell; mCTLA4Ig, murine CTLA4Ig.

In this study, we examined the role of CD28/B7 and LFA-1/ICAM interactions in the regulation of T cell proliferation and differentiation of naive T cells from DO11.10 transgenic mice that express a TCR specific to OVA 323–339 (13). The data suggest that the CD28/B7 and LFA-1/ICAM pathways have opposing roles in the regulation of naive T cell differentiation stimulated by "fresh" CD8$^-$ DCs, the most potent immunostimulatory cells of the spleen (9). Thus, DCs may promote Th1 development by suppressing Th2 development through the interaction of ICAM-1 and ICAM-2 with LFA-1.

Materials and Methods

Mice

BALB/c mice were purchased from Frederick Cancer Research Facility (Frederick, MD) and maintained in a specific pathogen-free barrier facility at the University of Chicago (Chicago, IL). DO11.10 transgenic mice expressing a TCR specific for OVA peptide 323–339 presented in the context of I-A^d (13) were bred to BALB/c mice and then to RAG-2-deficient-mice (gift from Fred Alt, Harvard Medical School, Boston, MA) to generate DO11.10 × RAG-2$^{-/-}$ mice. TCR transgene expression and B cell deficiency were analyzed by FACS.

Culture medium, Abs, blocking reagents

mAbs 145-2C11 (anti-CD3 (14)), J11d (anti-murine CD24, ATCC TIB-183; American Type Culture Collection (ATCC), Manassas, VA), and MKD6 (anti-murine I-A^d (15)) were prepared in our laboratory. Purified mAbs Yn1/1.7.4 (anti-ICAM-1, ATCC CRL-1878) and M17/4.2 (anti-LFA-1, ATCC TIB-127) were a gift from Dr. Robert Hendricks (University of Pittsburgh, PA), and ascites of Yn1/1.7.4 were a gift from Dr. Jim Miller (University of Chicago). MIC2/4 (anti-ICAM-2) mAb was purchased from PharMingen (San Diego, CA). The murine (m)CTLA4Ig was obtained from Genetics Institute (Cambridge, MA).

Purification of T cells and APCs

T cells from spleen and lymph nodes of DO11.10 × RAG-2$^{-/-}$ were purified after passage over nylon wool columns, depletion of cells expressing CD24 (J11d) and MHC class II (MKD6) with rabbit complement at 37°C for 45 min, and Ficol-Hypaque gradient separation. For DC purification, low density BALB/c spleen cells were prepared as previously described (16, 17). The recovered low density cells, preincubated with 2.4G2 mAb to reduce nonspecific binding, were stained with biotin-labeled N418 (anti-CD11c, ATCC HB224) revealed by streptavidin-phycoerythrin, FITC-labeled 14-4-4S mAb (anti-I-E, ATCC HB-32) and phycoerythrin-labeled 53–6.7 (anti-CD8α from PharMingen) mAbs in staining buffer (PBS/2% FCS/5 mM EDTA). The CD8$^-$ DC population (I-E$^+$, CD11c$^+$, CD8$^-$) was sorted on a FACStar^{Plus} (Becton Dickinson, Mountain View, CA). Cells were kept at 4°C in staining buffer throughout the procedure. DC-depleted splenocytes were prepared by double staining with biotin-labeled N418 revealed by streptavidin-phycoerythrin and FITC-labeled 14-4-4S mAb, and then CD11c$^-$ cells were sorted on a FACStar^{Plus}.

Proliferation assays

Six thousand T cells were cultured with 2,000 irradiated CD8$^-$ DCs or 20,000 irradiated splenocytes depleted of DCs (1000 rad) in round-bottom

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96-well culture plates in DMEM (Life Technologies, Grand Island, NY). The medium was supplemented with 10% FCS (Summit Biotechnology), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.29 mM L-glutamine, nonessential amino acids, 10 mM HEPES, 5×10^{-5} M 2-ME, and 1 μg/ml OVA 323–339 peptide (University of Chicago Peptide Synthesis Facility). Proliferative responses were assessed at 96 h by adding 1 μCi/well of [^{3}H]thymidine during the final 7–9 h of culture before harvesting and scintillation counting.

T cell stimulation for cytokine production

T cells (10^4/well) were stimulated in the same culture medium used for the proliferation assay with 1 μg/ml OVA peptide and purified CD8$^-$ DCs (2×10^3/well) in round-bottom 96-well culture plates. In some assays, supernatants (20 μl/well) were collected at 40 h and analyzed for IL-2 by ELISA. Alternatively, after 5 or 7 days, cells for each culture condition were pooled, and live cells were recovered by Ficol-Hypaque gradient centrifugation, washed twice in PBS, and replated (2×10^5 cells/well in 400 μl) onto anti-CD3-coated (145-2C11 at 1 μg/ml) 48-well plates. Supernatants were harvested after 44 h and analyzed for cytokines by ELISA (IL-2, IL-4, and IL-5 using commercial kits from Endogen, Cambridge, MA, and IFN-γ using reagents kindly provided by Dr. Robert Schreiber, Washington University (St. Louis, MO).

Results and Discussion

CD8$^-$ DCs are the major splenic APC for naive DO11.10 T cells

In the spleen, the CD8$^-$ DC subpopulation is considered the critical initiator of primary immune responses compared with the other DC subset and other APCs (9, 17). In this study, Ag-specific T cells from DO11.10 $\times$ RAG-2$^{-/-}$ transgenic mice were used in in vitro assays to compare DCs and other APCs in naive T cell proliferation and differentiation. DC subpopulations and other APCs of the spleen were purified by multiparameter flow cytometric cell sorting to avoid the necessity for in vitro culture, which could modify their physiologic properties. Naive CD4$^+$ T cells were very poorly stimulated by DC-depleted splenocytes. In contrast, proliferation of the TCR transgenic cells stimulated by 10-fold less CD8$^-$ DCs was increased by 50-fold. (Fig. 1*A*). These results indicate that DCs are the major, if not the only, APCs able to stimulate naive DO11.10 T cells in vitro. Thus, additional experiments utilized the CD8$^-$ DC population to study the role of costimulation in proliferation and differentiation of naive T cells.

CD4$^+$ T cell proliferation in the presence of reagents that block either CD28/B7 or LFA-1/ICAM interactions

The role of B7 in the proliferation of naive CD4$^+$ T cells was assessed by stimulating naive DO11.10 T cells with syngeneic DCs in the presence of 1 μg/ml OVA peptide and 5 μg/ml mCTLA4Ig (a soluble CD28 antagonist that binds with high affinity to the B7-1 and B7-2 molecules). IL-2 production at day 2 was inhibited by 80% by mCTLA4Ig, confirming the role of B7 costimulation in IL-2 production (18). In contrast, T cell proliferation was inhibited by only ~50% at day 4 with mCTLA4Ig (Fig. 1*B*). This result differs from previous reports in which late proliferation of T cells stimulated by total splenocytes was almost completely inhibited in the presence of CD28 blockade (19, 20). However, in those systems, macrophages and B cells may play a role in the stimulation of T cells, especially as mixed naive and memory T cell populations may be present in these RAG$^+$ mice. In fact, contrary to naive T cells, activated T cells can be efficiently stimulated by B cells (21). Thus, stimulation of pure naive T cells by DCs, as opposed to mixed T cell populations stimulated by the different splenic APCs, may be less dependent on CD28 signaling for proliferative responses. One explanation for the inability of mCTLA4Ig to fully block T cell proliferation is that DCs express other costimulatory molecules that stimulate the T cells.

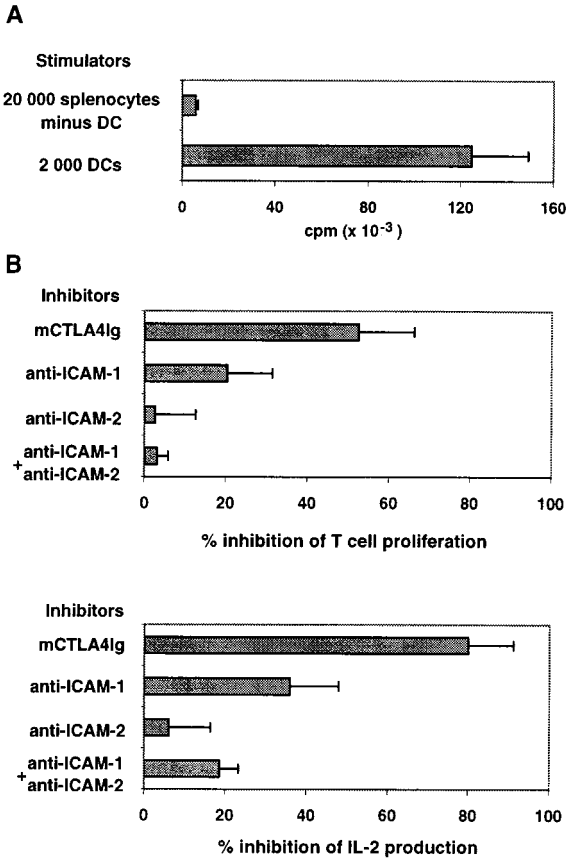

FIGURE 1. Role of CD28/B7 and LFA-1/ICAM interactions in proliferation of naive CD4$^+$ T cells stimulated by DCs. *A*, DO 11.10 T cells (6×10^3/well) were cultured with CD8$^-$ spleen DCs (2×10^3/well) or DC-depleted total splenocytes (2×10^4/well) and OVA peptide for 4 days (peak of T cell proliferation). Results are expressed as the mean cpm of three independent experiments. *B*, DO 11.10 T cells (6×10^3/well) were cultured with CD8$^-$ spleen DCs (2×10^3/well) without (control culture) or with inhibitors of CD28/B7 (mCTLA4Ig at 5 μg/ml), LFA-1/ICAM-1 (10% ascites or 5 μg/ml of anti-ICAM-1 mAb), or LFA-1/ICAM-2 (10 μg/ml of anti-ICAM-2 mAb) interactions. T cell proliferation was measured at day 4 and IL-2 production at 40 h. The percentage of inhibition was calculated as follows: [1 $-$ (cpm or IL-2 of culture with inhibitor/cpm or IL-2 of control culture)] $\times$ 100. These data are representative of four experiments for the culture with mCTLA4Ig and anti-ICAM-1 mAb and two experiments for the culture with anti-ICAM-2 and both anti-ICAM-1 and anti-ICAM-2 mAbs.

Blocking mAbs specific to ICAM-1 and ICAM-2 were used for the examination of the role of LFA-1/ICAM interactions in Ag-specific T cell proliferation in this model. The addition of blocking anti-ICAM-1 mAb had only a minimal effect on both proliferation and IL-2 growth factor production of DO11.10 T cells stimulated by DCs as compared with control cultures. Furthermore, blocking ICAM-2/LFA-1 interaction did not inhibit T cell activation. There were no additive effects of blocking both ICAM-1 and ICAM-2 (Fig. 1*B*). Conflicting results have been reported on the role of ICAM/LFA-1 in T cell proliferation. In artificial systems of T cell stimulation by insect cells or fibroblasts transfected with MHC and costimulatory molecules or by immobilized ICAM-1 and mAb to CD3, ICAM-1/LFA-1 seems to be a major costimulatory pathway (22–24). Other studies using blocking mAbs or ICAM-1- and LFA-1-deficient mice have concluded that ICAM-1/LFA-1 interactions are dispensable in many settings (25–28). Our results suggest that proliferation of naive DO11.10 CD4$^+$ T cell stimulated

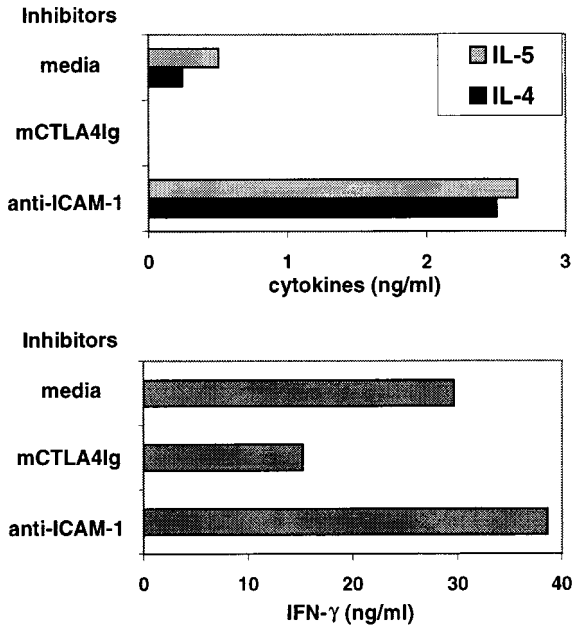

FIGURE 2. Opposite effects of blocking B7 and ICAM-1 costimulation on Th2 cytokine production. DO11.10 T cells (10^4/well) were cultured with $CD8^-$ spleen DCs (2×10^3/well), OVA peptide, and no blocking reagent (media) or either blocking CD28/B7 (mCTLA4Ig at 5 μg/ml) or LFA-1/ICAM-1 (anti-ICAM-1, 10% ascites of Yn1 mAb) interactions. After 5 days of primary stimulation, blast T cells were harvested and restimulated with immobilized anti-CD3. IL-4, IL-5, and IFN-γ secretion was measured after 44 h. A representative experiment from two independent experiments is shown.

by DCs is only partially dependent on LFA-1/ICAM-1 and LFA-1/ICAM-2 interactions (<20% inhibition in the presence of blocking mAbs). Anti-LFA-1 mAb blocked 40% of the T cell proliferative response and 80% of IL-2 production (data not shown). The results can be explained in several ways. First, a third ligand of LFA-1 that has been identified on human APCs may also exist in mice (29). Alternatively, a regulatory LFA-1 signaling pathway exists that is triggered by the anti-LFA-1 mAbs, as previously suggested (30). Finally, the anti-LFA-1 mAb blocks the LFA-1/ICAM interactions more efficiently than the combination of anti-ICAM-1 and anti-ICAM-2 mAbs.

Blocking CD28/B7 and LFA-1/ICAM costimulation inhibits and promotes Th2 cytokine production, respectively

As stated above, costimulatory events have been shown to effect the differentiation of Th cells both in vitro and in vivo. Thus, the role of CD28/B7 and LFA-1/ICAM-1 costimulation in Th differentiation was examined. Naive $CD4^+$ T cells from DO11.10 $\times$ $RAG2^{-/-}$ were cultured with DCs and 1 μg/ml (0.5 μM) OVA 323–339 for 5 days. Since levels of IFN-γ, IL-4, and IL-5 cytokines were not detected in the primary culture (data not shown), cells were harvested and restimulated with immobilized anti-CD3 for 2 days, and cytokines in the culture supernatant were quantified by ELISA. In the absence of blocking reagents, the T cells differentiated into a Th1-like phenotype, producing high levels of IFN-γ (15–30 ng/ml) and low levels of IL-4 and IL-5 (0.15–0.5 ng/ml) (Fig. 2). Blocking B7 costimulation during primary culture with mCTLA4Ig completely inhibited Th2 cytokine production, with less effect on IFN-γ production (Fig. 2), as previously observed (7, 31).

In contract, blocking LFA-1/ICAM-1 interaction had limited effects on IFN-γ and IL-2 (Fig. 1*B*) production but significantly enhanced Th2 responses. The production of IL-4 and IL-5 was increased by 25- to 40-fold in the presence of anti-ICAM-1 mAb in primary culture as compared with control cultures (Figs. 2 and 3). Furthermore, the addition of purified anti-ICAM-2 mAb resulted, on average, in a 15-fold increase in IL-4 and IL-5 production as compared with the control culture (Fig. 3). Thus, ICAM-2 regulated Th2 T cell differentiation, although apparently less efficiently than ICAM-1. Blocking both ICAM-1 and ICAM-2 in primary culture had a dramatic synergistic effect. Compared with control culture, 100-1000 times more IL-4 and IL-5 was produced in the presence of the combined mAb blockade. A similar increase of Th2 cytokines was observed in the presence of blocking anti-LFA-1 mAb (Fig. 3). Since the CD28/B7 blockade and ICAM/LFA-1 blockade resulted in opposite effects on Th cell development, combined treatments were examined. Addition of mCTLA4Ig to the culture with blocking anti-ICAM-1 mAb resulted in a significantly lower increase in Th2 cytokine production, suggesting that regulation of Th2 development by LFA-1/ICAM was dominant but CD28/B7 dependent (data not shown). The similar effect of anti-ICAMs and anti-LFA-1 mAbs strongly suggests that the mAbs are indeed acting by blocking LFA-1/ICAM interactions. However, ICAMs and LFA-1 molecules are expressed on T cells. Therefore, we tested whether the anti-ICAM-1 and anti-LFA-1 Abs have direct effects on T cells in the absence of APCs. Purified T cells were stimulated with immobilized anti-CD3 plus anti-CD28. The addition of soluble anti-ICAM-1 or anti-LFA-1 mAbs had no effect on Th2 cytokines in this assay, supporting the hypothesis that the mAbs were acting by blocking LFA-1/ICAM interactions between T cells and APCs (Table I).

The role of LFA-1/ICAM interactions in the regulation of Th2 cytokine production was unexpected. It has been suggested that the major function of LFA-1/ICAM interactions is to stabilize the APC/T cell conjugation, increasing signaling through the TCR (signal 1) or possibly costimulatory molecules. The hypothesis of an "adhesion only" role of LFA-1 has been emphasized in a recent publication showing that LFA-1 facilitates generation of signal 1 (32). This scenario predicts that blocking LFA-1/ICAM interactions should be equivalent to lowering OVA peptide dose in our system. In similar experimental conditions, Hosken et al. showed that intermediate levels (0.14–1.2 μM) of OVA peptide (the concentrations we used for experiments described in Figs. 2 and 3) drive Th1 differentiation, whereas very low levels of peptides (0.01–0.04 μM) induce Th2 differentiation (3). We do not believe that the increase of Th2 cytokines observed after blocking LFA-1/ICAM interaction is due to less signal 1 for several reasons. First, under conditions using lower doses of OVA peptide, there was a significant inhibition of DO11.10 T cell proliferation and a severe drop in IL-2 production (Ref. 3 and data not shown). Such inhibition of T cell stimulation was not observed with anti-ICAM-1 and anti-ICAM-2 blocking Abs (Fig. 1*B*). Second, the increase in Th2 cytokines is much more dramatic with blocking anti-ICAM Abs (100- to 1000-fold increase) than with lowering OVA peptide doses (4- to 10-fold increase; Ref. 3 and data not shown). Third, whatever the dose of OVA peptide (0.025, 0.1, 0.5, 1, or 50 μg/ml), blocking LFA-1/ICAM interactions always resulted in an increase of Th2 cytokines (data not shown). Finally, low doses of OVA peptide resulted in a decrease in IFN-γ production (Ref. 3 and data not shown) which was not observed after blocking both ICAM-1 and ICAM-2 molecules (Fig. 3). However, it is possible that the increase in Th2 cytokines following the addition of anti-LFA-1 Ab to the culture was due, in part, to a lower signal 1. Indeed, with anti-LFA-1 Ab, but not with anti-ICAM-1 and anti-ICAM-2 Abs, T cell proliferation was reduced in primary

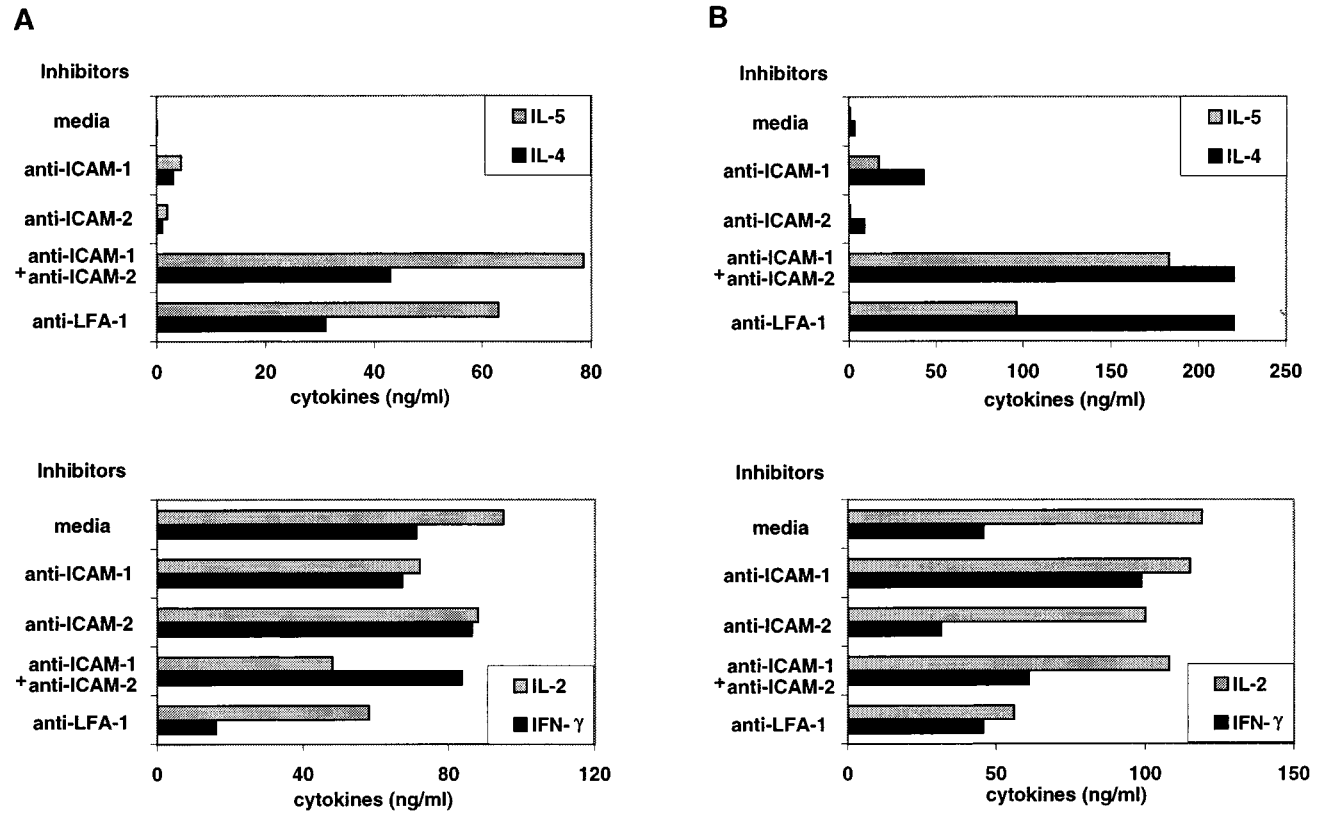

FIGURE 3. Synergistic up-regulation of Th2 cytokines by blocking ICAM-1/LFA-1 and ICAM-2/LFA-1 interactions. DO11.10 T cells (10^4/well) were cultured with $CD8^-$ spleen DCs (2×10^3/well), OVA peptide, and no blocking reagent (media) or a variety of mAbs blocking ICAM/LFA-1 interaction: anti-ICAM-1 (5 μg/ml), anti-ICAM-2 (10 μg/ml), or anti-LFA-1 (5 μg/ml) mAbs. After 5 days, blast T cells were restimulated with immobilized anti-CD3. IL-4, IL-5-, IL-2-, and IFN-γ-secreted cytokines were measured after 44 h. *A* and *B* represent two independent experiments. In *experiment B*, levels of IL-4 in culture with anti-ICAM-1 and anti-ICAM-2 Abs or with anti-LFA-1 Ab were >220 ng/ml.

culture, and IFN-γ and IL-2 inconstantly diminished in secondary culture (Fig. 3).

In summary, our findings show that the LFA-1/ICAM interactions have a major role in the regulation of IL-4 and IL-5 production by $CD4^+$ T cells. The effect appears to be dominant because the ligation of CD28/B7 alone promotes Th2 cytokines, while coligation of LFA-1/ICAM is inhibitory. It should be emphasized that the effect of LFA-1/ICAM blockade is not limited to the DO11.10 system described herein. In separate studies, we have observed that the Th2 cytokines produced by C57BL/6 T cells responding to BALB/c APCs in an allogeneic MLR are enhanced by the addition of either anti-LFA-1 or anti-ICAM-1 mAbs (data not shown). Thus, the regulation of Th1/Th2 balance by LFA-1/ICAMs is effected in at least three different systems: Ag-specific stimulation; MLR and anti-CD3 Ab stimulation. In addition, Th2 cytokine production was observed using T cells from the Th2-prone BALB/c mice, the Th1-prone C57BL/6 mice, and human T cells (33).

Table I. *Effects of anti-ICAM-1 and anti-LFA-1 mAbs on purified T cells stimulated by anti-CD3 and anti-CD28*[a]

	Cytokines (primary culture)			Cytokines (secondary culture)		
Inhibitors	IL-4	IL-5	IFN-γ	IL-4	IL-5	IFN-γ
Medium	1.6	1.1	0.9	32	79	9
Anti-ICAM-1	1.5	1.4	1.0	28	76	12
Anti-LFA-1	1.0	1.1	1.0	36	83	10

[a] Purified T cells (2×10^6/well) were stimulated with immobilized anti-CD3 (2C11) and anti-CD28 (pv1) in 24 wells. After 3 days, the cells were rested for 2 days with 20 U/ml IL-2 and restimulated with immobilized anti-CD3. The Abs to ICAM-1 and LFA-1 (10 μg/ml) were added during the primary culture and the rest periods. Supernatant were harvested at day 3 of primary culture and at day 5 of secondary culture, and cytokine content was measured by ELISA. The concentrations of cytokines are expressed in ng/ml.

The mechanism by which LFA-1/ICAM interactions suppress Th2 development is unclear. However, it is unlikely that decreased adhesion or the strength of signal 1 is responsible. We believe it is more likely that unique signals are delivered via this pathway, either into the T cells or perhaps the DCs that inhibits Th2 development. In a simple system in human, coimmobilized anti-CD3 and ICAM-1 resulted in the differentiation of naive T cells into IFN-γ-secreting cells consistent with the existence of LFA-1 signaling into T cells (33). Our results are particularly relevant to ongoing clinical studies in which the benefits of blocking LFA-1/ICAM interactions in allograft rejection and autoimmunity are being examined. Our findings suggest that part of the beneficial effects of these therapies may be mediated by an increase of Th2 cytokines, which have anti-inflammatory properties (1).

Note added in proof. Similar observations that Th2 cytokine production is regulated by ICAM/LFA-1 interaction has been reported by C. R. Luksch, O. Winqvist, N. E. Ozaki, L. Karlsson, M. R. Jackson, P. H. Peterson, and S. R. Webb.

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