Costimulation by Purified Intercellular Adhesion Molecule 1 and Lymphocyte Function-associated Antigen 3 Induces Distinct Proliferation, Cytokine and Cell Surface Antigen Profiles in Human "Naive" and "Memory" CD4+ T Cells

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Summary

Activation of resting human CD4+ "naive" (CD45RA+CD45RO⁻) and "memory" (CD45RA⁻CD45RO+) T cells requires costimulatory signals in addition to engagement of the T cell receptor/CD3 complex (TCR/CD3). The adhesion pathways mediated by lymphocyte function-associated antigen 1/intercellular adhesion molecule 1 (LFA-1/ICAM-1) and CD2/LFA-3 are capable of providing such costimulatory signals. Our work shows that these costimulatory adhesion pathways are critically involved in regulation of T cell differentiation/maturation. Evidence for subset-specific costimulatory requirements is demonstrated by the finding that only memory CD4+ T cells were costimulated by LFA-3, whereas both naive and memory CD4+ T cells were costimulated by ICAM-1. In addition, these costimulatory adhesion pathways regulated reciprocal cytokine secretion patterns for interleukin 5 (IL-5) and granulocyte/macrophage colony-stimulating factor (GM-CSF). Repeated costimulation of CD4+ memory T cells with LFA-3 led to secretion of high levels of IL-5, while repeated costimulation with ICAM-1 induced high levels of secreted GM-CSF. Significant interferon γ (IFN-γ) production was observed with either of the costimulatory ligands. Extensive cell surface analysis of these in vitro cultures of peripheral blood derived memory CD4+ T cells, with monoclonal antibodies obtained from the 5th Leucocyte Typing Workshop, revealed differential expression of a singular antigen, CD60. This antigen was preferentially expressed on LFA-3–costimulated cells suggesting a positive correlation between CD60 expression and a T helper type 2–like cytokine profile. In conclusion, this report demonstrates a new functional role for costimulatory adhesion molecules in regulating differential cytokine secretion in human memory CD4+ T cells.

V arious T cell accessory molecules have been shown to be capable of providing costimulatory signals required for T cell proliferation (1–8). Many of these costimulatory molecules are also involved in adhesive interactions of the T cells with apposing cells and extracellular matrix components (9–12). These shared adhesive properties have raised the question as to (a) whether a common intracellular signal is involved in the action of these costimulatory adhesion pathways (13); and (b) what might be the in vivo relevance of these seemingly redundant multiple T cell costimulatory pathways. We and others have proposed models predicting that the unique microenvironment of the site of antigen presentation will influence the differentiation/maturation of T cells into distinct functional effector populations upon antigen-specific

stimulation (8, 14–18). In particular, the locally available costimulatory pathways, ECM proteins, and cytokines may provide the specific combinatorial set of signals necessary for the development of specialized immune responses.

In this study we address, in a simplified in vitro model system, some of these issues by investigating the costimulatory role of adhesion molecules intercellular adhesion molecule 1 (ICAM-1¹; CD54) and leukocyte function-associated antigen 3 (LFA-3; CD58) in mature T cell activation and differentiation. We studied the activation requirements of human resting "naive" (CD45RA+CD45RO⁻) and "memory"

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¹Abbreviations used in this paper: ICAM-1, intercellular adhesion molecule 1; Thp, T helper precursors.

(CD45RA⁻CD45RO⁺) CD4⁺ T cell subpopulations (19). Differentiation into distinct effector populations was examined by determining the levels in the culture supernatants of the various cytokines e.g., IL-5, IFN-γ, and GM-CSF, after TCR/CD3-mediated activation with these multiple costimulatory conditions. FACS® analysis was simultaneously performed on the CD4⁺ T cells derived from these in vitro cultures. Our findings showed subset specific requirements for costimulation by ICAM-1 and LFA-3. Furthermore, repeated costimulation of memory CD4⁺ T cells led to reciprocal cytokine profiles for IL-5 and GM-CSF, and differential expression of the ganglioside antigen GD3 (CD60). These combined results demonstrate the role of costimulatory adhesion molecules in regulation of T cell differentiation.

Materials and Methods

Cells. Human PMBCs from normal donors were separated by Ficoll-Hypaque density-gradient centrifugation. Resting CD4⁺ T cells were subsequently obtained by rigorous immunomagnetic negative selection with Advanced Magnetic Particles (Advanced Magnetic, Cambridge, MA) bound to goat anti–mouse IgG. Negative selection was performed as described (20), using a cocktail of mAbs consisting of anti-HLA class II mAb (IVA12), CD19 mAb (FMC 63), CD16 mAb (3G8), CD11b mAb (NIH11b-1), CD14 mAb (63D3), CD8 mAb (B9.8), and mAb against glycophorin (10F7). For isolation of the reciprocal subsets of CD45RO⁺ and CD45RA⁺ CD4⁺ T cells the CD45RA mAb (FMC71) and the CD45RO mAb (UCHL1), respectively, were added to the cocktail of mAbs. Purity of the isolated cells was >98%. The selected CD4⁺ T cells were free of monocytes based on the criterion that there is no proliferative response to optimal concentrations (1/200 dilution) of PHA (M form) (GIBCO BRL, Gaithersburg, MD).

Proliferation Assays. Proliferation assays were performed using standard techniques as described (1). Briefly 50,000 purified CD4⁺ T cells/microtiter well were cultured in 96-well tissue culture clusters with flat bottom wells (model no. 3596; Costar Corp., Cambridge, MA) for 3 d in culture medium (RPMI 1640 supplemented with 20 mM glutamine, 10% heat-inactivated FCS, and 100 IU/ml of penicillin, 100 µg/ml streptomycin [all from Biowhittaker, Gaithersburg, MD]) under various conditions as mentioned in the experiment description, and pulsed (25 µl/well) with a [³H]thymidine solution (5 mCi/ml, 2 mCi/mmol specific activity; New England Nuclear, Boston, MA) during the last 24 h before being harvested on glass fiber filters. Incorporation of radioactive label was measured by liquid scintillation counting. Results are expressed as the arithmetic mean cpm of triplicate cultures. Monocyte-independent CD4⁺ T cell proliferation was obtained by stimulation with the combination of PMA (1 ng/ml) and PHA (1/200 dilution). For prolonged culture through repeated stimulation, 24-well flat bottom tissue culture cluster plates (model no. 3524; Costar Corp.) were used. Restimulation of the cell cultures was performed 6 d after the last stimulation.

Antibody Reagents and Other Reagents. mAbs were used as dilutions of ascites fluid unless indicated otherwise in the following list. CD3 mAb: murine derived mAb mOKT3 (IgG2a) (American Tissue Culture Collection [ATCC], Rockville, MD) and a humanized version hOKT3 (CDR grafted on human IgG4 with alanine substitutions in its Fc-receptor binding site (21) (both used as purified immunoglobulin) (Dr. J. A. Bluestone, University of Chicago); anti-HLA class II mAb: IVA12 (IgG1) (Dr. J. D. Capra,

University of Texas Southwestern Medical Center, Dallas, TX); CD19 mAb; FMC63 (IgG2a) (Dr. H. Zola, Flinders Medical Centre, Bedford Park, South Australia); CD16 mAb; 3G8 (IgG1) (Dr. D. M. Seal, National Cancer Institute, Bethesda, MD); CD14 mAb: 63D3 (IgG1) (Dr. J. D. Capra); glycophorin mAb: 10F7 (ATCC); CD8 mAb: B9.8 (Dr. B. Malissen, Institut National de la Santé et de la Recherche Medicale–Centre National de la Recherche Scientifique [INSERM-CNRS], Marseille, France); CD45RA mAb: FMC71 (IgG2b) (Dr. H. Zola); CD45RO mAb: UCHL-1 (Dr. P. Beverley, Imperial Cancer Research Fund, London, UK), CD60 mAb: UM4D4 (IgM) (Dr. D. Fox, University of Michigan, Ann Arbor, MI), and T cell panel of the 5th Leukocyte Typing Workshop.

Affinity-purified ICAM-1 was isolated from a Hodgkin's lymphoma cell line, L428, as described (1). Affinity-purified multimeric LFA-3 is isolated from red blood cells as described (22). The purified proteins ICAM-1, LFA-3, and the CD3 mAbs mOKT3 and hOKT3 were immobilized on the plastic well bottom by dilution in PBS. After overnight incubation at 4°C wells were washed with PBS. The amount of purified protein applied to each well is indicated in the figures.

Mitogen-driven T cell activation was performed with PHA-M (GIBCO-BRL) at 1/200 final dilution and phorbol ester PMA at 1 ng/ml.

Recombinant human IL-1β and IL-4 were purchased from Collaborative Biomedical Products (Becton Dickinson Labware, Bedford, MA) and recombinant human IL-2, IL-6, and IL-7 were purchased from GIBCO-BRL.

Cytokine Assays. To obtain culture supernatant for cytokine analysis the cells were cultured in culture medium (see above) (10⁶ cells/ml, final volume 2 ml) in 24-well flat bottom tissue culture plates (model no. 3524; Costar Corp.). Preparation of the tissue culture plates were identical to those used for proliferation and prolonged cultivation through repeated restimulation. The culture supernatants were harvested 24 or 48 h after (re)stimulation of the cells. IL-4, GM-CSF, IL-5, and IFN-γ levels in those experiments indicated with the letter "A," as depicted in Table 1, Figs. 2 and 3 (e.g., Exp. 1 A and 2 A) were determined in ELISA assays as described (23); minimum sensitivities of those assays were >1 ng/ml for GM-CSF, >30 pg/ml for IL-5 and >0.1 ng/ml for IFN-γ. The levels of GM-CSF, and IL-5 in the culture supernatants of those experiments indicated with the letter "B", as depicted in Figs. 2 and 3 (e.g., Exp. 1 B and 2 B) were determined using commercially available mAb based ELISA assays (PharMingen, San Diego, CA) and performed according to the suppliers instructions. Minimum sensitivities of these assays were >1 ng/ml for GM-CSF, and >75 pg/ml for IL-5. Levels of IFN-γ in the "B" series of experiments were determined with an ELISA assay developed in our laboratory using a human IFN-γ–specific mouse mAb and a human IFN-γ–specific rabbit antiserum, both purchased from Biosource International (Camarillo, CA). Goat anti–rabbit horseradish peroxidase (HRP) with specific substrate was used for readout. The sensitivity for IFN-γ detection was >0.1 ng.

Flow Microfluorometry. Cells (3–4 × 10⁵) were washed with FACS® (Becton Dickinson & Co., Mountain View, CA) media (HBSS without phenol red and without Ca⁺/Mg²⁺ [Biowhittaker] containing 0.2% human serum albumin and 0.2% sodium azide) before incubation with human IgG (10 mg/ml) for 10 min at 4°C to inhibit subsequent binding of mAb to FcR. Cells were then incubated with specific mAb at saturating concentrations for 30 min at 4°C, washed twice with FACS® media, and stained with FITC-conjugated f(ab')₂ goat anti–mouse IgG (Southern Biotechnology Associates, Inc., Birmingham, AL) for another 30 min at 4°C. Finally the cells were washed twice and analyzed using a

FACScan®. Fluorescence intensity is expressed as arbitrary units on a log scale. Histogram overlays were made with the use of the flow analyze program WinMDI provided through Internet by Joseph Trotter (The Salk Institute, La Jolla, CA).

Results

Differential Costimulatory Requirements for Naive and Memory CD4+ T Cells. In our study of various costimulatory requirements of human subpopulations of CD4+ T cells we used an adaptation of a previously described simplified culture model for T cell activation (1). Briefly, a CD3 mAb (humanized OKT3, see Materials and Methods) is coimmobi-

lized with either of the purified costimulatory adhesion ligands, ICAM-1, or LFA-3. These molecules, ICAM-1 and LFA-3, costimulate through interaction with receptors LFA-1(CD11a/CD18) and CD2 on the T cell, respectively. Highly purified populations of resting CD4+CD45RA+CD45RO− (naive) and CD4+CD45RA−CD45RO+ (memory) T cells, obtained through rigorous negative-selection, were stimulated in this model in a 4-d proliferation assay. The in vitro culture conditions included the addition of human recombinant cytokines at the start of the culture. The proliferation results of four representative experiments are shown in Fig. 1, *A* and *B*. Immobilized CD3 mAb alone or in combination with
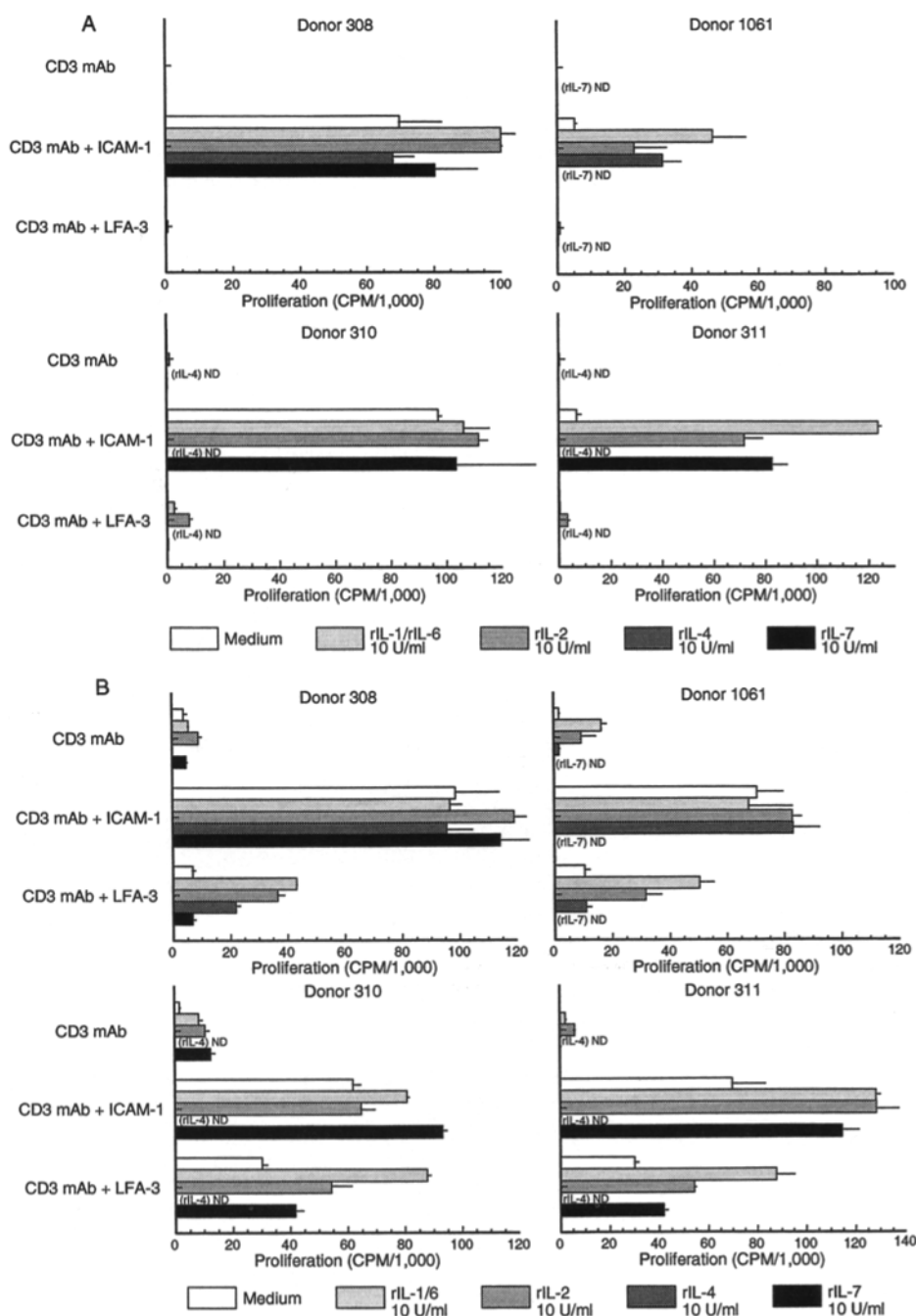
Figure 1. Differential costimulation of naive and memory CD4+ T cells with ICAM-1 and LFA-3. (*A*) Naive CD4+ T cells, (*B*) Memory CD4+ T cells. Human CD4+ T cells were isolated (see Materials and Methods) and stimulated in a 4-d proliferation assay with CD3 mAb alone (1 μg/ml), CD3 mAb + ICAM-1 (∼50 ng/ml) or CD3 + LFA-3 (2 μg/ml) in the presence of various cytokines such as rIL-1/rIL-6, rIL-2, rIL-4, and rIL-7 at a final concentration of 10 U/ml. Murine OKT3 was used for donors 308, 310, 311, and humanized OKT3 for donor 1061.

the various cytokines (rIL-1/6, rIL-2, rIL-4, and rIL-7) did not lead to proliferation of naive cells (Fig. 1 A) and only minimal proliferation with memory cells (Fig. 1 B). ICAM-1 costimulation induced proliferation both in naive and memory cells. In some donors naive cells required the addition of cytokines to achieve strong proliferation (donors 1061 and 311; Fig. 1 A). The results with rIL-1/6 and rIL-2 confirm previous published observations (2).

It is interesting to note that costimulation with LFA-3 was restricted to the memory cells. Addition of the exogenous cytokines rIL-1/6 and rIL-2 consistently enhanced this proliferation (Fig. 1 B), whereas a lesser effect was observed after addition of rIL-4 and rIL-7 in these assays.

Naive and Memory CD4+ T Cells Have Different Cytokine Profiles. To investigate whether different costimulatory conditions may result in generation of distinct effector functions we tested the cytokine profiles of the two subpopulations of human CD4+ T cells after costimulation with ICAM-1 or LFA-3. Supernatant was collected after 24 h of stimulation and assayed for the presence of a variety of cytokines (Table 1); cell proliferation (day 4) in parallel cultures was also determined. The results indicated that naive cells produce significant amounts of IL-2, but fail to secrete detectable levels of IL-4, IFN-γ, or GM-CSF (Table 1). Furthermore, IL-2 production correlated with subsequent proliferation of naive cells, and was only observed with ICAM-1.

Memory CD4+ T cells differed in producing not only more IL-2, but also significant levels of IFN-γ and GM-CSF (Table 1). Occasionally, even low levels of IL-4 could be de-
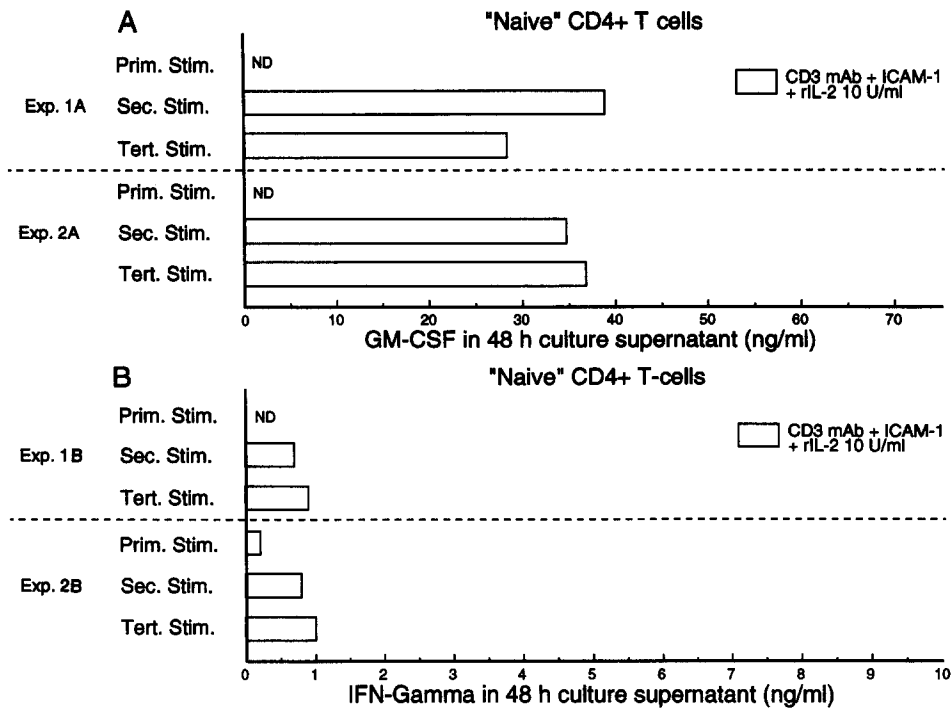
tected. Primary stimulations of memory cells did not, however, show a significant difference in the pattern of cytokine secretion between costimulation through ICAM-1 or LFA-3.

Repeated Costimulation of Memory CD4+ T Cells with ICAM-1 or LFA-3 Leads to Different Cytokine Profiles, Resembling Th1- and Th2-like Patterns. The studies shown in Table 1 were restricted to primary stimulations of resting cells. We subsequently performed studies on both naive and memory cells using repeated stimulations at 6-d intervals with the same costimulatory condition. Initial experiments indicated that the addition of rIL-2 to the cultures resulted far better cell yields after repeated stimulation than addition to the cultures of a combination of rIL-1β/rIL-6, and that supernatant collected after 48 h was optimal (data not shown). Less IL-2 and IL-4 were detected in the culture supernatants of secondary and tertiary stimulations (after 24 and 48 h) than in primary stimulations suggesting a higher cytokine consumption rate. Indeed 6 d after each stimulation cells expressed high levels of IL-2 receptor α (CD25) (data not shown). Therefore, we focused on the cytokine IFN-γ, GM-CSF, and IL-5 which (*a*) appeared unaffected by consumption and (*b*) whose secretion profiles would allow us to make comparisons with Th1-like and Th2-like cytokine patterns. The results shown for naive cells (Fig. 2, A and B) are restricted to repeated stimulations with ICAM-1, because naive cells fail to be stimulated by LFA-3. Only the results for IFN-γ and GM-CSF are shown, because IL-5 was not detectable under any condition or at any time point in naive cell cultures. While naive cells did not produce much GM-CSF (Table 1, and Fig. 2

Table 1. *Cytokine Profile of Naive and Memory CD4+ T Cells*

		IL-2 U/ml 24 h	IL-4 pg/ml 24 h	IFN-γ ng/ml 24 h	GM-CSF ng/ml 24 h	Proliferation CPM/1,000 96 h
	Naive CD4+ T cells					
Exp. 1A	CD3 mAb + ICAM-1 + rIL-1/6	8.4	<50	<0.1	<0.8	108.0
	CD3 mAb + LFA-3 + rIL1/6	<1.0	<50	<1.0	<0.8	0.1
	PHA/PMA + rIL-1/6	55.6	<50	1.6	<0.8	70.0
Exp. 2A	CD3 mAb + ICAM-1 + rIL-1/6	>87.0	<50	0.6	<0.8	57.0
	CD3 mAb + LFA-3 + rIL-1/6	3.8	<50	0.2	<0.8	0.1
	PHA/PMA + rIL-1/6	>87.0	<50	5.7	1.2	83.0
	Memory CD4+ T cells					
Exp. 1A	CD3 mAb + ICAM-1 + rIL-1/6	59.9	96	2.1	17.2	96.0
	CD3 mAb + LFA-3 + rIL-1/6	51.4	<50	2.1	2.7	43.0
	PHA/PMA + rIL-1/6	54.4	<50	1.8	2.0	55.0
Exp. 2A	CD3 mAb + ICAM-1 + rIL-1/6	>87.0	94	2.4	3.8	52.0
	CD3 mAb + LFA-3 + rIL-1/6	>87.0	54	4.6	3.0	48.0
	PHA/PMA + rIL-1/6	>87.0	129	3.9	3.9	34.0

Cytokine levels were determined in culture supernatants, 24 h after primary stimulation with CD3 mAb (murine OKT3) + ICAM-1 or CD3 mAb + LFA-3, or PHA/PMA (as described in Materials and Methods). Proliferation data are from parallel cultures at day 4 (see Materials and Methods).

A

"Naive" CD4+ T cells

Exp. 1A — Prim. Stim. | ND
Sec. Stim.
Tert. Stim.

Exp. 2A — Prim. Stim. | ND
Sec. Stim.
Tert. Stim.

☐ CD3 mAb + ICAM-1
+ rIL-2 10 U/ml

GM-CSF in 48 h culture supernatant (ng/ml)

B

"Naive" CD4+ T-cells

Exp. 1B — Prim. Stim. | ND
Sec. Stim.
Tert. Stim.

Exp. 2B — Prim. Stim.
Sec. Stim.
Tert. Stim.

☐ CD3 mAb + ICAM-1
+ rIL-2 10 U/ml

IFN-Gamma in 48 h culture supernatant (ng/ml)

Figure 2. Cytokine profiles of naive CD4+ T cells after ICAM-1 costimulation. (*A*) GM-CSF, (*B*) IFN-γ. Culture supernatants were collected 48 h after each (re)stimulation. The level of cytokine production was determined in ELISA assay (see Materials and Methods). Costimulatory conditions of ICAM-1 are described in Materials and Methods. ND, Not detectable above background.

A) or IFN-γ (Table 1 and Fig. 2 *B*) in the primary stimulations, they did produce significant levels of both cytokines after secondary and tertiary stimulation (Fig. 2, *A* and *B*). The levels of GM-CSF produced by naive cells were comparable to those of memory cells (Figs. 2 *A* and 3 *C*), whereas levels of IFN-γ never reached those of memory cells (Figs. 2 *B* and 3 *B*). Analysis on a FACScan® of these repeatedly stimulated naive cells showed that complete phenotypic conversion from CD45RA+CD45RO− to CD45RA−CD45RO+ had taken place after the tertiary stimulation (data not shown).
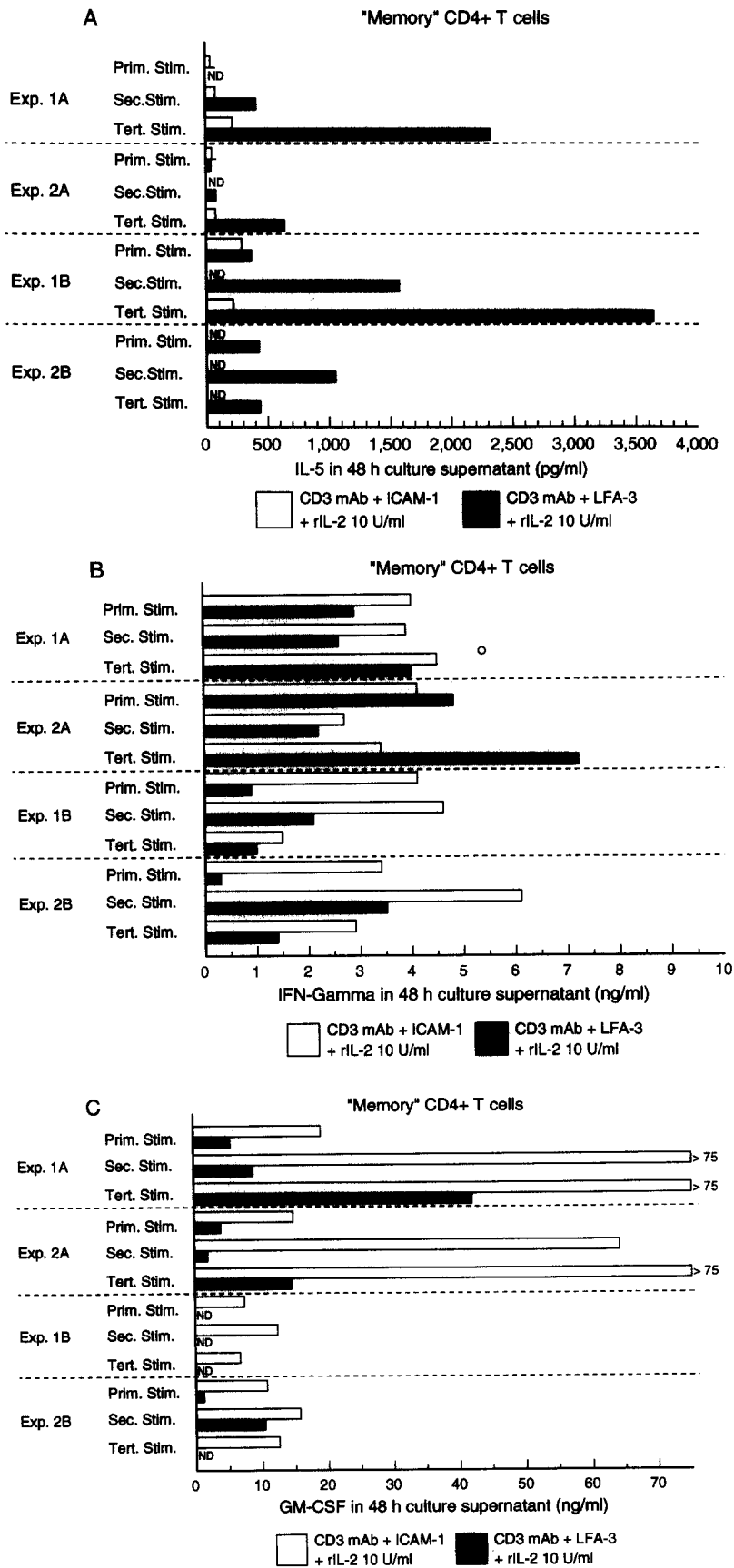
Most interestingly, repeated costimulation of memory cells with ICAM-1 or LFA-3 did lead to differential cytokine secretion. Specifically, the levels for IL-5 produced clearly showed a positive correlation with LFA-3 costimulation (Fig. 3 *A*). In contrast, GM-CSF levels produced were markedly higher with ICAM-1 costimulated memory cells (Fig. 3 *C*). IFN-γ was only marginally higher with ICAM-1 costimulated memory cells (Fig. 3 *B*).

Repeated Costimulation of Memory CD4+ T Cells with ICAM-1 or LFA-3 Leads to Phenotypically Different Cell Populations as Defined by the Cell Surface Molecule CD60. To establish whether different costimulatory in vitro culture conditions influence the phenotype of the stimulated cells we performed FACScan® analysis with a large series of mAbs including the T cell panel of mAbs from the Fifth Leukocyte Typing Workshop. We specifically focused on memory cells in the hope of identifying staining patterns that might correlate with the differential cytokine profiles observed when memory cells are repeatedly stimulated with ICAM-1 or LFA-3. From a panel of over 90 different mAbs only one mAb showed consistently a different staining pattern. The mAb UM4D4 (CD60-specific) stained resting CD4+ memory T cells in a

bimodal pattern (40–60% positive cells) (Fig. 4, *A* and *D*). After repeated costimulation with ICAM-1 only very low levels of CD60-positive cells could be found (Fig. 4, *B* and *E*). In contrast, memory cells repeatedly costimulated with LFA-3 maintained significant levels of CD60 expression cells (Fig. 4, *C* and *F*). Resting naive cells are negative for CD60 and repeated stimulation of naive cells with ICAM-1 did not affect this staining pattern (data not shown).

Discussion

This study was initiated to determine the role of the costimulatory adhesion pathways LFA-1/ICAM-1 and CD2/LFA-3 in human T cell activation and differentiation/maturation. The function of accessory molecules in T cell activation and differentiation/maturation has been widely studied using a variety of approaches (reviewed in 15, 16). The design of our present study is unique in several respects. First, we have used natural ligands, rather than mAbs, to study the contribution of costimulatory adhesion pathways in isolation. Second, the T cells used in our experiments are highly purified, and comparisons are made between two well-established functionally distinct subpopulations of resting human CD4+ T cells, distinguished by their differential expression of isoforms of the CD45 gene; specifically, we compared the CD45RA+CD45RO− subset, consisting of naive cells, and the CD45RA−CD45RO+ subset, which includes putative memory cells. Third, we used a multifaceted approach to examine T cell activation and differentiation/maturation by analyzing not only cell proliferation and cytokine secretion patterns, but also the cell surface phenotype of the activated cells.

A

"Memory" CD4+ T cells

Exp. 1A — Prim. Stim. / Sec.Stim. / Tert. Stim.
Exp. 2A — Prim. Stim. / Sec.Stim. / Tert. Stim.
Exp. 1B — Prim. Stim. / Sec.Stim. / Tert. Stim.
Exp. 2B — Prim. Stim. / Sec.Stim. / Tert. Stim.

IL-5 in 48 h culture supernatant (pg/ml)

☐ CD3 mAb + ICAM-1 + rIL-2 10 U/ml   ■ CD3 mAb + LFA-3 + rIL-2 10 U/ml

B

"Memory" CD4+ T cells

Exp. 1A — Prim. Stim. / Sec. Stim. / Tert. Stim.
Exp. 2A — Prim. Stim. / Sec. Stim. / Tert. Stim.
Exp. 1B — Prim. Stim. / Sec. Stim. / Tert. Stim.
Exp. 2B — Prim. Stim. / Sec. Stim. / Tert. Stim.

IFN-Gamma in 48 h culture supernatant (ng/ml)

☐ CD3 mAb + ICAM-1 + rIL-2 10 U/ml   ■ CD3 mAb + LFA-3 + rIL-2 10 U/ml

C

"Memory" CD4+ T cells

Exp. 1A — Prim. Stim. / Sec. Stim. / Tert. Stim.
Exp. 2A — Prim. Stim. / Sec. Stim. / Tert. Stim.
Exp. 1B — Prim. Stim. / Sec. Stim. / Tert. Stim.
Exp. 2B — Prim. Stim. / Sec. Stim. / Tert. Stim.

GM-CSF in 48 h culture supernatant (ng/ml)

☐ CD3 mAb + ICAM-1 + rIL-2 10 U/ml   ■ CD3 mAb + LFA-3 + rIL-2 10 U/ml

Figure 3. Cytokine profiles of memory CD4+ T cells after ICAM-1 or LFA-3 costimulation. (A) IL-5, (B) IFN-γ, and (C) GM-CSF. Culture supernatants were collected 48 h after each (re)stimulation. The level of IL-5, GM-CSF, and IFN-γ production was determined in ELISA assay (see Materials and Methods). Costimulatory conditions of ICAM-1 and LFA-3 are described in Materials and Methods. ND, Not detectable above background. NT, Not tested.
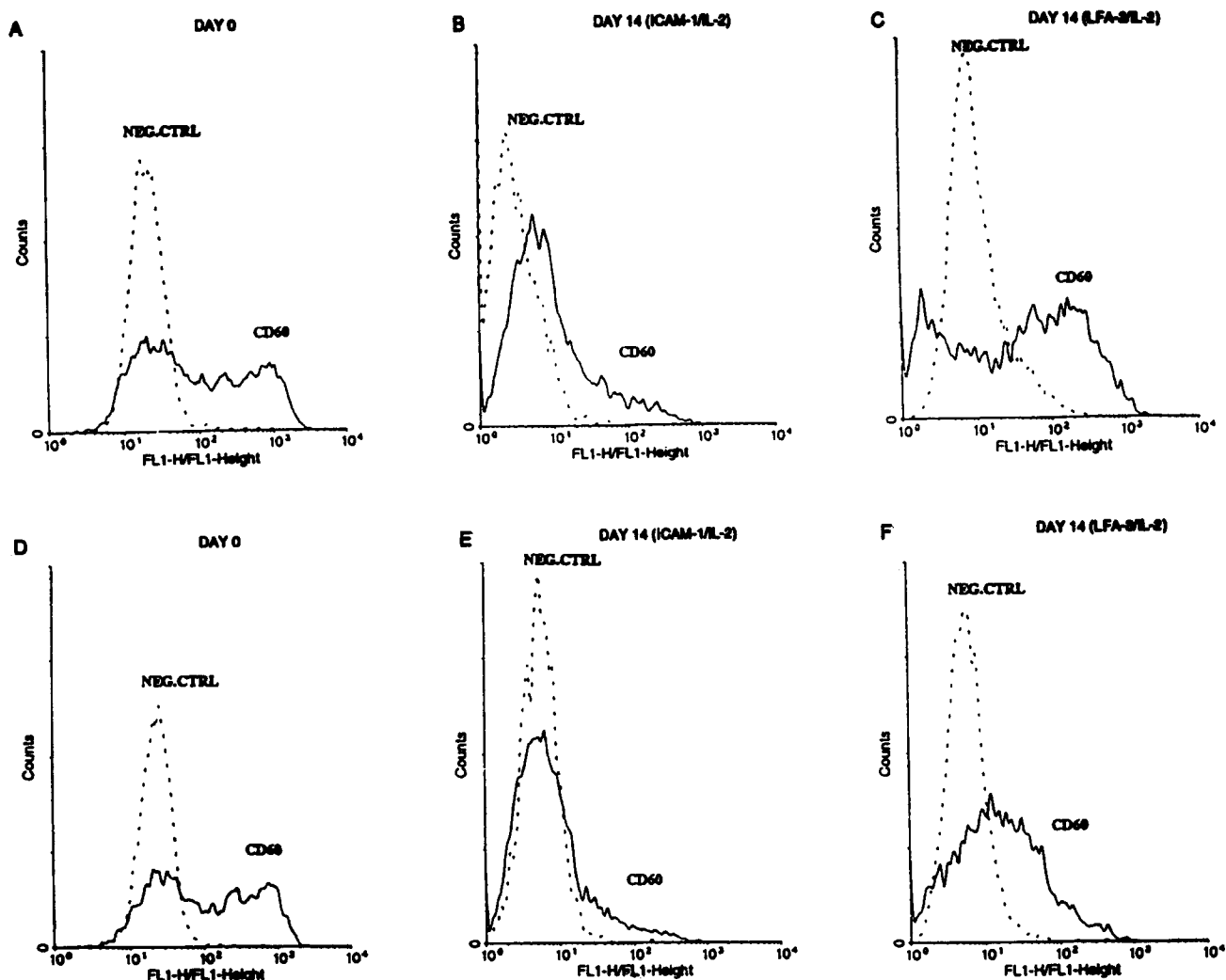
Figure 4. Differential CD60 expression after repeated costimulation of memory CD4⁺ T cells with ICAM-1 or LFA-3. Two representative donors are depicted in figure: donor A: (*A*, *B*, and *C*); and donor B: (*D*, *E*, and *F*). *Dotted lines* are negative control anti-CD19 mAb (FMC63) and *solid lines* are anti-CD60 mAb (UM4D4). (*A* and *D*) starting population at day 0 (resting cells); (*B* and *E*), ICAM-1 costimulated population day 14 (2 d after tertiary stimulation). The percentage CD60⁺ cells is 15% in (*B*) and 8% in (*E*); (*C* and *F*), LFA-3 costimulated population day 14. The percentage CD60⁺ cells is 51% in (*C*) and 27% in (*F*).

The proliferation studies described in Fig. 1 (*A* and *B*) showed the surprising finding that only memory CD4⁺ T cells can be costimulated with natural ligand for CD2, LFA-3, while both naive and memory CD4⁺ T cells readily respond to costimulation with ICAM-1, the natural ligand for LFA-1. Several possibilities exist for the lack of response of naive cells to LFA-3 costimulation including an inadequate interaction with the ligands and/or absence of cofactors in the intracellular signal transduction pathways. Naive CD4⁺ T cells express less of the specific receptor CD2 than memory CD4⁺ T cells (24). Nevertheless both naive and memory T cells can be isolated from PBL by E-rosetting, which is predominantly an adhesive interaction through CD2/LFA-3, indicating at least a certain degree of functional binding through CD2 occurring in both subsets. It was recently shown that the avidity of CD2 for LFA-3, in analogy with integrins, is also upregulated by TCR/CD3 complex–mediated activa-

tion (25). One can, therefore, suggest that naive CD4⁺ T cells may not have the requirements to obtain a high avidity CD2 receptor. Indeed, in studies with coimmobilized CD3 and CD2 mAbs, both naive and memory CD4⁺ T cells could be induced to proliferate (data not shown). This suggests that if one circumvents the issue of strength of interaction by using mAbs one can overcome the lack of responsiveness in naive CD4⁺ T cells for LFA-3 costimulation. These findings also demonstrate the distinct advantage of the usage of natural ligand in reproducing in vivo stimulatory conditions. In addition to LFA-3, there are two alternative (low affinity) ligands for CD2, namely CD59 (26–28) and CD48 (29–32). Deckert et al. (26) reported that CD59 showed both synergy in CD2-mediated adhesion and activation of T cells using LFA-3 and CD59 transfectants (26). We, however, were unable to confirm any stimulatory effect of purified CD59 (kindly provided by Dr. P. Sims, Milwaukee Blood Center,

Milwaukee, WI) alone or in combination with LFA-3 in our in vitro system. For optimal adhesion and activation of T cells through these ligands of CD2 one may need sufficient lateral mobility of these ligands (33), which can more easily be achieved in cell transfectants.

Human CD4+ T cells, by analogy to the murine system (34, 35), can be divided into at least four functional subsets: Thp, Th0, Th1, and Th2, based on their pattern of cytokine secretion (36–40). The T helper precursors (Thp) are mainly limited to the production of IL-2. Activated Thp cells most likely differentiate initially into Th0-like cells (41) producing IL-2, IL-4, and GM-CSF, but not TNF-β or IL-5. Subsequent maturation can either lead to Th1-like cells secreting cytokines including IL-2, IFN-γ, TNF-β, and GM-CSF or Th2-like cells capable of producing IL-4, IL-5, IL-10, and low levels of GM-CSF (34, 36, 38, 40, 42). In contrast to the mouse, human Th1-like cells appear to secrete low levels of IL-10 (43). Th1-like cells are in general associated with delayed type sensitivity and cell-mediated immunity (44), while Th2-like cells are effective in assisting humoral responses (44). To assess differentiation/maturation upon costimulation with ICAM-1 or LFA-3 we focused initially on cytokine secretion patterns, which would help to distinguish these putative T helper subsets. We found that naive CD4+ T cells predominantly secreted IL-2 in the primary stimulations (Table 1) and therefore resemble strongly a Thp-like population. This confirms data from other laboratories in other systems (16, 45, 46). The broad range of cytokines readily produced in a primary stimulation by memory CD4+ T cells with either ICAM-1 or LFA-3 costimulation (Table 1) suggest, as expected, that memory cells contain a mixture of cell populations with Th0- (i.e., IL-2, IL-4), Th1- (i.e., IL-2, IFN-γ), and Th2-like (i.e., IL-4, IL-5) characteristics.

Repeated stimulation, however, of both naive and memory CD4+ T cells led to distinct different cytokine profiles. Naive cells were now capable of producing GM-CSF and IFN-γ (Fig. 2, A and B), although the secreted IFN-γ levels did not reach those of memory cells (Fig. 3 B). Most interestingly, memory CD4+ T cells showed costimulatory signal-dependent regulation of cytokine secretions patterns after repeated in vitro stimulation. ICAM-1 costimulation induced significant levels of both GM-CSF (Fig. 3 C) and IFN-γ (Fig. 3 B), but only minor amounts of IL-5 (Fig. 3 A). In contrast, LFA-3 costimulation led to high levels of IL-5 (Fig. 3 A) and low levels of IFN-γ (Fig. 3 B). The yield of GM-CSF, while substantial, was still significantly less than obtained with ICAM-1 costimulation (Fig. 3 C). These results indicate that LFA-3 costimulation of memory CD4+ T cells results in a preferential Th2-like pattern (IL-5 and lower amounts of GM-CSF), while ICAM-1 costimulation gives rise to a Th1-like pattern (IFN-γ and high amounts of GM-CSF, no IL-5). The clearly detectable levels of IFN-γ after LFA-3 costimulation differ from the usual Th2-like cytokine pattern. There are two possibilities for the effect of LFA-3 costimulation on IL-5 secretion: (a) direct regulation of IL-5 secretion or (b) preferential outgrowth of Th2-like cells. We favor the latter hypothesis in which LFA-3–stimulated cells

are gradually enriched for Th2-like subset of cells, but may still contain some Th1-like cells. Evidence for heterogeneity among the LFA-3–costimulated cells can also be seen in the FACScan® analysis of CD60 expression (Fig. 4, C and F). Similarly, ICAM-1 costimulation could enrich for Th1-like cells. Others scenarios are certainly possible, as can be deduced from a recent publication from Schandene et al. (47), in which they found evidence for suppression of T cell IL-5 secretion by IL-10. Our preliminary results show that IL-10 is produced in (both) primary stimulations, but not thereafter (data not shown). One could therefore postulate that there is indeed an inverse correlation between IL-10 and IL-5, but this is, nevertheless, still LFA-3 costimulation dependent.

T cell differentiation/maturation was also examined by determining cell surface phenotype of the differentially stimulated cells. These cytofluorometric studies showed remarkable consistency of phenotype; with one singular exception, expression of the cell surface antigen CD60 on LFA-3–costimulated memory cells. The CD60 expression which is present on 40–60% of the starting population of resting memory CD4+ T cells is almost completely lost after ICAM-1 costimulation. In contrast, LFA-3 costimulated cells maintain significant levels of CD60+ cells.

The CD60 antigen is expressed by a variety of different hematopoietic lineages, such as T cells, monocytes, and platelets. Surface expression on T cells is restricted to subsets of CD4+ and CD8+ T cells. The CD60+CD4+ subset contains the cells that can provide help for B cell differentiation (48). In CD4+ T cells, IL-4 production is higher in the CD60+CD4+ subset, while the CD60−CD4+ cells secrete more IL-2 (49). Similarly, CD60+CD8+ T cells produce the majority of the IL-4 and are able to drive B cell differentiation, whereas CD60−CD8+ T cells secrete most of the IFN-γ (50). The CD60 antigen has been identified as the O-acetylated disialosyl group which is expressed on ganglioside GD3 (51). Differential membrane expression of glycolipids on Th1 and Th2 cells has also been observed in mouse. Presence of the ganglioside GD1a was found restricted to Th2 cells, whereas the ganglioside GD1α was found on Th1 cells (52). These combined results point to a distinct pattern of expression of various gangliosides correlating with Th1-like and Th2-like cytokine profiles.

A wide series of other cell surface markers has been suggested in the literature for identifying functional human T helper subsets including CD26 (53, 54), CD27 (55, 56), CD30 (57), and CD31 (58). The panel of antibodies with which we screened for differential expression, after ICAM-1 or LFA-3 costimulation, included at least two different mAbs directed against each of these markers, but yielded no differences in our hands. It should be mentioned, however, that cell surface expression of subset-specific markers will be regulated both in vivo and in vitro by a variety of factors, such as accessory pathways, presence of cytokines and growth factors, and the downregulatory effects on receptor expression after interaction with their counter-receptor.

The regulatory effects of cytokines such as IL-4 (39, 59, 60), IFN-γ (59, 61, 62) and IL-12 (63–67) on T helper cell

differentiation are well established and extensively described. In contrast, up until now, there have been no reports describing the role of costimulatory adhesion ligands in this process of T helper cell differentiation/maturation. Our studies are the first to demonstrate specific costimulatory adhesion pathway-dependent CD4$^+$ T cell differentiation, both at the level of cytokine secretion and at the level of cell surface phenotype. Although our results establish the important principle of costimulatory adhesion pathway-dependent CD4$^+$ T cell differentiation, it is unclear under what physiological conditions this might operate. Since IFN-γ is a potent inducer of ICAM-1, this may be part of feedback by which Th1-like patterns are reinforced. Further evidence for such a combined role of cytokine and costimulatory adhesion molecule comes from preliminary data using similar type experiments with LFA-3 and ICAM-1 costimulation of memory cells, but now in the presence of rIL-4 (100 U/ml). It is interesting to note that LFA-3 costimulation in the presence of rIL-4 failed to

produce any detectable IFN-γ, while the induction of IL-5 was unaffected. In contrast, rIL-4 added to ICAM-1 costimulated memory cells only partially suppressed IFN-γ secretion (data not shown). Furthermore, the presence of rIL-4 did correlate with a slight increase of CD60-positive cells (data not shown).

In conclusion, we present here data showing differential requirements for ICAM-1- and LFA-3–mediated costimulation of the naive and memory CD4$^+$ T cell subsets. Moreover, repeated costimulation with ICAM-1 or LFA-3 leads within the memory CD4$^+$ T cell population to reciprocal cytokine secretion patterns for the lumphokines IL-5 and GM-CSF, and to differential cell surface expression of the CD60 antigen. The combined results strongly indicate a regulatory role for the costimulatory adhesion pathways LFA-1/ICAM-1 and CD2/LFA-3 in T cell activation and differentiation/maturation.

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