

LFA-1 co-stimulation inhibits T_H2 differentiation by down-modulating IL-4 responsiveness

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Keywords: cytokine receptors, GATA-3, signal transduction, STAT6, T_H1/T_H2 cells

Abstract

Initial T cell activation in the context of different co-stimulatory receptors can influence subsequent lineage commitment into T_H effector cell subtypes. Specifically, CD28 co-stimulation promotes T_H2 differentiation, whereas leukocyte function-associated antigen-1 (LFA-1) co-stimulation promotes T_H1 differentiation and inhibits T_H2 differentiation. In this report, we have addressed the mechanism of LFA-1-mediated inhibition of T_H2 responses. We show that co-stimulation through LFA-1 does not decrease early IL-4 secretion, but rather induces a loss in IL-4 responsiveness. T cells primed in the context of LFA-1 co-stimulation require a 5-fold increase in the concentration of IL-4 required to drive T_H2 differentiation, which is not mediated by a loss in IL-4R expression. To determine whether LFA-1 co-stimulation impacts on proximal signaling from the IL-4R, we first identified a kinetic window where we could separate IL-4-driven T_H2 differentiation from initial T cell priming. T cells were primed for 2 days under different co-stimulation conditions and re-cultured in the presence of IL-4. Subsequent T_H2 differentiation was absolutely dependent on addition of IL-4. Proximal IL-4R signaling, as evidenced by tyrosine phosphorylation of signal transducer and activator of transcription-6 (STAT6), was not inhibited by initial co-stimulation through LFA-1, yet these T cells still required higher amounts of IL-4 and corresponding higher levels of STAT6 activation to up-regulate GATA-3 and induce T_H2 differentiation. Thus, LFA-1 co-stimulation appears to interfere with GATA-3 expression downstream of STAT6. These results suggest that LFA-1 co-stimulation functions as a threshold modulator of T_H2 differentiation, increasing the effective concentration of IL-4 required to drive T_H2 responses.

Introduction

Vertebrates have evolved mechanisms of specific immune recognition in response to a diverse array of pathogens. CD4-positive T cells are essential coordinators of this response and can broadly be divided into two classes of effector cells, T_H1 and T_H2 cells (1). T_H1/T_H2 differentiation can result in effective, ineffective or destructive immune responses depending on the context of the response. T_H1 cells produce pro-inflammatory cytokines, such as IL-2, IFN- γ and tumor necrosis factor- α , and are necessary for the clearance of intracellular pathogens. T_H1 cells have also been implicated in the progression of organ-specific autoimmune diseases. In contrast, T_H2 cells can secrete IL-4, IL-5, IL-6, IL-10 and IL-13, which antagonize inflammatory responses and promote B cell survival, expansion and antibody class switching to IgE, a response that is

necessary for effective host defense against many extra-cellular parasites. T_H2 cells have also been found to be important mediators of a wide range of hypersensitivity diseases. Elucidating the factors that regulate effector T cell differentiation will increase our understanding and ability to manipulate these divergent immune responses, promoting effective immune responses and inhibiting destructive immune responses.

The best-defined signals that regulate T_H1/T_H2 differentiation are derived from cytokines (2–4). Upon initial activation, T cells express both T_H1- and T_H2-associated cytokines (5). External signals, primarily cytokines, then shift the balance of the master regulators of T_H cell differentiation, T-bet for T_H1 cells and GATA-3 for T_H2 cells (6, 7). For T_H2 cells, the primary

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Transmitting editor: K. Murphy

Received 20 October 2004, accepted 28 December 2004

cytokine that promotes differentiation is IL-4. Engagement of the IL-4R induces activation of the transcription factor, STAT6, through tyrosine phosphorylation and nuclear translocation (8). T_H2 cell differentiation is severely compromised in the absence of STAT6 (9–11) and selective activation of STAT6 early during T_H differentiation results in increased T_H2 responses (12, 13). One of the downstream targets of STAT6 is GATA-3. GATA-3 was implicated in T_H2 differentiation when it was isolated in a differential screen of T_H1 and T_H2 cells (14). There is a perfect correlation between GATA-3 and T_H2 cell development, and recent data suggest that deletion of GATA-3 in naive T cells abrogates T_H2 differentiation (15). Furthermore, forced expression of GATA-3 in T_H1 cells can induce a lineage switch to a T_H2 cell phenotype (16). GATA-3 binds to the 3' IL-4 enhancer and can induce chromatin re-modeling of the T_H2 effector cytokine gene cluster, including induction of DNase I hypersensitive sites and histone acetylation (17–21). Thus, GATA-3 is both necessary and sufficient to induce the genetic profile associated with T_H2 cells and so appears to be the master regulator of T_H2 differentiation.

In addition to cytokines, the overall strength of TCR signaling can influence T_H cell differentiation (22–29). However, the precise mechanisms associated with this effect have been difficult to pinpoint, in part because of the complexity of TCR signaling. Also, many studies have utilized knockout mice and it has been difficult to distinguish between developmental changes during thymic maturation, effects on T_H differentiation and modulation of T_H effector cytokine expression. Nevertheless, one interesting correlation is the role of calcium signaling in T_H cell differentiation. T_H2 cells rapidly undergo a loss in sustained calcium signaling (30) and altered peptide ligands that promote T_H2 differentiation induce dramatically reduced calcium responses (31, 32). This correlates with an increase in the ratio of nuclear factor of activated T cells-2 (NFAT2) to NFAT1 in the nucleus and an increase in the induction of early IL-4 transcription (32). Likewise, genetic disruption of NFAT1 expression can enhance T_H2 responses (33). Thus, one mechanism whereby the strength of TCR signaling can modulate T_H cell differentiation may be through calcium-mediated changes in NFAT isoform activation.

The overall strength of TCR signaling can also be modulated by co-stimulatory molecules and co-stimulation through many receptors, including CD28 (34) and leukocyte function-associated antigen-1 (LFA-1) (35–42), can differentially induce T_H1 or T_H2 response. CD28 is the most potent co-stimulatory molecule, promoting T cell responses through a variety of mechanisms, and plays a key role in the generation of T_H2 responses (34). CD28 signaling has been suggested to promote T_H2 differentiation through the up-regulation of IL-2 (43, 44), increased IL-4R sensitivity (45) or up-regulation of GATA-3 and histone acetylation of the T_H2 cytokine locus (46, 47). In contrast to CD28 co-stimulation, engagement of LFA-1, which is also associated with enhanced T cell signaling, inhibits T_H2 differentiation and can promote T_H1 differentiation (35–42). Importantly, LFA-1 can inhibit T_H2 differentiation even in the presence of normal antigen-presenting cells (APC) and CD28 co-stimulation, suggesting that co-stimulation through LFA-1 actively inhibits the differentiation of T_H2 responses (37, 38, 40, 41).

Because all professional APC express intracellular adhesion molecule-1 (ICAM-1), the ligand for LFA-1, it is difficult to

explain how LFA-1-mediated, dominant inhibition of T_H2 differentiation is circumvented during *in vivo* T_H2 responses. In this study, we show that LFA-1 co-stimulation does not inhibit the early IL-4 production necessary for T_H2 differentiation, but rather decreases the ability of the T cells to respond to IL-4. This decrease in responsiveness is not mediated by a loss in IL-4R-induced STAT6 phosphorylation, but rather reflects a failure to up-regulate GATA-3 expression in the presence of STAT6. Thus, LFA-1 co-stimulation may function as a threshold modulator of IL-4-driven T_H2 differentiation, increasing the dependence of T cells on exogenous sources of IL-4.

Methods

Cells

Cell lines derived from the fibrosarcoma 6132A-PRO cell line transfected with I-A^d and ICAM-1 (ProAd-ICAM) or I-A^d and B7-1 (ProAd-B7) have been previously described (39, 48). The ProAd-B7/ICAM cells were generated by super-transfection of the ProAd-B7 cells with ICAM-1. All cell lines were maintained in DMEM supplemented with 10% FCS, 2 mM glutamine, 0.1 mM non-essential amino acids, 40 µg ml⁻¹ gentamycin and 50 µM 2-mercaptoethanol. G418 (200 µg ml⁻¹) and/or MXH (6 µg ml⁻¹ mycophenolic acid, 250 µg ml⁻¹ xanthine and 15 µg ml⁻¹ hypoxanthine) were included in the culture media during routine passage of the cells, but were removed at least 24 h prior to use in T cell assays. The continual expression of I-A^d, ICAM-1 and B7-1 was monitored by flow cytometric analysis. CD4-positive T cells were purified from lymph nodes of DO11.10 TCR transgenic mice by negative selection as previously described (48). Briefly, class II-positive cells and CD8-positive cells were depleted by complement lysis followed by magnetic separation of any remaining antibody-coated cells with sheep-anti-rat and anti-mouse IgG magnetic beads (Dyna, Oslo, Norway). Purity of the remaining CD4-positive population was routinely confirmed by flow cytometry. Purified CD4-positive T cells (1×10^5 ml⁻¹) were stimulated with 2 µg ml⁻¹ of the 323–339 ovalbumin peptide presented by an equal number of mitomycin C-treated APC. Recombinant human IL-2 (Genzyme, Cambridge, MA, USA) was added to provide for T cell expansion (48). To assay for IL-4 production at 24 h, cell concentration was increased to 1×10^6 ml⁻¹. Recombinant mouse IL-4 was from R&D Systems (Minneapolis, MN, USA).

Immunoprecipitation and western blot analysis

Whole-cell lysates were prepared to assay for GATA-3 expression as described (16). Briefly, T cells were lysed in 5% SDS, 0.5 M Tris, pH 6.8, 0.5 mM EDTA, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride (PMSF) for 10 min at room temperature. Lyzed cells were centrifuged at 100 000 r.p.m., and supernatants were separated by electrophoresis on 10% SDSP. After electrotransfer to nitrocellulose, blots were probed with anti-GATA-3 mAb (Santa Cruz Biotech, Santa Cruz, CA, USA). Tyrosine phosphorylation of STAT6 was assayed as described (49). Briefly, 5×10^6 T cells were lysed in 10 mM Tris, pH 7.4, 150 mM NaCl, 0.1% NaN₃, 5 mM EDTA, 0.2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.5% Nonidet P-40, 50 mM NaF, 10 mM Na

pyrophosphate, 1 mM Na orthovanadate, 1 mM PMSF and 25 $\mu\text{g ml}^{-1}$ aprotinin for 30 min at 4°C. STAT6 was immunoprecipitated from lysates using anti-Stat6 antisera (R&D) and separated on 8% SDSP. After electrotransfer to nitrocellulose, parallel blots were probed with either anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology, Lake Placid, NY, USA) or anti-Stat6 (R&D). Blots were visualized using enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA) and the image was captured using the FluorChem 8800 Imaging System (Alpha Innotech, San Leandro, CA, USA).

Results

LFA-1 co-stimulation does not inhibit early IL-4 production

Co-stimulation through LFA-1 has been shown to lead to sustained calcium responses (50, 51). As discussed above, sustained calcium responses do not seem to support T_H2 development and this change in initial T cell signaling appears to reflect a change in NFAT isoform utilization that impacts on the level of early IL-4 transcription (30–33). T_H2 differentiation is dependent on this early IL-4 expression; so, one potential explanation for the ability of LFA-1 to inhibit T_H2 differentiation is that LFA-1 co-stimulation reduces the production of this initial burst of IL-4. To test this hypothesis, we measured IL-4 production 24 h after T cells were stimulated by antigen presented in the context of LFA-1 co-stimulation (by ProAd-ICAM cells), in the context of CD28 co-stimulation (by ProAd-B7 cells) or in the context of both LFA-1 and CD28 co-stimulation (by ProAd-B7/ICAM cells). While IL-4 production by 24 h differed modestly after antigen presentation under different co-stimulation conditions, these differences did not correlate with T_H2 differentiation (Fig. 1). In particular, antigen presentation by the ProAd-B7/ICAM cells reproducibly resulted in the greatest amount of IL-4 production (Fig. 1A) but did not result in T_H2 differentiation (Fig. 1B). When anti-IL-4R antibody was added to inhibit cytokine consumption, the overall levels of detectable IL-4 increased, but the pattern of IL-4 production did not

change (data not shown). These results indicate that the ability of LFA-1 co-stimulation to inhibit T_H2 differentiation is not mediated through inhibition of early IL-4 secretion.

LFA-1 co-stimulation reduces the sensitivity of T cells to IL-4-induced T_H2 differentiation

The ability of LFA-1 to inhibit T_H2 responses in spite of normal levels of early IL-4 production suggests that T cells simulated in the context of LFA-1 co-stimulation may become less responsive to IL-4-induced T_H2 differentiation. To test this possibility, various amounts of exogenous IL-4 were added to the initial T cell activation cultures. Consistent with previous results (39, 41), high concentrations of IL-4 could restore T_H2 differentiation to T cells primed with antigen in the context of LFA-1 co-stimulation (ProAd-ICAM, Fig. 2). When the levels of IL-4 were reduced, the T_H2 response rapidly dropped off and significant secondary IL-4 responses required an IL-4 concentration of 3–5 ng ml^{-1} (100–200 pM), which approximates the K_d of the IL-4R. In contrast, when T cells were primed in the context of CD28 co-stimulation (ProAd-B7, Fig. 2), they were responsive to very low levels of exogenous IL-4 and addition of 0.05 ng ml^{-1} (3 pM) resulted in enhanced T_H2 differentiation. Thus, LFA-1 co-stimulation appears to inhibit IL-4 responsiveness after initial T cell activation, increasing the threshold required to drive T_H2 differentiation above the level of IL-4 produced during T cell priming.

IFN- γ has been shown to antagonize T_H2 differentiation (2), raising the possibility that the ability of LFA-1 to inhibit T_H2 differentiation is indirect, due to enhanced secretion of IFN- γ . To test this possibility, we included anti-IFN- γ -blocking antibodies at the onset of the IL-4 dose responses as described above in Fig. 2. We found that anti-IFN- γ had no effect on the sensitivity to IL-4-induced T_H2 differentiation in T cells primed by ProAd-ICAM cells (data not shown). Interestingly, we did detect a significant increase in T_H2 responses in T cells primed by ProAd-B7 cells by the addition of anti-IFN- γ , indicating that CD28 co-stimulation does promote IFN- γ secretion at a level

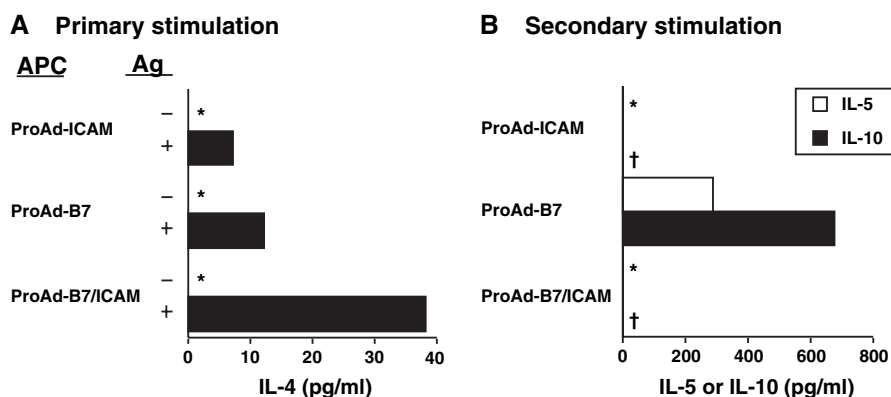


Fig. 1. LFA-1 co-stimulation does not inhibit IL-4 production during the first 24 h after antigen presentation. (A) CD4-positive T cells purified from DO11.10 transgenic mice were stimulated with or without 2 $\mu\text{g ml}^{-1}$ ovalbumin peptide (OVA) (antigen) presented by an equal number of ProAd-ICAM, ProAd-B7 or ProAd-B7/ICAM cells (APC). After 24 h, supernatants were assayed for the presence of IL-4 by capture ELISA. IL-4 levels less than the limit of detection (5 pg ml^{-1}) are indicated (asterisk). These data are representative of three independent experiments. (B) CD4-positive T cells purified from DO11.10 TCR transgenic mice were stimulated with 2 $\mu\text{g ml}^{-1}$ OVA presented by an equal number of ProAd-ICAM, ProAd-B7 or ProAd-B7/ICAM cells for 7–10 days. T cells were harvested and re-stimulated with plate-bound anti-CD3 (1 $\mu\text{g ml}^{-1}$) for 24 h and supernatants were assayed by capture ELISA for the presence of T_H2 cytokines, IL-5 and IL-10 (B). Cytokine levels below the limit of detection of 120 pg ml^{-1} for IL-5 (asterisk) and IL-10 (dagger) are indicated. One of three representative independent experiments is shown.

that inhibits T_H2 differentiation. However, we did not detect this effect following LFA-1 co-stimulation, suggesting that the ability of LFA-1 to inhibit T_H2 responses may be a direct effect on the signals that promote T_H2 differentiation.

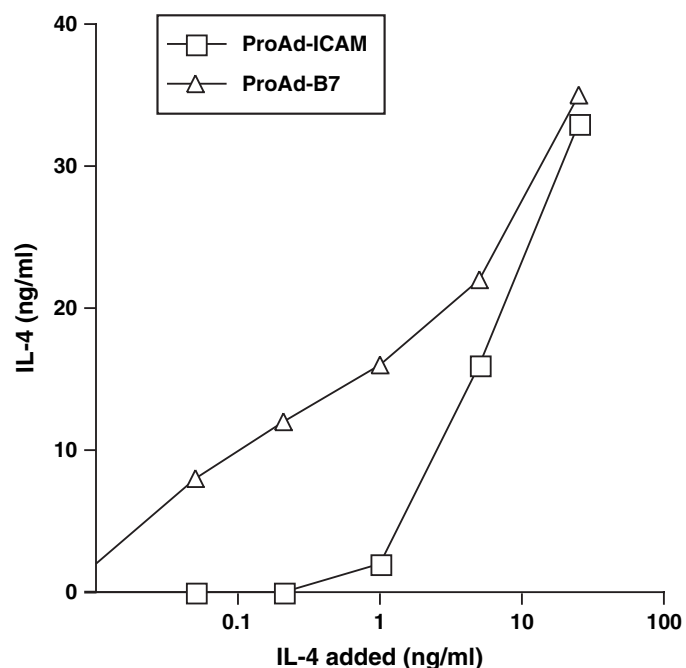


Fig. 2. LFA-1 co-stimulation reduces IL-4 responsiveness. CD4-positive T cells purified from DO11.10 TCR transgenic mice were stimulated with $2 \mu\text{g ml}^{-1}$ ovalbumin peptide presented by an equal number of ProAd-ICAM or ProAd-B7 cells. The indicated concentrations of IL-4 shown on the x-axis were added to parallel wells at the initiation of culture. After 7 days, T cells were harvested and re-stimulated with plate-bound anti-CD3, and supernatants were assayed 24 h later for the presence of IL-4 by capture ELISA (y-axis).

LFA-1 inhibits the early amplification of IL-4 responses

LFA-1-mediated inhibition of T_H2 differentiation is not mediated through down-modulation of IL-4R expression (data not shown). This suggests that LFA-1 might modulate signaling through the IL-4R. However, the functional assays for T_H2 differentiation rely on assaying cytokine expression during secondary T cell stimulation 7 days after initial priming. Before we could assay IL-4R signaling events, it was critical to determine the kinetic window, when activated T cells were responsive to IL-4-induced T_H2 differentiation. We identified this window through two different approaches. First, we evaluated the amplification of T_H2 responses directly, by measuring the production of IL-4 (Fig. 3) and up-regulation of GATA-3 (Fig. 4) during an ongoing response. We measured IL-4 levels over time after antigen presentation by ProAd-ICAM, ProAd-B7 or ProAd-B7/ICAM cells with or without the addition of low levels of IL-4 (200 pg ml^{-1}). This concentration of IL-4 was chosen because it promoted T_H2 differentiation in ProAd-B7-primed T cells, but not in ProAd-ICAM- or ProAd-B7/ICAM-primed cells (Fig. 2 and data not shown). As shown in Fig. 3A, IL-4 was undetectable 2 days after stimulation under any conditions. This is not surprising; even at a 10-fold higher cell density, IL-4 is difficult to detect early after stimulation (Fig. 1A). After antigen presentation by ProAd-B7 cells, IL-4 is detectable on days 3 and 4. In contrast, IL-4 was not detectable at all within the first 4 days after antigen presentation by ProAd-ICAM or ProAd-B7/ICAM cells (Fig. 3A). Addition of 200 pg ml^{-1} IL-4 enhanced IL-4 production 10-fold after antigen stimulation by ProAd-B7 cells, but did not significantly increase IL-4 production above the baseline of IL-4 initially added to ProAd-ICAM- or ProAd-B7/ICAM-primed T cells (Fig. 3B). These results support the idea that LFA-1 co-stimulation, even in the presence of CD28 co-stimulation, inhibits IL-4 responsiveness after initial T cell activation. Furthermore, these results indicate that IL-4 is inducing T_H2 cell differentiation on or about day 2 after initial T cell activation. Similar kinetics and co-stimulation

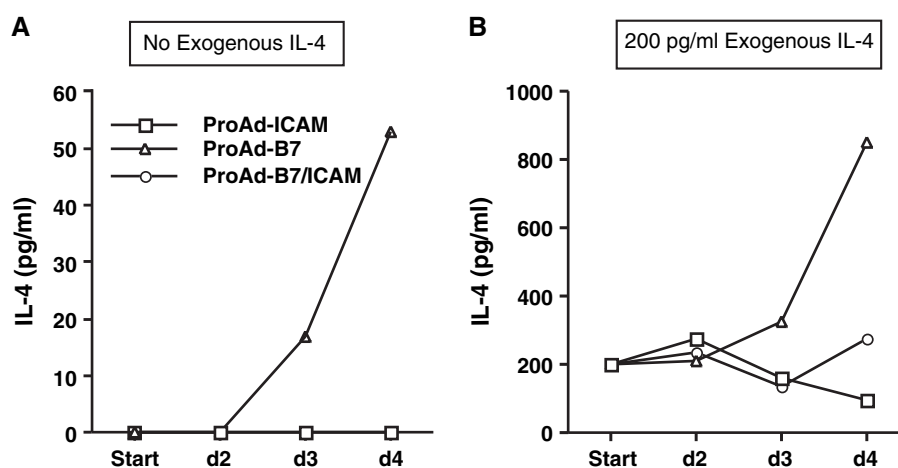


Fig. 3. LFA-1 co-stimulation inhibits the IL-4-dependent amplification of T_H2 responses during T cell activation. (A) CD4-positive T cells purified from DO11.10 transgenic mice were stimulated with $2 \mu\text{g ml}^{-1}$ ovalbumin peptide presented by an equal number of ProAd-ICAM, ProAd-B7 or ProAd-B7/ICAM cells. At various time points after the initiation of the culture, parallel wells were assayed for the presence of IL-4 by capture ELISA. (B) CD4-positive T cells were stimulated and assayed as described above except that 200 pg ml^{-1} IL-4 was added at the initiation of culture. Data shown are representative of three independent experiments.

dependence were seen when the level of GATA-3 expression was assayed by western blots (Fig. 4).

To further clarify the time when activated T cells were responsive to IL-4-induced T_H2 differentiation, we varied the

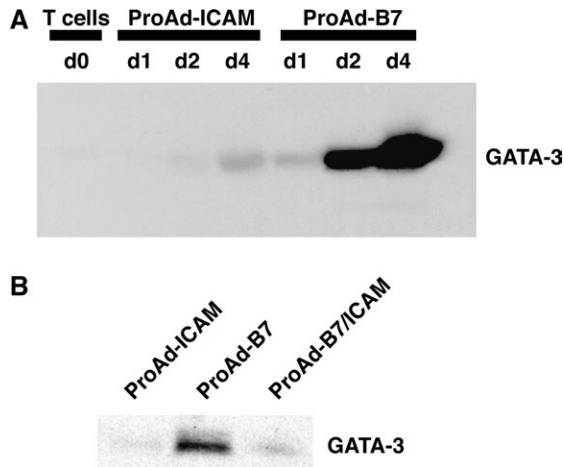


Fig. 4. LFA-1 co-stimulation prevents up-regulation of GATA-3 expression. (A) CD4-positive T cells purified from DO11.10 transgenic mice were stimulated with $2 \mu\text{g ml}^{-1}$ ovalbumin peptide (OVA) presented by an equal number of ProAd-ICAM or ProAd-B7 cells. At 1, 2 and 4 days after initiation of the culture (d1, d2 and d4, respectively), whole-cell lysates were prepared, normalized for T cell number and analyzed for GATA-3 expression by western blot. Lysates from unstimulated T cells (d0) were analyzed in parallel. (B) CD4-positive T cells purified from DO11.10 transgenic mice were stimulated with $2 \mu\text{g ml}^{-1}$ OVA presented by an equal number of ProAd-ICAM, ProAd-B7 or ProAd-B7/ICAM cells. After 7 days, whole-cell lysates were prepared, normalized for T cell number and analyzed for GATA-3 expression by western blot. The position of GATA-3 is indicated in both panels on the right.

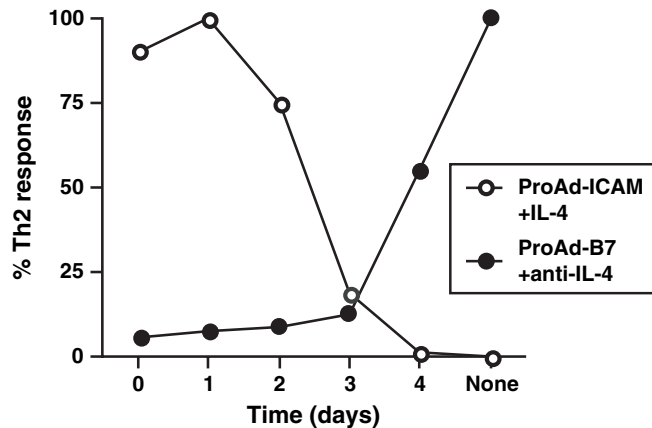


Fig. 5. Exogenous IL-4 and anti-IL-4 define a kinetic window of IL-4-dependent T_H2 differentiation. CD4-positive T cells purified from DO11.10 TCR transgenic mice were stimulated with $2 \mu\text{g ml}^{-1}$ ovalbumin peptide presented by an equal number of ProAd-ICAM (open circles) or ProAd-B7 (closed circles). IL-4 was added to the ProAd-ICAM-stimulated cells and anti-IL-4 was added to the ProAd-B7-stimulated cells at the initiation of culture (day 0), at 1–4 days after the initiation of culture or not at all (none). On day 7, T cells were harvested and re-stimulated with plate-bound anti-CD3, and supernatants were assayed 24 h later for the presence of IL-4 by capture ELISA. Data are represented as percent maximum T_H2 (IL-4) response.

time of IL-4 addition to the T cell cultures stimulated with antigen presented in the context of LFA-1 co-stimulation (Fig. 5). Addition of IL-4 anytime within the first 2 days significantly enhanced T_H2 differentiation, but by day 3, the enhancement was reduced, suggesting that by this time the T cells were losing responsiveness to IL-4. When T cells are primed in the context of CD28 co-stimulation, endogenous IL-4 drives T_H2 differentiation and this is blocked by the addition of anti-IL-4. When we varied the time of addition of anti-IL-4, we also found that the key window of T cell responsiveness to IL-4 was between days 2 and 3, and by day 4 the ability of anti-IL-4 to inhibit T_H2 differentiation was diminished. Similar results showing a loss in IL-4-induced T_H2 development following initial TCR activation have recently been reported (52). Taken together, these results indicate that co-stimulation-dependent and IL-4-driven amplification of T_H2 responses occurs between days 2 and 4 after T cell activation.

LFA-1-mediated inhibition of IL-4 responsiveness is not mediated through inhibition of STAT6 phosphorylation

The kinetic analysis described above indicate that T cells that have been primed for 2 days should be responsive to IL-4-induced T_H2 differentiation. We, therefore, chose this time point to determine whether initial priming through LFA-1 would inhibit IL-4R signal transduction. T cells were primed in the context of

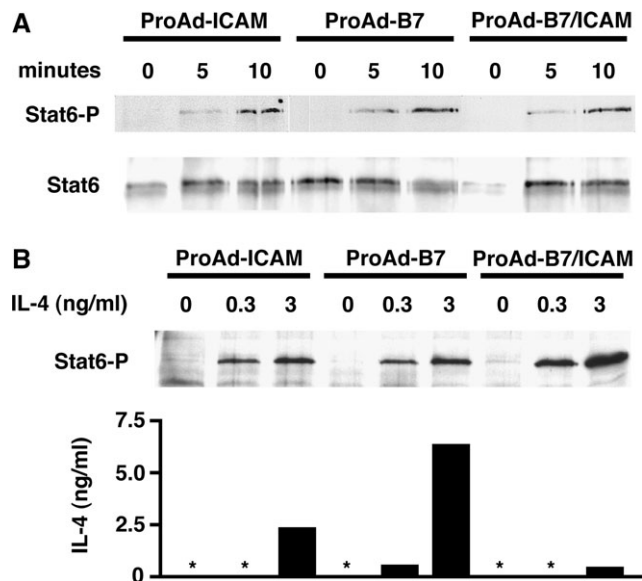


Fig. 6. LFA-1 co-stimulation does not induce long-term inhibition of STAT6 activation. (A) CD4-positive T cells purified from DO11.10 TCR transgenic mice were stimulated with $2 \mu\text{g ml}^{-1}$ ovalbumin peptide presented by an equal number of ProAd-ICAM, ProAd-B7 or ProAd-B7/ICAM cells. After 2 days, the cells were harvested, starved for 2 h in low serum media and stimulated with 0.3 ng ml^{-1} of IL-4 for 5 or 10 min. STAT6 was immunoprecipitated from cellular lysates and assayed for tyrosine phosphorylation (Stat6-P) and total STAT6 (Stat6) by western blot. (B) T cells were primed and stimulated with 0.3 or 3.0 ng ml^{-1} IL-4 as in (A). STAT6 was immunoprecipitated from cellular lysates and assayed for tyrosine phosphorylation by western blot (top panel). An aliquot of the same cells was re-cultured and re-stimulated on day 7 with plate-bound anti-CD3 to measure secondary IL-4 production (bottom).

LFA-1 and/or CD28 co-stimulation for 2 days and the T cells were purified and stimulated with 0.3 ng ml^{-1} of IL-4. This concentration of IL-4 is sufficient to drive T_H2 proliferation only in the absence of LFA-1 co-stimulation (see Figs 2 and 3). IL-4R signaling was assayed by tyrosine phosphorylation of STAT6 at 5 and 10 min after activation (Fig. 6A). No differences in the magnitude or kinetics of STAT6 phosphorylation were detected, suggesting that LFA-1 co-stimulation does not inhibit IL-4R signaling.

To confirm that the T cells in which we were assaying IL-4R signaling were still responsive to IL-4-driven T_H2 differentiation and had retained their reduced sensitivity to IL-4 following initial priming in the context of LFA-1 co-stimulation, aliquots from the same primed T cells were assayed for IL-4R signaling and for subsequent T_H2 differentiation (Fig. 6B). In this experiment, T cells were primed in the context of LFA-1 and/or CD28 co-stimulation in the presence of anti-IL-4 to eliminate any possible T_H2 promoting effects of endogenous IL-4 during the initial priming. Under these conditions, T_H2 differentiation was entirely dependent on addition of IL-4 when the T cells were re-cultured on day 2. Importantly, as we had detected earlier, T cells stimulated in the presence of LFA-1 co-stimulation (ProAd-ICAM- and ProAd-B7/ICAM-primed T cells) were less sensitive to IL-4-driven T_H2 differentiation than T cells stimulated in the absence of LFA-1 co-stimulation (ProAd-B7-primed T cells) (Fig. 6B, bottom panel). In contrast, when aliquots of the same T cells were assayed, IL-4R-induced STAT6 phosphorylation was not effected by LFA-1 co-stimulation (Fig. 6B, top panel). These data indicate that LFA-1-mediated inhibition of T_H2 responses is not mediated through down-modulation of IL-4R-induced STAT6 activation, and rather suggest that initial priming of T cells in the context of LFA-1 inhibits a downstream event that regulates STAT6-induced expression of GATA-3.

Discussion

T_H cell lineage commitment is driven by positive amplification loops that reinforce early signals and establish new thresholds, effectively driving lineage commitment. Although most of these signals are derived by cytokine signaling, co-stimulation can have both positive and negative influences on T_H cell differentiation. In this report, we have examined the inhibition of T_H2 differentiation induced by T cell priming in the context of LFA-1 co-stimulation. We found that this function of LFA-1 is not mediated through inhibition of initial production of IL-4. Rather, LFA-1 co-stimulation suppresses the IL-4-dependent amplification of T_H2 differentiation that occurs 2–4 days after stimulation. LFA-1 shifts the dose of IL-4 responsiveness, increasing the threshold required to drive T_H2 differentiation above the level of IL-4 produced during T cell priming. This correlates with a loss in GATA-3 up-regulation. Surprisingly, this loss in IL-4 responsiveness is not mediated through a decrease in proximal IL-4R signaling events, as evidenced by tyrosine phosphorylation of STAT6. These data indicate that LFA-1-mediated inhibition of T_H2 differentiation may result in part through the dissociation of STAT6-induced GATA-3 expression.

LFA-1 could act through several different mechanisms to inhibit T_H2 differentiation. The suppressors of cytokine signaling (SOCS) family of proteins have been implicated in T_H cell

differentiation (53). However, SOCS proteins inhibit Janus kinase (JAK) activation of STAT proteins and so if LFA-1 co-stimulation was enhancing SOCS expression, it would have led to the reduction of STAT6 phosphorylation, which was not detected. One possibility is that LFA-1 co-stimulation could inhibit STAT6 activity. For example, several STAT family members can undergo secondary serine phosphorylation at a consensus mitogen activated protein (MAP) kinase site and this has been shown to enhance their transcriptional activity (54). Although there is evidence for serine and threonine phosphorylation of STAT6, the consensus MAP kinase site is not found in STAT6 and the functional relevance of serine/threonine phosphorylation of STAT6 has not been established (55).

Alternatively, LFA-1 engagement could function through the induction of factors that prevent activated STAT6 from binding its consensus DNA element. For example, BCL-6 inhibits STAT6 transcriptional activity by competing for its consensus DNA element (56). BCL-6-deficient mice show increased T_H2 responses, implying that BCL-6 may regulate the threshold of STAT6 activation required to bring about T_H2 differentiation (57, 58). Likewise, members of the protein inhibitor of activated STAT (PIAS) family bind to activated STAT family dimers and target them for small ubiquitin-like modifier (SUMO)-dependent degradation (59–61). These mechanisms are attractive because they could be long lasting, persisting after initial T cell activation in the presence of LFA-1 co-stimulation and could be overcome by high concentrations of STAT6, induced by exogenous IL-4.

Finally, LFA-1 co-stimulation may inhibit T_H2 responses independent of STAT6 activation. Dissociation of STAT6 and GATA-3 regulation has also been shown for the inhibition of T_H2 development by transforming growth factor- β (62, 63). Although it has been clearly shown that signaling through STAT6 can lead to up-regulation of GATA-3 expression, there is no evidence that STAT6 directly regulates GATA-3 expression. Regulation of GATA-3 expression at the molecular level is not well understood. In general, the GATA family of proteins functions by controlling cell lineage commitment in different cell types at different stages of development. Specificity is maintained through tissue-specific and developmentally regulated control of GATA transcription, and because other factors regulate the accessibility of target genes to GATA proteins. The GATA-3 gene is encoded in a large locus; all the regulatory elements associated with developmentally regulated, tissue-specific expression of the GATA-3 gene have not been identified (64–67). In naive T cells, GATA-3 expression and function are held in check by the transcriptional silencer, Runx1, and by the GATA interacting protein, friend of GATA (FOG), respectively (68–70). It is possible that LFA-1 modulates the expression or activity of these regulatory proteins, and inhibits the autoinduction of GATA-3 (19). Alternatively, LFA-1 co-stimulation may inhibit the induction of another transcription factor that works in concert with STAT6 to up-regulate GATA-3 expression.

The ability of LFA-1 to shift the dose response of IL-4-mediated T_H2 differentiation may be consequential for *in vivo* immune responses. First, this provides an explanation of how T_H2 responses can be generated by antigen presentation by conventional APC that typically express ICAM-1. Co-stimulation through LFA-1 impacts on the threshold of IL-4

that promotes T_H2 differentiations. Interestingly, APC vary in their relative expression of ICAM-1 and B7-1 and B7-2 both dynamically over time and between different types of APC. Resting B cells for example express ICAM and low levels of B7 that increase after mitogen stimulation or T cell help. As a result, the state of B cell activation during antigen presentation can change T_H cytokine production (71). Classically defined by morphology, dendritic cells are being increasingly recognized as a heterogeneous population of cells and it is clear that different types of dendritic cells can elicit different T_H cytokine responses (72). While the influence of dendritic cells has been explained primarily in terms of their cytokine production, it is notable that dendritic cell subsets differ in their B7 and ICAM expression (41, 73) and the high level of ICAM-1 expression on poly(inosinic-cytidylic)acid-activated DC1 cells contributes to their ability to promote T_H1 differentiation (41).

Because *in vivo* IL-4 acts in a paracrine fashion on cells in a defined anatomical space, it is difficult to generalize about IL-4 concentrations *in vivo*. However, the bulk culture concentrations we have used as a model are appropriate in relation to the binding characteristics of the IL-4R. At an estimated K_d of 300 pM (8), the lower doses of IL-4 at which we detect differences in IL-4 responsiveness are well below saturation of the receptor. With only a small percentage of the IL-4R engaged, changes in receptor sensitivity could dramatically alter the threshold of IL-4 required for differentiation. As a consequence, co-stimulation may couple small differences in IL-4 concentration to the amplification of IL-4 production through positive feedback. In this regard, the ability of LFA-1 to inhibit IL-4 responsiveness may be an important determinate factor in T_H2 differentiation. There are several important consequences of this model. First, unlike a model in which co-stimulation acts directly on T cell differentiation events, this effect of LFA-1 modulates the response of T cells to their environment. Thus, LFA-1 co-stimulation will influence T_H differentiation regardless of the relevant *in vivo* source of IL-4. Secondly, modulation of T_H2 responses through IL-4 and IL-4 antagonists has been proposed as a potential immunomodulation therapy for autoimmune and atopic diseases, respectively. The ability of LFA-1 co-stimulation to alter the threshold of IL-4 responsiveness may be an important factor in designing efficacious strategies based on this approach.

Acknowledgements

The authors thank Tim Mossman for comments on the manuscript and Anne Sperling and Julie Auger for providing reagents. This work was supported by NIH grant AI 48237 and a grant from the Juvenile Diabetes Fund International to J.M. S.A.J. and B.J.E. were supported by NIH training grant GM-07183.

Abbreviations

APC	antigen-presenting cells
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
ICAM-1	intracellular adhesion molecule-1
JAK	Janus kinase
LFA-1	leukocyte function-associated antigen-1
MAP	mitogen activated protein
PIAS	protein inhibitor of activated STAT
PMSF	phenylmethylsulfonylfluoride
SOCS	suppressors of cytokine signaling

STAT	signal transducer and activator of transcription
SUMO	small ubiquitin-like modifier

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