

Interleukin 2 Induces the Expression of CD45RO and the Memory Phenotype by CD45RA⁺ Peripheral Blood Lymphocytes

By Michael D. Roth

From the Department of Medicine and the Jonsson Comprehensive Cancer Center, University of California at Los Angeles School of Medicine, Los Angeles, California 90024

Summary

The CD45RA and CD45RO isoforms of the leukocyte common antigen identify functionally distinct "naive" and "memory" T cell subsets. While antigenic and mitogenic stimuli are known to initiate transition from the naive to memory state, little is known about the role of cytokines in this process. This report demonstrates that *in vitro* exposure of purified CD45RA⁺/CD45RO⁻ peripheral blood lymphocytes (PBL) to interleukin 2 (IL-2) promotes their conversion to the CD45RA⁻/CD45RO⁺ phenotype. Conversion to CD45RO occurs for both the CD3⁺ and CD3⁻/CD56⁺ lymphocyte subsets, but occurs more rapidly, and at lower IL-2 concentrations, in the CD3⁻/CD56⁺ population. Expression of CD45RO was observed only in response to IL-2 and was not observed during long-term culture in IL-4, IL-6, or IL-7. We also examined the effect of IL-2 on the expression of adhesion molecules by T cells. The expression of CD2, CD11a, and CDw29 increased, and expression of Leu-8 (LAM-1) decreased, on cultured CD45RA⁺/CD45RO⁻ cells after they converted to expression of CD45RO. In contrast, lymphocytes that remained CD45RA⁺/CD45RO⁻ after 10 d in culture exhibited no change from their baseline adhesion molecule profile. Finally, to test the role of endogenous IL-2 during T cell activation we stimulated CD45RA⁺/CD45RO⁻ PBL with immobilized anti-CD3 in the presence of neutralizing anti-IL-2 antibody and/or cyclosporin A. Both agents significantly reduced the expression of CD45RO and the effect of cyclosporin A was reversed by exogenous IL-2. We conclude that IL-2 promotes CD45RA⁺ cells to express the memory phenotype and is a mediator of CD45RO expression after stimulation of the T cell receptor/CD3 complex.

The leukocyte common antigens (CD45) are a family of 180–220-kD transmembrane glycoproteins present in high abundance on the surface of all leukocytes (1). They share a common intracellular domain that exhibits protein tyrosine phosphatase activity (2). Their different extracellular domains are produced through alternate mRNA splicing of three exons (A, B, C) present on a common parent gene (1, 3). While the exact function and role of the different CD45 proteins remains to be determined, the CD45RA (RA,¹ identified by mAb Leu-18) and CD45RO (RO, identified by mAb UCHL1) isoforms have proven to be reliable markers for identifying functionally distinct "naive" and "memory" T cell subsets (4–7). T cells that express the RA⁺/RO⁻ phenotype are small, resting lymphocytes that secrete a limited repertoire of cytokines (5, 8, 9); that express low levels of specific adhesion molecules (5, 10, 11); and that respond slowly and almost exclusively to primary antigenic stimuli (7, 12, 13). This

phenotype predominates in peripheral blood, especially in the blood of neonates (14). In contrast, T cells that express the RA⁻/RO⁺ phenotype are larger, more activated lymphocytes that express activation markers such as CD25; that secrete a large repertoire of cytokines including IL-2, IL-4, IL-5, IL-6, GM-CSF, TNF, and IFN- γ ; that express high levels of adhesion molecules including CD2, LFA-1, and CDw29; and that respond briskly to recall antigens (4–13). CD45RO⁺ lymphocytes predominate at sites of inflammation (15), within mucosal tissues (16, 17), and among the population of tumor-infiltrating lymphocytes (TIL) (18, 19).

Antigen presentation is believed to be the primary *in situ* signal that induces RA⁺/RO⁻ lymphocytes to transform into memory cells and concordantly express the RO isoform. Several *in vitro* models have been developed to study this transition process including activation of RA⁺/RO⁻ T cells with alloantigen (13) or with mitogens such as Con A, PHA, or anti-CD3 mAb (10, 13, 20). However, little is known about the role of specific cytokines in the phenotypic conversion of naive to memory cells. While investigating the *in vitro* propagation of RO⁺ lymphocytes from TIL, we discovered

¹ Abbreviations used in this paper: CM, complete medium; CsA, cyclosporin A; RA, CD45RA; RO, CD45RO; TIL, tumor-infiltrating lymphocytes.

that culture of lymphocytes in IL-2 induces RA⁺/RO⁻ lymphocytes to convert to expression of RO (18). This report investigates the capacity for IL-2, as compared with other lymphotrophic cytokines, to promote highly enriched RA⁺/RO⁻ PBL to convert to expression of RA⁻/RO⁺ phenotype. We also examine the effects of IL-2 on the expression of adhesion molecules during the transition of purified RA⁺ T cells to the RO⁺ phenotype. Finally, using neutralizing anti-IL-2 antibody and cyclosporin A (CsA), we examine the role of IL-2 as a mediator of memory conversion after stimulation of the TCR/CD3 complex. Our results suggest that IL-2 is a specific mediator involved in the conversion of naive T cells to the memory phenotype.

Materials and Methods

Study Design. Highly purified RA⁺/RO⁻ peripheral blood lymphocytes were activated *in vitro* with either IL-2, IL-4, IL-6, IL-7, or with immobilized anti-CD3 mAb. The effects of these different activating agents on the expression of CD45 isoforms, adhesion molecules and proliferation were sequentially evaluated after 4–21 d of culture.

Media and Cytokines. Complete medium (CM) was composed of RPMI 1640 (ICN Biomedicals, Inc., Costa Mesa, CA) supplemented with 10% heat-inactivated filtered human AB serum (Irvine Scientific, Santa Ana, CA), 0.01 M HEPES buffer, and antibiotic-antimycotic mixture (Gibco Laboratories, Grand Island, NY). Recombinant IL-2 (1.8 × 10⁷ U/mg protein) was the generous gift of Cetus Corp. (Berkeley, CA); recombinant IL-4 was obtained from Immunex Corp. (Seattle, WA); recombinant IL-6 was purchased from R & D Systems, Inc. (Minneapolis, MN); and recombinant IL-7 was purchased from Sterling Drug, Inc. (Malvern, PA). CsA in a stock solution of 50 mg/ml was purchased from Sandoz Pharmaceuticals (East Hanover, NJ).

mAbs. mAbs used for cell depletion and activation included preservative-free purified anti-CD45RA (Leu-18; Becton Dickinson & Co., Mountain View, CA), anti-CD45RO (UCHL1 clone; Dako Corp., Carpinteria, CA), and anti-CD3 (X35 clone; Amac, Inc., Westbrook, ME). Polyclonal rabbit anti-human IL-2 IgG (Collaborative Biomedical, Bedford, MA) with a neutralizing activity of 9.4 IU/μg of antibody was used to block the effects of IL-2. FACS[®] analysis was performed with the following panel of monoclonal antibodies: CD45RA-FITC (Leu-18), CD4-FITC, CD8-PE, CD56-PE, and Leu-8-FITC (LAM-1) from Becton Dickinson & Co.; CD45RO-PE and CD45RO-FITC from Dako Corp.; CD5-FITC, CD11a-FITC, and CDw29-FITC from Amac, Inc.; and CD3-TC (Tri-Color[®] fluorochrome) and CD2-FITC from Caltag Laboratories (S. San Francisco, CA).

Isolation of RA⁺/RO⁻ and RA⁻/RO⁺ Lymphocytes from PBL. Peripheral blood mononuclear cells from normal volunteers were isolated by Ficoll-Paque (Pharmacia LKB Biotechnology, Piscataway, NJ) density gradient centrifugation and depleted of monocytes by two rounds of plastic adherence, each for 1 h at 37°C. The resulting nonadherent PBLs were enriched for cells expressing either the RA⁺ or RO⁺ isoform by labeling with isoform-specific mAb (2 μg/10⁶ cells) and negatively depleting with two rounds of goat anti-mouse immunoglobulin-conjugated magnetic beads (Dyna, Inc., Great Neck, NY) as previously described (18).

Activation with IL-2, IL-4, IL-6, and IL-7. To determine the direct effects of different cytokines on CD45 isoform expression, we cultured RA⁺/RO⁻ lymphocytes at 10⁶ cells/ml for up to 21 d in either various concentrations of IL-2, ranging from 20 to 500

U/ml, or in 500 U/ml IL-4, 100 U/ml IL-6, or 4,000 U/ml IL-7. Aliquots of these cell cultures were analyzed at different timepoints for the expression of CD45 isoforms, the expression of cell surface adhesion molecules and for proliferation. RA⁻/RO⁺ PBL were also cultured in 500 U/ml of IL-2 for comparison.

Activation with Immobilized Anti-CD3. To stimulate lymphocytes via the TCR/CD3 complex we incubated 12-well microtiter plates overnight at 4°C with a 5-μg/ml solution of anti-CD3 mAb in PBS. Nonabsorbed anti-CD3 was washed out of the wells with PBS and RA⁺/RO⁻ lymphocytes were then cultured with the immobilized anti-CD3 and CM for 4 d at 10⁶ cells/ml (37°C). These conditions maximally stimulate CD3⁺ lymphocytes (20). The role of IL-2 secretion as a mediator of anti-CD3-induced proliferation and CD45 isoform conversion was evaluated by adding either neutralizing anti-IL-2 antibody, CsA, or their combination, to duplicate culture wells at the beginning of the 4-d culture. Titration experiments demonstrated that 200 μg/ml of anti-IL-2 antibody was capable of completing neutralizing the effects of >500 U/ml of Cetus IL-2 with respect to cell activation, proliferation, and expression of LAK activity.

Proliferation Assay. Lymphokine proliferation was assessed by the uptake of [³H]thymidine. After various periods of incubation, 10⁵ viable lymphocytes from the different activation groups were placed in 96-well microtiter plates in triplicate. Wells were pulsed with 1 μCi of [³H]thymidine (Amersham International, Amersham, UK) and incubated overnight in the presence of the indicated cytokines at 37°C. Cells were then harvested on a PhD harvester (Cambridge Technology, Inc., Cambridge, MA), mixed with 5 ml of scintillation cocktail (Research Products International Corp., Mt. Prospect, IL), and counted on a liquid scintillation counter (Beckman Instruments Inc., Fullerton, CA).

FACS[®] Analysis. Lymphocytes were labeled with fluorescently conjugated mAb specific for the various cell surface markers as described by the manufacturers. Cell surface phenotype was then determined by flow cytometry using a FACSCAN[®] II flow cytometer that was calibrated daily with "Calibrate beads" and Autocount software (all from Becton Dickinson & Co.). 5,000–25,000 events were acquired for each sample using FACScan[®] Research software (Becton Dickinson & Co.) that simultaneously acquired data for forward-scatter, side-scatter, fluorescence channel 1 (FITC label, FL1), fluorescence channel 2 (PE label, FL2), and fluorescence channel 3 (TC label, FL3). The settings for all of these parameters were optimized at the initiation of the study and maintained constant during all analyses. Gates were set with respect to the forward- and side-scatter to exclude red blood cells and cell debris and to include all other viable cell events. Individual sample data was analyzed on a computer (9000 series model 310; Hewlett-Packard, Palo Alto, CA) using the FACScan[®] Research software. This system allowed data to be gated with respect to two of the fluorescent markers (such as CD45RO-PE and CD3-TC) and analyzed with respect to a third fluorescent marker (such as CD11a-FITC).

Statistics. Comparisons between treatment groups was performed with a paired Student's *t* test with a significant difference accepted as *p* ≤ 0.05. Results presented as mean values ± SEM, unless otherwise stated.

Results

IL-2 Promotes RA⁺/RO⁻ Lymphocytes to Express RO in Culture. PBL from normal volunteers contained 53 ± 2.9% RA⁺/RO⁻ cells, 15 ± 4.5% RA⁺/RO⁺ cells, and 31 ± 5.0% RA⁻/RO⁺ cells (*n* = 4). Negative selection using anti-CD45RO or anti-CD45RA mAb and immunomagnetic

Table 1. Effect of IL-2 Culture on the Distribution of CD3⁺ and CD56⁺/CD3⁻ Lymphocytes

	Day 0*	Day 15
Percent CD3 ⁺ †	80.8 ± 4.0	70.6 ± 4.9
Percent CD56 ⁺ /CD3 ⁻	19.2 ± 4.0	30.4 ± 6.2

* RA⁺/RO⁻ PBLs were cultured in 500 U/ml of IL-2 for 0 or 15 d (*n* = 5).

† Relative percentage of lymphocytes expressing either the CD3⁺ or the CD3⁻/CD56⁺ phenotype as determined by two-color FACS[®] analysis.

beads routinely produced a 97–99% pure population of either RA⁺/RO⁻ or RA⁻/RO⁺ lymphocytes, respectively. Viability was >90%. The RA-enriched population averaged 80.8 ± 4% CD3⁺ lymphocytes and 19.2 ± 4% CD3⁻/CD56⁺ cells (Table 1). The RO-enriched population contained only CD3⁺ lymphocytes. When cultured in CM containing IL-2 both the RA-enriched and RO-enriched populations proliferated and maintained their viability for >3 wk. Sequential FACS[®] analysis demonstrated a stepwise conversion of RA⁺/RO⁻ cells from expression of only the RA isoform, to cells expressing both the RA and RO isoforms, and finally to cells exhibiting only the RA⁻/RO⁺ phenotype (Fig. 1; *n* = 6). In contrast, purified RA⁻/RO⁺ PBL that were incubated in IL-2 maintained their RA⁻/RO⁺ phenotype throughout a 20-d culture period (Fig. 2; *n* = 3). Culture of RO⁺ cells in IL-2 was associated with an increase in their size (as measured by forward-scatter), their intracellular complexity (as measured by side-scatter), and their autofluorescence (background fluorescent intensity). The ap-

parent dim expression of CD45RA after 10 d of culture (Figs. 1 and 2) was actually due to this increased background autofluorescence and not to specific expression of RA.

The Kinetics and IL-2 Dependence of RO Expression Is Different for CD3⁺ and CD3⁻/CD56⁺ Lymphocytes. The emergence of CD45RO in response to titrating concentrations of IL-2 was determined for the whole cell population and independently for the CD3⁺ and CD3⁻/CD56⁺ cell subsets (Fig. 3). The overall response to IL-2 was concentration dependent with an average of 74 ± 2.3% of the cells converting to RO in response to 20 U/ml IL-2 (day 21) and 92 ± 3.5% converting to RO in response to 500 U/ml of IL-2 (*p* < 0.01, *n* = 3). Analysis of the CD3⁺ subset demonstrated a similar IL-2-dependent expression of RO. However, the response by CD3⁻/CD56⁺ cells was characteristically different. Maximal expression of RO was observed with as little as 20 U/ml of IL-2. In addition, the rate of conversion of CD3⁻/CD56⁺ cells to the RA⁻/RO⁺ phenotype was significantly more rapid than that observed with CD3⁺ cells. In response to 500 U/ml of IL-2, only 26 ± 5.4% of CD3⁺ cells expressed RO by day 7, whereas 84 ± 4.0% of CD3⁻/CD56⁺ lymphocytes expressed RO at the same timepoint (Fig. 3; *p* < 0.01). After 2 wk of culture the CD3⁻/CD56⁺ subset had expanded to account for ~30.4 ± 6% of the total cell population (Table 1).

IL-4, IL-6, and IL-7 Do Not Promote the Expression of RO. The elaboration of lymphotropic cytokines by RO⁺ lymphocytes is postulated to be important in the activation of RA⁺ lymphocytes during antigenic stimulation. To determine whether cytokines other than IL-2 promote the expression of RO, we set up simultaneous cultures of purified RA⁺/RO⁻ PBL in high concentrations of either IL-2 (500 U/ml), IL-4 (500 U/ml), IL-6 (100 U/ml), or IL-7 (4,000 U/ml). Expression of the RO isoform after 10 d in culture

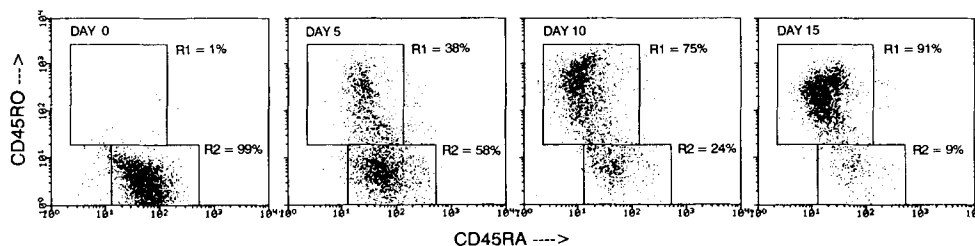


Figure 1. IL-2 promotes RA⁺/RO⁻ lymphocytes to express RO in culture. RA⁺/RO⁻ PBL purified by negative selection were cultured in complete medium containing 500 U/ml IL-2 and analyzed on days 0, 5, 10, and 15 for intensity of staining with anti-CD45RA-FITC (horizontal axis) and anti-CD45RO-PE (vertical axis) by FACS[®] analysis. A stepwise conversion from expression of RA⁺/RO⁻, to expression of RA⁺/RO⁺, to expression of the RA⁻/RO⁺ phenotype was observed. The apparent low level expression of CD45RA on day 15 cells is due to the increased background autofluorescence of cultured cells and not to specific staining with anti-CD45RA. Representative experiment, *n* = 6.

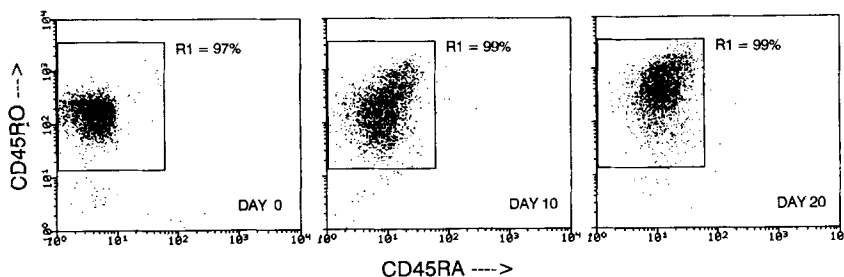


Figure 2. RA⁻/RO⁺ PBL cultured in 500 U/ml of IL-2 maintain their phenotype for up to 20 d in culture. Representative experiment, *n* = 3.

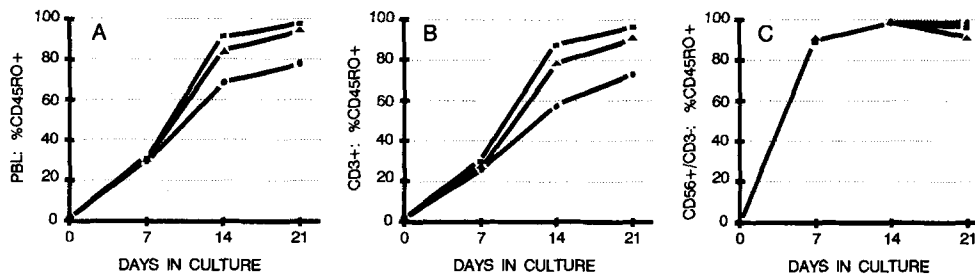


Figure 3. The kinetics and concentration dependence of IL-2-induced expression of RO is different for CD3⁺ and CD3⁻/CD56⁺ lymphocytes. RA⁺/RO⁻ PBL were cultured in either 20 U/ml (●), 100 U/ml (▲), or 500 U/ml (■) of IL-2 for 21 d and analyzed by FACS[®] for the percentage of lymphocytes expressing RO. Results are shown for the whole lymphocyte population (A), and for the CD3⁺ subset (B), and for the CD3⁻/CD56⁺ subset (C). Representative experiment, *n* = 3.

was essentially restricted to those cells cultured in IL-2 (*n* = 2). As shown in Fig. 4, 52% of cells cultured in IL-2 for 10 d expressed the RA⁻/RO⁺ phenotype, whereas only 1, 3, or 6% of cells cultured in IL-4, IL-6, or IL-7, respectively, expressed the RA⁻/RO⁺ phenotype. This lack of RO expression was not a correlate of decreased cell viability, since viability remained ≥89% in all of the 10-d cytokine cultures with the exception of IL-6 (92% for IL-2, 89% for IL-4, 90% for IL-7, and 56% for IL-6).

IL-2 Alters Adhesion Molecule Expression Only on Cultured RA⁺/RO⁻ Lymphocytes That Express RO. Characteristic patterns of adhesion molecule expression have been described for both the RA⁺ and RO⁺ T cell subpopulations (5, 8, 11), as well as for IL-2 activated lymphocytes (21). Three-color FACS[®] analysis, which allowed selective gating on either CD3⁺/RO⁺ or CD3⁺/RO⁻ cells, was used to determine whether the alterations in adhesion molecule expression pro-

duced by IL-2 occur equally in all T cells, or preferentially in those converting to expression of RO. Analyses were performed after 10 d of IL-2 culture, a time-point at which ~60% of T cells express RO and 40% still express only RA. As demonstrated in Fig. 5, freshly isolated RA⁺/RO⁻ lymphocytes express intermediate levels of CD2, intermediate levels

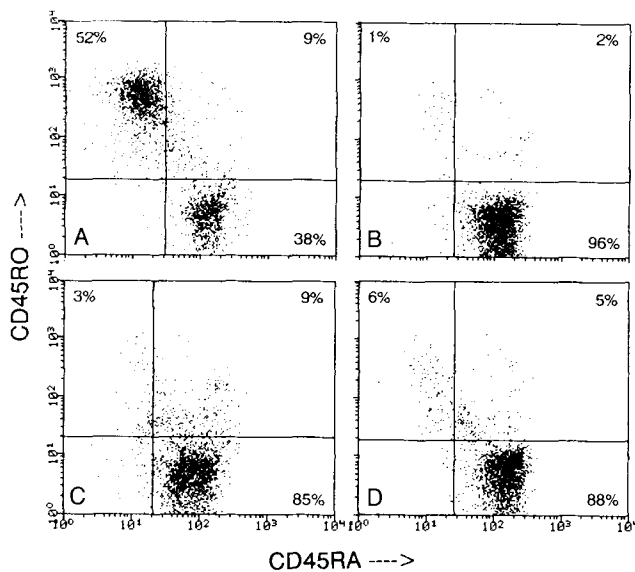


Figure 4. IL-2 alone, but not IL-4, IL-6, or IL-7, promotes the expression of RO. RA⁺/RO⁻ PBLs were cultured for 10 d in either 500 U/ml of IL-2 (A), 500 U/ml of IL-4 (B), 100 U/ml of IL-6 (C), or 4,000 U/ml of IL-7 (D), and analyzed by FACS[®] for the expression of RA (horizontal axis) and RO (vertical axis). Representative experiment, *n* = 2.

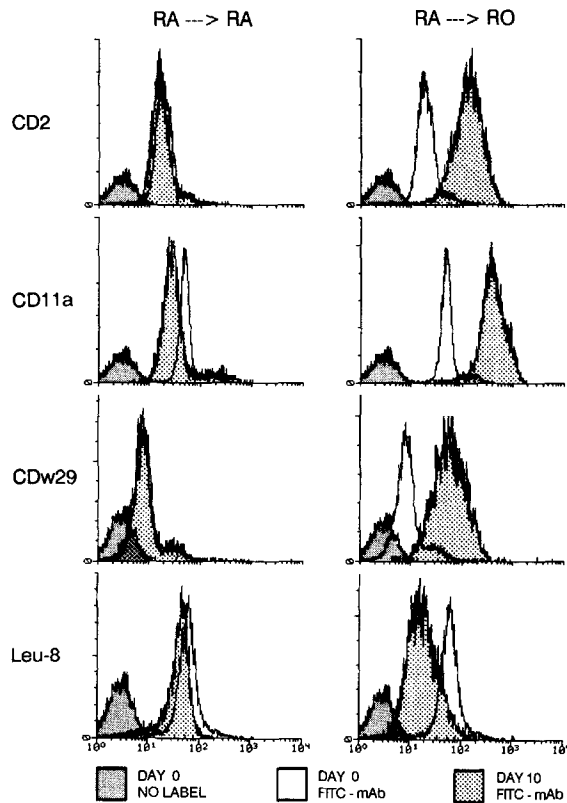


Figure 5. IL-2 alters adhesion molecule expression only on cultured RA⁺/RO⁻ lymphocytes that express RO. RA⁺/RO⁻ PBL were cultured for 10 d in 500 U/ml of IL-2 and analyzed on days 0 and 10 with FITC-labeled mAbs for expression of the following adhesion molecules: CD2, CD11a, CDw29, and Leu-8. Histograms on the left show the results for day 10 cells that remained RA⁺/RO⁻ (RA → RA) and histograms on the right show results for day 10 cells that developed expression of RO⁺ (RA → RO).

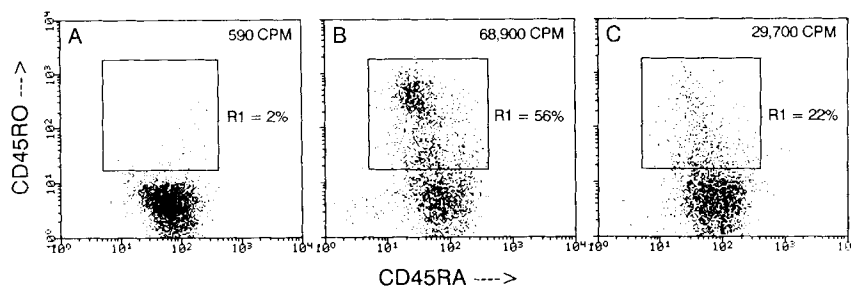


Figure 6. IL-2 is involved in the anti-CD3-induced expression of RO. Fresh RA⁺/RO⁻ PBL (A) were cultured for 4 d in culture wells precoated with 5 μg/ml of anti-CD3 mAb (B), or in anti-CD3-coated culture wells containing 200 μg/ml of anti-IL-2 antibody (C). The effects of these treatments on the expression of RA and RO and on [³H]thymidine incorporation (in cpm) is shown for a representative experiment (n = 4).

of the β₂-integrin CD11a, negligible levels of β₁-integrins as identified by CDw29, and high levels of the lymph node high endothelial venule ligand, LAM-1 (Leu-8). Cells that remained RA⁺/RO⁻ after 10 d of culture demonstrated no change from this baseline adhesion molecule profile. In contrast, T cells that developed expression of RO⁺ demonstrated a marked increase in their expression of CD2, CD11a, and CDw29, and a decrease in their expression of Leu-8. This latter adhesion molecule profile is similar to that observed on fresh and IL-2 activated RA⁻/RO⁺ lymphocytes.

IL-2 Is a Mediator in the Anti-CD3-induced Expression of RO. Since exposure to IL-2 alone promoted lymphocytes to express RO, we used neutralizing anti-IL-2 antibody and/or CsA to determine the role of IL-2 as a mediator of RO expression after cross-linking of the CD3 receptor. As demonstrated in Fig. 6, stimulation of RA⁺/RO⁻ lymphocytes with immobilized anti-CD3 produced a rapid expression of RO by day 4 of culture. The addition of 200 μg/ml of anti-IL-2 antibody to these cultures, a concentration sufficient to completely neutralize the effects of >500 U/ml of exogenous IL-2, significantly reduced the number and intensity of cells expressing RO (44 ± 10.4% reduction in RO, n = 4). The effect of anti-IL-2 antibody on proliferation was more variable, ranging from 0.0 to 56% suppression. Similar to the effects of anti-IL-2, CsA at 5 μg/ml significantly reduced the

anti-CD3-stimulated expression of RO (38.4 ± 3.5% reduction, n = 3) and this effect was only slightly more pronounced when CsA, anti-IL-2, and anti-CD25 antibody were used in combination (Table 2). CsA blocks several early activation genes including IL-2, IL-4, and IFN-γ (22, 23). To confirm the role of IL-2 in CsA-mediated inhibition we therefore simultaneously added 500 U/ml of exogenous IL-2 to some of the CsA-treated cultures. Exogenous IL-2 restored RO expression to a level indistinguishable from that expressed by control anti-CD3 stimulated cells (89 ± 1% vs. 92 ± 1%, respectively; Table 2). These results suggest that endogenous IL-2 production plays a significant role in the expression of RO after cross-linking of the CD3 receptor.

Discussion

While the exact functions of the different CD45 isoforms remain to be determined, these molecules appear to modulate lymphocyte activation, effector function, and signal transduction in a variety of cell types ranging from NK cells (24, 25), to B cells (25, 26), to T cells (27, 28). In T cells, CD45 molecules may complex with CD2 and/or CD3 and their protein tyrosine phosphatase activity may modulate signal transduction via these molecules (29-31). Regardless of their exact function, specific expression of the RA and RO iso-

Table 2. Role of IL-2 in Anti-CD3-stimulated Expression of CD45RO

Treatment	Exp. 1		Exp. 2		Exp. 3	
	Percent RO ⁺	Proliferation	Percent RO ⁺	Proliferation	Percent RO ⁺	Proliferation
		<i>cpm</i>		<i>cpm</i>		<i>cpm</i>
Anti-CD3 only*	93	24,493 ± 1,504	93	23,753 ± 779	90	16,660 ± 1,357
Anti-CD3 + CsA†	59	3,981 ± 101	60	1,595 ± 666	51	2,215 ± 112
Anti-CD3 + CsA						
+ anti-IL-2/anti-CD-25‡	43	3,474 ± 130	53	583 ± 151	35	3,149 ± 272
anti-CD3 + CsA + IL-2	nd	nd	90	20,719 ± 1,330	88	24,715 ± 383

* RA⁺/RO⁻ PBL cultured for 4 d with 5 μg/ml immobilized anti-CD3 and evaluated for RO expression (FACS[®] analysis) and proliferation ([³H]thymidine uptake).

† RA⁺/RO⁻ PBL cultured with anti-CD3 and 5 μg/ml CsA.

‡ RA⁺/RO⁻ PBL cultured with anti-CD3, 5 μg/ml CsA, 200 μg/ml anti-IL-2, and 20 μg/ml anti-CD25.

|| RA⁺/RO⁻ PBL cultured with anti-CD3, 5 μg/ml CsA, and 500 U/ml Cetus IL-2.

forms by T cells has been invaluable in phenotypically identifying naive and memory populations, respectively. Cells expressing the RA⁻/RO⁺ phenotype, whether purified directly from peripheral blood or induced by activation of the TCR/CD3 complex, are distinguished from naive T cells by their expression of activation markers, by their elaboration of cytokines, by specific alterations in their expression of adhesion molecules, and by their capacity to provide T cell help and to respond rapidly and specifically to a secondary antigen challenge (4–13).

Antigen presentation is believed to be the primary *in situ* signal responsible for transition from the naive to memory state and can be mimicked *in vitro* by stimulating the TCR/CD3 complex with either alloantigens or nonspecific mitogens such as Con A, PHA, or anti-CD3 mAb (10, 13, 20). While it is readily appreciated that such stimulation induces RA⁺ cells to secrete primarily IL-2 (8, 9, 20, 32), little has been previously reported about the role of this or other cytokines in the conversion from RA to RO. De Jong et al. (20) reported that exogenous IL-2 augments the proliferation of both resting and anti-CD3 activated RA⁺ T cells, but that anti-CD25 mAb does not reduce proliferation if these cells are optimally stimulated with immobilized anti-CD3. They imply from these results that both IL-2-dependent and IL-2-independent proliferation occurs when RA⁺ cells are stimulated at the TCR/CD3 complex. Wasik and Morimoto (33) observed that IL-2, IL-4, and IL-6, either alone or in combination, augment anti-CD3-induced proliferation of RA⁺/CD4⁺ T cells. Neither study examined the effects of these cytokine manipulations on RO⁺ expression or memory-like function. Braakman et al. (34) were the first to suggest that stimulation by IL-2 might be directly involved in the expression of RO. They described the development of RO on a percentage of NK cells and γ/δ T cells that were cultured for 1 wk in IL-2, but this effect was not well delineated. While examining the effects of IL-2 culture on TIL, we observed a progressive enrichment of the RA⁻/RO⁺ population (18). To evaluate this further, we cultured purified RA⁺/RO⁻ PBL in IL-2 and observed their stepwise conversion to the RA⁻/RO⁺ phenotype over 1–3 wk in culture. In this report, we confirm that IL-2 alone, in the absence of antigens or mitogens, induces RA⁺ lymphocytes to switch to expression of RO. IL-2 also maintained the expression of RO on purified RA⁻/RO⁺ PBL that were cultured *in vitro* for up to 3 wk. Conversion of RA⁺ cultures to expression of RO cannot be explained by simple expansion of contaminating RO⁺ lymphocytes. Not only did our RA⁺ population routinely contain <1% RA⁻/RO⁺ cells, but the pattern of expression was a stepwise conversion from RA⁺/RO⁻, to RA⁺/RO⁺, and finally to RA⁻/RO⁺. In addition, we and others (32, 35) have not observed any significant growth advantage of the RO population, over the RA⁺ population, when cultured in IL-2. A negative depletion technique was used in this study in order to avoid direct activation of the cells being studied (36), and a two-phase adherence was performed to remove monocytes and dendritic cells that might have induced an autologous MLR. In some studies we also used autologous serum to control for nonspecific TCR activation. No differ-

ence was observed between autologous and allogeneic serum. The efficacy of all of these precautions is born out by the fact that only cultures containing IL-2 progressed to expression of RO.

IL-2-induced transition to the RA⁻/RO⁺ phenotype was observed for both the CD3⁺ and CD3⁻/CD56⁺ subpopulations, but with different kinetics and dose responses for each subgroup. CD3⁺ cells converted in a step-wise and concentration-dependent manner, while CD3⁻/CD56⁺ lymphocytes rapidly and maximally converted to the RA⁻/RO⁺ phenotype in response to as little as 20 U/ml of IL-2. Essentially all circulating CD3⁻/CD56⁺ lymphocytes express the RA⁺/RO⁻ phenotype and function as NK cells (34). Their culture in IL-2 is not only associated with the rapid expression of RO, but also with the rapid upregulation of adhesion molecules, enhanced cytokine secretion, and induction of LAK activity (21). These functional alterations are remarkably similar to those described for T cells converting to the memory state. The fact that IL-2 induces both CD3⁺ and CD3⁻/CD56⁺ lymphocytes to express RO, even though CD3⁻/CD56⁺ cells lack a TCR, supports our hypothesis that IL-2 is directly involved in the process of CD45 isoform conversion.

The expanded cytokine production of RO⁺ lymphocytes, and the apparent role of these cytokines in promoting T cell growth and activity, has been taken to suggest a synergistic interaction between naive and memory T cells during immune responses (5, 7). We therefore examined if cytokines other than IL-2 might similarly promote cultured cells to express RO. However, while IL-4, IL-6, and IL-7 maintained, or even promoted cell growth in culture, we did not observe any significant expression of RO in response to any of these cytokines. Since Wasik and Morimoto (33) report that IL-4 and IL-6 augment proliferation of RA⁺ T cells when added to IL-2, it is possible that combinations of these cytokines might augment maturation to a memory cell state; we did not specifically test for such a response in this study. Alternatively, Brod et al. (37) observed that IL-6 upregulates RA expression in some RO⁺ T cell clones and it is possible that some cytokines may promote expression of RA rather than RO.

As another measure of lymphocyte maturation we correlated the changes in RA and RO expression during culture to the effects of IL-2 on adhesion molecule expression. RA⁺ and RO⁺ T cells typically express different adhesion molecule profiles (5, 6, 11). RA⁺ lymphocytes do not express β_1 integrins, whereas RO⁺ cells do; RA⁺ lymphocytes express moderate levels of β_2 integrins, whereas RO⁺ lymphocytes express high levels; and RA⁺ lymphocytes express high levels of the LAM-1, whereas populations of RO⁺ lymphocytes lose expression of this high endothelial venule ligand. This differential expression of adhesion molecules by naive and memory T cells is believed to be intimately related to their different trafficking and effector functions (5, 6). We hypothesized that IL-2-induced alterations in adhesion molecule expression, which are known to occur when T cells are cultured in IL-2 (21), might be restricted to those T cells converting to expression of RO. This hypothesis was

confirmed by examining IL-2 cultures on day 10, a time-point at which only 50–60% of the cells express RO. Enhanced expression of CD2, CD11a, and CDw29, and a decrease in expression of LAM-1, was observed only in the RO⁺ subset. These results suggest that IL-2 not only promotes the expression of CD45RO, but that it is responsible for the phenotypic profile of memory T cells.

Stimulation of the TCR/CD3 complex is the primary *in vivo* signal that results in T cell activation and the acquisition of memory during an immune response. To examine the role of endogenous IL-2 during T cell activation we stimulated RA⁺/RO⁻ lymphocytes with immobilized anti-CD3 mAb in the presence or absence of neutralizing antibody to IL-2. Similar to the report by de Jong et al. (20), anti-IL-2 antibody had a variable effect on proliferation with no suppression in several cases. In contrast, anti-IL-2 antibody uniformly suppressed (but did not eliminate) the number and intensity of cells expressing RO. To confirm these findings we also stimulated RA⁺/RO⁻ lymphocytes in the presence of either CsA or a mixture of CsA and anti-IL-2 and anti-CD25 antibodies. CsA reduced the acquisition of RO to a level similar to that produced by anti-IL-2, and the combination reduced the expression of RO slightly better than either agent alone. CsA exerts its effects by blocking the activation

of several genes including those coding for IL-2, IL-4, and IFN- γ (22, 23). We therefore examined the specific role of IL-2 by supplementing CsA-treated cultures with exogenous IL-2. As hypothesized, exogenous IL-2 abrogated the effects of CsA and completely restored both proliferation and RO expression. The implications of these studies are that IL-2 plays a direct role in mediating RO expression, that IL-2 may further augment RO expression through its effects on proliferation, and that factors other than IL-2 must also be involved in promoting RO expression since stimulation with anti-CD3 produced RO⁺ cells even in the presence of CsA, anti-IL-2, and anti-CD25. The exact nature, role and interaction of these other signals remain to be elucidated.

In summary, IL-2 promotes highly purified RA⁺/RO⁻ lymphocytes to convert to the RA⁻/RO⁺ phenotype *in vitro*. This conversion occurs for both the CD3⁺ and the CD3⁻/CD56⁺ subsets and is associated with the acquisition of an adhesion molecule profile identical to that found on classic memory cells. Blocking experiments with anti-IL-2 and CsA likewise suggest that endogenous production of IL-2 is one of the factors mediating RO expression when T cells are stimulated via the TCR/CD3 complex. CD45RO expression, especially in cultured lymphocytes, may not always indicate prior antigenic exposure.

Excellent technical assistance was provided by Elana Saks-Rosenthal and flow cytometry was performed by the JCCC Flow Cytometry Core Facility (grant CA-16402) with the technical assistance of Negoita Neagos, M.D.

This work was supported by National Cancer Institute Clinical Investigators Award CA-01549.

Address correspondence to Michael D. Roth, M.D., Division of Pulmonary & Critical Care, Department of Medicine, UCLA School of Medicine, Los Angeles, CA 90024.

Received for publication 9 July 1993 and in revised form 13 October 1993.

References

1. Thomas, M.L. 1989. The leukocyte common antigen family. *Annu. Rev. Immunol.* 7:339.
2. Tonks, N.K., C.D. Diltz, and E.H. Fischer. 1990. CD45, an integral membrane protein tyrosine phosphatase. *J. Biol. Chem.* 265:10674.
3. Streuli, M., L.R. Hall, Y. Saga, S.F. Schlossman, and H. Saito. 1987. Differential usage of three exons generates at least five different mRNAs encoding human leukocyte common antigens. *J. Exp. Med.* 166:1548.
4. Beverley, P.C.L. 1992. Functional analysis of human T cell subsets defined by CD45 isoform expression. *Semin. Immunol.* 4:35.
5. Akbar, A.N., M. Salmon, and G. Janosy. 1991. The synergy between naive and memory T cells during activation. *Immunol. Today.* 12:184.
6. Mackay, C.R. 1991. T-cell memory: the connection between function, phenotype and migration pathways. *Immunol. Today.* 12:189.
7. Clement, L.T. 1992. Isoforms of the CD45 common leukocyte antigen family: markers for human T-cell differentiation. *J. Clin. Immunol.* 12:1.
8. Salmon, M., G.D. Kitas, and P.A. Bacon. 1989. Production of lymphokine mRNA by CD45R⁺ and CD45R⁻ helper T cells from human peripheral blood and by human CD4⁺ T cell clones. *J. Immunol.* 143:907.
9. Kristensson, K., C.A.K. Borrebaech, and R. Carlsson. 1992. Human CD4⁺ T cells expressing CD45RA acquire the lymphokine gene expression of CD45RO⁺ T-helper cells after activation *in vitro*. *Immunology.* 76:103.
10. Wallace, D.L., and P.C.L. Beverley. 1990. Phenotypic changes associated with activation of CD45RA⁺ and CD45RO⁺ T cells. *Immunology.* 69:460.
11. Sanders, M.E., M.W. Makgoba, S.O. Sharrow, D. Stephany, T.A. Springer, H.A. Young, and S. Shaw. 1988. Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (UCHL1, CDw29, and Pgp-1) and have enhanced IFN- γ production. *J. Immunol.* 140:1401.

12. Plebanski, M., M. Saunders, S.S. Burtles, S. Crowe, and D.C. Hooper. 1992. Primary and secondary human *in vitro* T-cell responses to soluble antigens are mediated by subsets bearing different CD45 isoforms. *Immunology*. 75:86.
13. Akbar, A.N., L. Terry, A. Timms, P.C.L. Beverley, and G. Janossy. 1988. Loss of CD45R and gain of UCHL1 reactivity is a feature of primed T cells. *J. Immunol.* 140:2171.
14. Paoli, P.D., S. Battistin, and G.F. Santini. 1988. Age-related changes in human lymphocyte subsets: progressive reduction of the CD4 CD45R (suppressor inducer) population. *Clin. Immunol. Immunopathol.* 48:290.
15. Sterry, W., S. Bruhn, N. Kunne, B. Lichtenberg, M.K. Weber, J. Brasch, and V. Mielke. 1990. Dominance of memory over naive T cells in contact dermatitis is due to differential tissue immigration. *Br. J. Dermatol.* 123:59.
16. Harvey, J., D.B. Jones, and D.H. Wright. 1989. Leukocyte common antigen expression on T cells in normal and inflamed human gut. *Immunology*. 68:13.
17. Marathias, K.P., F.I. Preffer, C. Pinto, and R.L. Kradin. 1991. Most human pulmonary infiltrating lymphocytes display the surface immune phenotype and functional responses of sensitized T cells. *Am. J. Respir. Cell Mol. Biol.* 5:470.
18. Nguyen, Q.H., R.L. Moy, M. Roth, R. Yamamoto, S. Tomono, and S.M. Dubinett. 1993. Expression of CD45 isoforms in fresh and IL-2-cultured tumor-infiltrating lymphocytes from basal cell carcinoma. *Cell. Immunol.* 146:421.
19. Bukowski, R.M., W. Sharfman, S. Murthy, P. Rayman, R. Tubbs, J. Alexander, G.T. Budd, J.S. Sergi, L. Bauer, V. Gibson, et al. 1991. Clinical results and characterization of tumor-infiltrating lymphocytes with or without recombinant interleukin 2 in human metastatic renal cell carcinoma. *Cancer Res.* 51:4199.
20. de Jong, R., M. Brouwer, F. Miedema, and R.A.W. van Lier. 1991. Human CD8⁺ T lymphocytes can be divided into CD45RA⁺ and CD45RO⁺ cells with different requirements for activation and differentiation. *J. Immunol.* 146:2088.
21. Robertson, M.J., M.A. Caligiuri, T.J. Manley, H. Levine, and J. Ritz. 1990. Human natural killer cell adhesion molecules: differential expression after activation and participation in cytotoxicity. *J. Immunol.* 145:3194.
22. Gergely, P., and A. Aszalos. 1990. Effect of cyclosporin A on the anti-CD3 antibody- and concanavalin A-induced activation and membrane potential of human T lymphocytes. *Immunopharmacology*. 20:191.
23. Terada, N., R. Or, K. Weinberg, J. Domenico, J.J. Lucas, and E.W. Gelfand. 1992. Transcription of IL-2 and IL-4 genes is not inhibited by cyclosporin A in competent T cells. *J. Biol. Chem.* 267:21207.
24. Starling, G.C., S.E. Davidson, J.L. McKenzie, and D.N.J. Hart. 1987. Inhibition of natural killer-cell mediated cytotoxicity with monoclonal antibodies to restricted and non-restricted epitopes of the leucocyte common antigen. *Immunology*. 61:351.
25. Mittler, R.S., R.S. Greenfield, B.Z. Schacter, N.F. Richard, and M.K. Hoffmann. 1987. Antibodies to the common leukocyte antigen (T200) inhibit an early phase in the activation of resting human B cells. *J. Immunol.* 138:3159.
26. Lin, J., V.K. Brown, and L.B. Justement. 1992. Regulation of basal tyrosine phosphorylation of the B cell antigen receptor complex by the protein tyrosine phosphatase, CD45. *J. Immunol.* 149:3182.
27. Martorell, J., R. Vilella, L. Borche, I. Rojo, and J. Vives. 1987. A second signal for T cell mitogenesis provided by monoclonal antibodies CD45 (T200). *Eur. J. Immunol.* 17:1447.
28. Ledbetter, J.A., L.M. Rose, C.E. Spooner, P.G. Beatty, P.J. Martin, and E.A. Clark. Antibodies to common leukocyte antigen p220 influence human T cell proliferation by modifying IL-2 receptor expression. *J. Immunol.* 135:1819.
29. Schraven, B., Y. Samstag, P. Altevogt, and S.C. Meuer. 1990. Association of CD2 and CD45 on human T lymphocytes. *Nature (Lond.)*. 345:71.
30. Volarevic, S., C.M. Burns, J.J. Sussman, and J.D. Ashwell. 1990. Intimate association of Thy-1 and the T-cell antigen receptor with the CD45 tyrosine phosphatase. *Proc. Natl. Acad. Sci. USA.* 87:7085.
31. Kanner, S.B., J.P. Deans, and J.A. Ledbetter. 1992. Regulation of CD3-induced phospholipase C-gamma1 (PLCγ1) tyrosine phosphorylation by CD4 and CD45 receptors. *Immunology*. 75:441.
32. Salmon, M., G.D. Kitis, J.S. Hill Gaston, and P.A. Bacon. 1988. Interleukin-2 production and response by helper T-cell subsets in man. *Immunology*. 65:81.
33. Wasik, M.A., and C. Morimoto. 1990. Differential effects of cytokines on proliferative response of human CD4⁺ T lymphocyte subsets stimulated via T cell receptor-CD3 complex. *J. Immunol.* 144:3334.
34. Braakman, E., E. Sturm, K. Vijverberg, B.A. van Krimpen, J.W. Gratama, and R.L.H. Bolhuis. 1991. Expression of CD45 isoforms by fresh and activated human γδ T lymphocytes and natural killer cells. *Int. Immunol.* 3:691.
35. Dohlsten, M., G. Hedlund, H.-O. Sjögren, and R. Carlsson. 1988. Two subsets of human CD4⁺ T helper cells differing in kinetics and capacities to produce interleukin 2 and interferon-γ can be defined by the Leu-18 and UCHL1 monoclonal antibodies. *Eur. J. Immunol.* 18:1173.
36. Manyonda, I.T., A.J. Soltys, and F.C. Hay. 1992. A critical evaluation of the magnetic cell sorter and its use in the positive and negative selection of CD45RO⁺ cells. *J. Immunol. Methods*. 149:1.
37. Brod, S.A., C.E. Rudd, M. Purvee, and D.A. Hafler. 1989. Lymphokine regulation of CD45R expression on human T cell clones. *J. Exp. Med.* 170:2147.