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The T Cell Antigen Receptor CD3:CD4 Molecular Complex Is Diminished on the Surface of Pulmonary Lymphocytes

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CD4, a 55-kd cell surface glycoprotein, binds to class II major histocompatibility complex (MHC) (Ia) antigens and functions as a coreceptor for the T cell antigen receptor (Tiαβ)-CD3 complex. We have observed that critical elements of the T cell antigen multireceptor complex, including Tiαβ, CD3, CD4, but not CD8, were diminished on CD45RO$^+$ pulmonary T lymphocytes but not CD45RO$^+$ peripheral blood T lymphocytes (PBL). Epitopes mapping from the first (D1) to the fourth (D4) extracytoplasmic Ig-like domains of CD4 were expressed to a lesser degree on pulmonary T cells than on PBL (P = 0.002). CD4 expression on pulmonary T cells did not increase after 72 hours of ex vivo culture in complete medium but was restored toward control levels by stimulation with phytohemagglutinin, anti-CD3, or interleukin-2. CD4 mRNA isolated from lung T cells and PBL co-migrated on Northern blots and the total levels of CD4 mRNA were comparable, suggesting that diminished CD4 expression by pulmonary T cells might reflect a posttranscriptional change. To determine whether CD4^{bright} T cells convert with mitogen stimulation to CD4^{dim} cells, PBLs were stimulated with immobilized anti-CD3, anti-CD4, or a molecularly engineered anti-CD3:CD4 bispecific monoclonal antibody and the ratio of the CD4:CD3 mean fluorescence staining intensities was calculated at days 3 and 13. The CD4:CD3 ratio decreased primarily for cells stimulated with anti-CD3:CD4, suggesting that co-ligation of CD3 and CD4 is required for the generation of CD4^{dim} T cells. We conclude that diminished Tiαβ-CD3:CD4 expression is a characteristic of T cells in lung that is not shared by peripheral blood T cells in vivo, and speculate that this change reflects T cell activation in a millieu of limited interleukin-2 availability. ***(Am J Pathol 1994, 145:1219–1227)***

The T cell antigen receptor (Tiαβ)-CD3 receptor complex plays a critical role in the recognition and response of T lymphocytes to antigen. CD4 is a 55-kd cell surface glycoprotein that binds to class II major histocompatibility complex (MHC) antigens[1] and functions as part of the T cell antigen multireceptor complex.[2, 3] CD4 also binds to the gp120 envelope protein of the human immunodeficiency virus (HIV)-1 and facilitates its entry into cells.[4–6] In addition to its role as a ligand receptor, CD4 plays a role in signal transduction and T cell activation.[7–12]

The magnitude of CD4 surface membrane expression is subject to regulation at both transcriptional and posttranscriptional levels. CD4 expression is substantially diminished on the surface membrane of T lymphocytes after exposure to antigen-presenting cells, infection with HIV-1, or incubation with phorbol esters.[13–16] Recently, we have observed that surface membrane expression of the Tiαβ-CD3:CD4 multireceptor is normally substantially less on human pulmonary T lymphocytes than on peripheral blood T cells. In this report, we describe how CD4 expression may be regulated by mitogens and explore the possibility that co-ligation of CD3 and CD4 during activation and limited availability of interleukin (IL)-2 may be required to generate CD4^{dim} lymphocytes.

Materials and Methods

Preparation and Culture of Cells

Grossly normal appearing fresh lung tissue was harvested from 12 patients undergoing pulmonary lo-

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bectomy or pneumonectomy for limited nonsmall cell lung carcinoma. All patients had a greater than 20 pack-year history of cigarette consumption. A saline bronchoalveolar lavage was performed on two patients with sarcoidosis, one with obstructive lung disease, and two normal volunteers; ~100 ml of bronchoalveolar lavage fluid was retrieved. Pulmonary T lymphocytes were purified after enzymatic digestion or mechanical disruption of lung tissue by sequential isolymph density gradient centrifugation and adherence to plastic and nylon wool column separation, as previously reported from our laboratory.[17] Pulmonary lymphocytes were maintained in culture at 37 C for 72 hours in complete medium (RPMI 1640 [JRH Biosciences, Lenexa, KS]; with 10% heat-inactivated human serum [Sigma Corp., St. Louis, MO], 2 mmol/L L-glutamine, 1% penicillin-streptomycin, 50 μg/ml gentamicin, and 5 mmol/L HEPES buffer [all GIBCO BRL, Gaithersburg, MD]). In some experiments, T cells were stimulated with recombinant human (rh) IL-2 (100 U/ml; Cetus Corporation, Emeryville, CA), a mitogenic murine anti-CD3 monoclonal antibody (MAb) (12F6, 0.1 μg/ml; R. Colvin), PHA (5 μg/ml; Sigma), phytohemagglutinin (PHA; 1 μg/ml), or rhIL-4 (100 U/ml; DNAX, Burlingame, CA). Peripheral blood (20 ml) from the same patients was drawn into glass tubes containing 3.8% acid citrate dextrose, mononuclear cells were isolated on isolymph gradients, and subsequent purification of T lymphocytes was conducted as described above. In some experiments, peripheral blood T lymphocytes (PBLs) were exposed for up to 30 minutes to graded dilutions of collagenase and/or DNAse (Worthington Co.) to determine whether digestive enzymes affected surface membrane antigen expression. Mitogen responses were determined at 72 hours after pulsing the wells for 4 hours with 1 μCi/well of [^{3}H]thymidine (specific activity 81 Ci/mmol; DuPont/New England Nuclear, Boston, MA).

$CD45RA^+$ cells (>90% purity) were isolated from peripheral blood by subjecting citrated blood to isolymph separation and then incubating the mononuclear cells on tissue culture plates coated with anti-CD45RO for two cycles of adherence, 45 minutes each at room temperature. An aliquot of the nonadherent cells was stained with anti-CD45RA and FITC-conjugated goat anti-mouse IgG to assess purity.

The surface membrane expression of CD4 on pulmonary T lymphocytes of other species was also examined quantitatively by cytofluorimetry. C57/BL mice (Charles River Laboratories, Wilmington, MA) were sacrificed by nembutal overdose, the lungs and spleens were mechanically disrupted, lymphocytes were stained with rat anti-murine CD4 (GK1.5; Becton Dickinson, San Jose, CA), and mean fluorescence staining intensities were analyzed in a FACS 440, as described below.

Immunofluorescent Staining and Analysis

CD4 surface membrane expression was examined by direct and indirect immunostaining with a panel of murine anti-human CD4 MAbs (Table 1). These included Leu3a (Becton Dickinson), OKT4, OKT4B, OKT4C, OKT4D, OKT4E, and OKT4F (Ortho Pharmaceutical Research Institute, Raritan, NJ). Sodium azide (0.1%) was present during the staining procedure. Fresh or cultured T cells (1×10^6 cells) were incubated in 50 μl phosphate-buffered saline (PBS) with 5 μl of FITC-conjugated, phycoerythrin (PE)-conjugated or unconjugated MAbs for 30 minutes at 4 C.

Cells treated with unconjugated MAbs were stained with $F(ab')_2$ goat anti-mouse IgG-FITC (1:40). The stained cells were fixed in 2% paraformaldehyde/PBS and stored in the dark at 4 C until analyzed. Fluorescence controls included mouse IgG1-FITC, IgG2a-PE, or $F(ab')_2$ goat anti-mouse IgG-FITC (Becton Dickinson). T lymphocytes were electronically gated based on their characteristic forward and (90°) side light scatter and were detected by anti-CD3 (Leu4) staining. Fluorescence staining intensities were analyzed in a Becton Dickinson FACS 440 cytofluorimeter equipped with a Consort-30 computer system. Identical cytofluorimeter settings were used for the analysis of pulmonary and peripheral blood lymphocytes. Antigen density was assessed indirectly by comparing the mean fluorescence intensities of the analyzed cells for each MAb using matched staining techniques.

RNA Isolation and Northern Blot

Total cellular mRNA was isolated from fresh pulmonary T lymphocytes and homologous blood T lymphocytes by acid guanidium thiocyanate-phenol-chloroform extraction of 5×10^6 cells.[18] The mRNA

Table 1. *MAbs Used to Examine CD4 Expression*

Clone	Source	Isotype	Putative Domain Binding Site[30]
Leu3a	Mouse	IgG1	D1
OKT4	Mouse	IgG2b	D4
OKT4B	Mouse	IgM	D2
OKT4C	Mouse	IgG2a	D1 + D2
OKT4D	Mouse	IgG1	D1
OKT4E	Mouse	IgG1	D1 + D2
OKT4F	Mouse	IgG1	D1 + D2

was subjected to electrophoresis under denaturing conditions in a 1% agarose/formamide gel and then transferred to nitrocellulose filters. In other experiments 10 µg of mRNA was applied to nitrocellulose filters in a Bio-Rad slot blot apparatus. The filters were baked, prehybridized, and hybridized to ^{32}p cDNA probes for human CD4 (gift of B. Seed) and β-actin (gift M. Vermeulen), exposed overnight to Kodak X-OMAT film,[19] and the autoradiographs were analyzed by gel densitometry.

Stimulation of T Cells

CD3 and CD4 surface membrane expression was examined after stimulation with anti-CD3 (12F6), OKT4, or the molecularly engineered bispecific murine anti-human CD3:CD4 (12F6:OKT4) MAb developed in our laboratories.[20] The MAbs were separately immobilized on plastic dishes[21] at predetermined optimal dilutions; $\sim 1 \times 10^6$ blood T cells were stimulated for 3 days and transferred to 24-well culture plates containing complete medium but no added IL-2. The cultured cells were harvested, washed twice in PBS, and stained on days 3 and 13 with anti-CD3 (Leu4-FITC) and anti-CD4 (Leu-3-PE) or with F(Ab')$_2$ goat anti-mouse Ig-FITC. Fluorescence staining intensities were measured in a Becton Dickinson FACS 440 and the ratio of the mean fluorescence staining intensities of CD4:CD3 was calculated.

Statistics

Mean fluorescence staining intensities were compared by paired *t*-test statistics.

Results

Surface Immune Phenotype of Pulmonary T Cells

The surface membrane fluorescence staining intensities of Tiαβ (anti-TCR, Becton Dickinson) and CD3 (Leu4; Becton Dickinson) antigens were significantly diminished ($P = 0.02$ and $P = 0.03$, respectively) on human pulmonary lymphocytes compared with PBL. More than 90% of Tiαβ-CD3$^+$ pulmonary T lymphocytes also expressed CD45RO,[17] a transmembrane protein functionally associated with T cell activation and the acquisition of immunological memory.[22–26]

The level of CD4 expression by pulmonary T lymphocytes was ~25% that of homologous PBL ($P < 0.001$) (Figure 1). Diminished CD4 expression (Figure 2) was also observed for murine pulmonary lymphocytes compared with splenic T cells (278 ± 35 *versus* 563 ± 60, $P = 0.001$). By contrast, the CD8 surface staining intensities of pulmonary T cells was comparable to those of PBL in all species (data not shown).

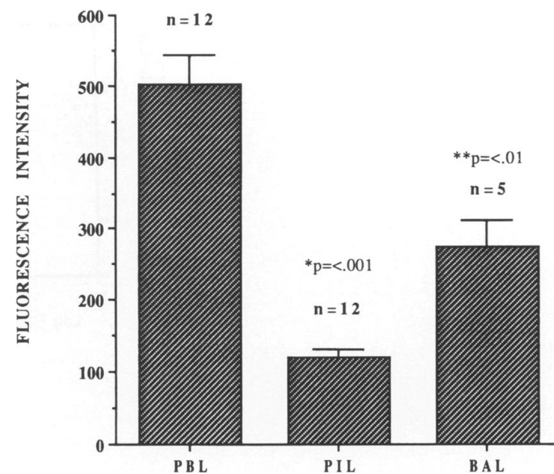

Figure 1. *CD4 expression by pulmonary and blood T cells. T cells isolated from enzymatically digested lung, pulmonary infiltrating lymphocytes (PIL), BAL, and PBL were stained with anti-CD4 (Leu3a)-FITC and analyzed by cytofluorimetry. Data represents the mean fluorescence intensities ± SD.*

Because digestive enzymes can decrease the expression of certain lymphocyte surface antigens,[27] T cells were also purified from human lungs by mechanical disruption and bronchoalveolar lavage (BAL). In both cases, the CD4 staining intensities of pulmonary lymphocytes were still significantly less than those of homologous PBL (all $P < 0.01$) (Figures 1 and 3). In addition, when human PBL were exposed to graded dilutions of collagenase and/or DNAse for up to 30 minutes, they showed staining intensities for Tiαβ, CD3, and CD4 comparable to those of untreated PBL.

A putative epitope map of the human CD4 molecule is shown in Figure 4.[28–30] Human pulmonary lymphocytes and PBL were stained with a panel of six anti-CD4 MAbs to determine whether their expression of all CD4 epitopes was diminished.[31] Staining of the OKT4, OKT4B, OKT4C, OKT4D, and OKT4E epitopes of pulmonary T cells was decreased (range, 39 to 64%) from PBL (Figure 5), whereas the magnitude of decreased OKT4F expression was small (~5%).

Regulation of CD4 Expression by Pulmonary Lymphocytes

A variety of local factors in the lungs can suppress the activities of T cells. We examined the possibility that CD4 expression might be transiently down-regulated *in vivo* by a factor within the lung but found that when

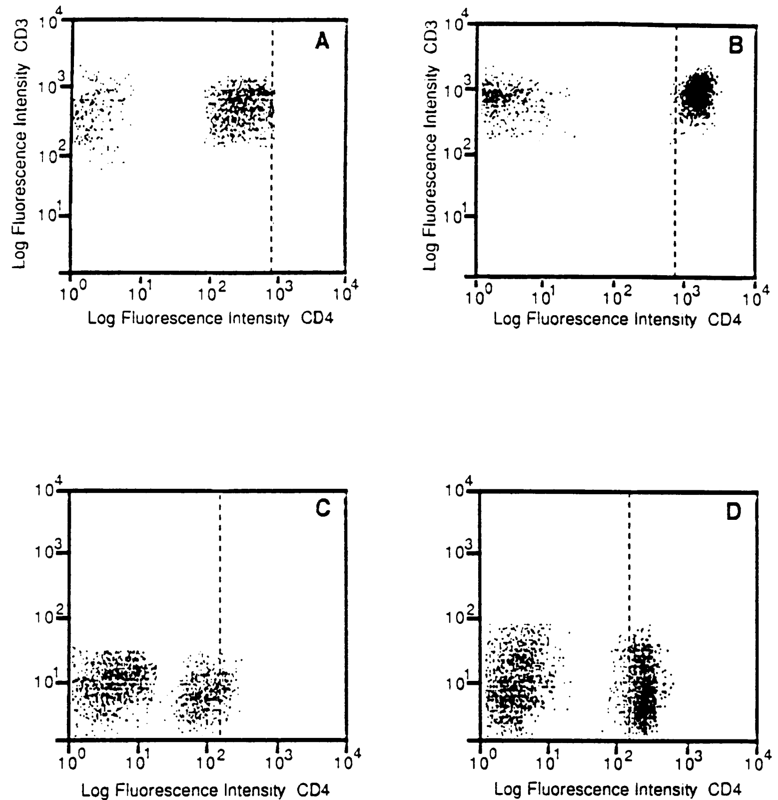

Figure 2. *Cytofluorimetric dot plots of human T cells. PIL* (A) *and PBL* (B) *stained with anti-CD4 (Leu3a-FITC) and anti-CD3 (Leu4-PE) shows diminished CD4 staining intensities for PIL. Murine PIL* (C) *and spleen* (D) *stained with anti-CD4 (GK1.5-FITC) shows decreased CD4 staining by PIL.*

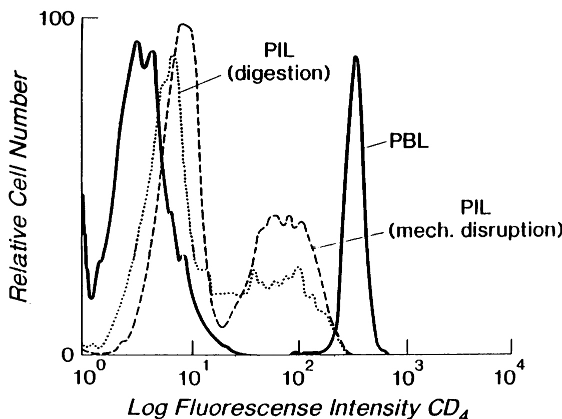

Figure 3. *Effect of enzymatic digestion on CD4 surface expression of blood lymphocytes. T cells isolated from enzymatically digested lung (dotted line), mechanically disrupted lung (dashed line), and peripheral blood (solid line) were stained with anti-CD4 (Leu3a-FITC) and analyzed by cytofluorimetry.*

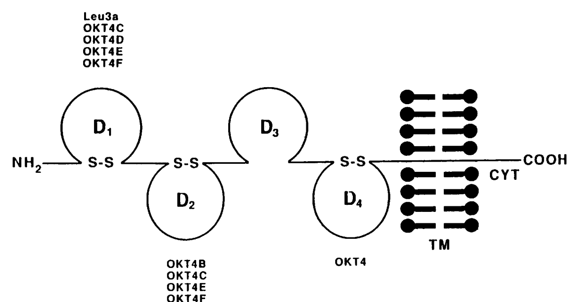

Figure 4. *Putative epitope mapping of CD4 antigen. The schematic diagram of CD4 shows important structural features, including the four Ig-like domains, D1 to D4 (three with intraloop disulfide bonds), the transmembrane (TM) portion, the intracytoplasmic tail (CYT), and the mapping of MAbs to specific domains.*

pulmonary lymphocytes were washed extensively with saline, and cultured in complete medium for 72 hours, their CD4 surface membrane expression did not increase.

The majority of pulmonary T cells are in G_o/G_1 phase of cell cycle and do not display IL-2 (TAC) receptors.[17] We investigated whether mitogen stimulation could augment CD4 surface membrane expression *in vitro*. Human pulmonary and blood lymphocytes were stimulated with PHA (5 µg/ml), anti-CD3 (0.1 µg/ml), rhIL-2 (100 µ/ml) (Cetus Corporation), or rhIL-4 (100 µ/ml) (DNAX) for up to 72 hours and CD4 surface membrane staining intensities were monitored by cytofluorimetry. With the exception of IL-4, which proved to be a weak mitogen for both blood and pulmonary T cells, as judged by [^{3}H]thymidine incorporation (data not shown), each of the other mitogens yielded a marked increase in the CD4 surface staining intensity on pulmonary T lymphocytes (Figure 6). However, CD4 surface membrane expression by PBL was not increased further by mitogen stimulation.

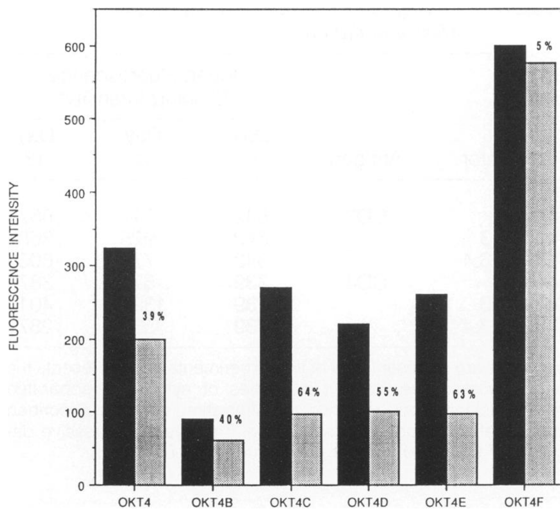

Figure 5. *Cell surface immune staining of PBL and PIL with a panel of murine MAbs that react with human CD4. Lymphocytes from enzymatically digested lung or blood were stained with the OKT4 panel of murine MAbs that react with epitopes distributed along the CD4 molecule. PBL, solid bars; PIL, shaded bars. Percentage of decrease of control staining intensities are shown.*

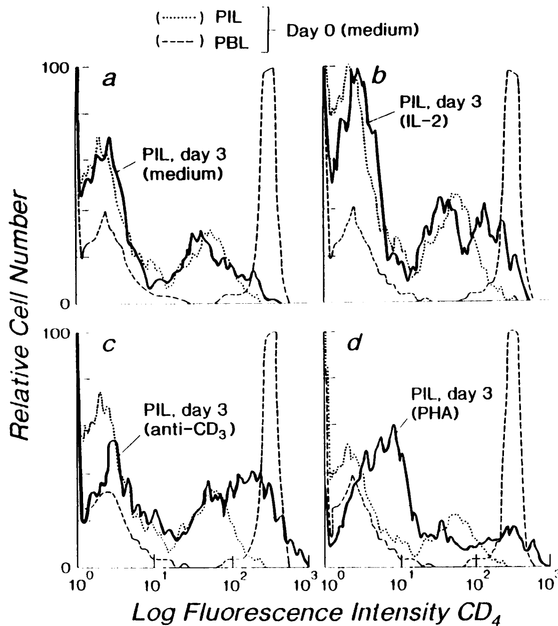

Figure 6. *Modulation of CD4 surface antigen expression in* in vitro. *Pulmonary lymphocytes isolated from enzymatic digests of lung were incubated in* (a) *complete medium,* (b) *rhIL-2 (100 Cetus) U/m,* (c) *anti-CD3 (12F6, 0.1 µg/ml), and* (d) *PHA (5 µg/ml) for up to 3 days stained with anti-CD4 on day 0 (before incubation) and day 3 and analyzed by cytofluorimetry. The fluorescence staining intensities were compared with those of freshly isolated PBL. Incubation of PBL in medium or with mitogens had little effect on CD4 staining intensity at day 3 (data not shown).*

Because dividing cells in the G2-M phase of the cell cycle have a larger cell volume than cells in the G_0/G_1 phase, the possibility that increased CD4 staining intensities might be a reflection of the greater volume of dividing T cells was examined. Forward light scatter, which approximates Coulter cell volume,[32] was slightly increased after mitogen stimulation, but this change in cell size accounted for only ~25% of the observed increase in CD4 expression.

Functional Analysis

We next examined whether $CD4^+$ cells from the lung were functionally distinct from $CD4^+$ cells in the peripheral blood. $CD4^+$ lymphocytes were purified from lung and blood by sequential nylon wool separation and complement lysis with OKT8 (Ortho Pharmaceuticals). The resulting cells were more than 90% $CD4^+$. The cells were next incubated with either IL-2 (100 Cetus U/ml), PHA (1 µg/ml), or anti-CD3 (1 µg/ml) for 72 hours. The results shown in Figure 7 show that $CD4^+$ cells from the lung can respond to mitogens, however, the magnitude of their responses to PHA and anti-CD3 stimulation was diminished compared with $CD4^+$ blood lymphocytes.

CD4 mRNA Levels

Decreased CD4 surface membrane expression by pulmonary T cells *in vivo* may be due to either diminished CD4 mRNa transcription or posttranscriptional changes. When examined by Northern blotting, CD4 mRNA isolated from freshly harvested pulmonary lymphocytes, pulmonary lymphocytes stimulated with IL-2, and blood lymphocytes all co-migrated at ~3000 kb. The total level of CD4 mRNA from freshly harvested pulmonary lymphocytes was 1.5- to 3-fold greater than that of purified $CD4^+$ PBL, as judged by gel densitometry (Figure 8), suggesting that diminished CD4 surface expression could not be accounted for by decreased CD4 mRNA transcription.

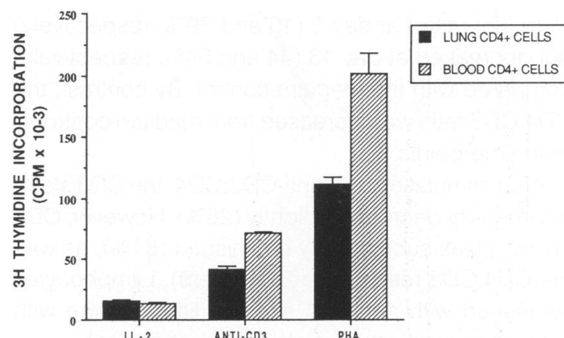

Figure 7. *Mitogen responses of $CD4^+$ blood and lung lymphocytes. $CD4^+$ blood and lung lymphocytes were purified (>90%) by sequential treatment with nylon wool and OKT8 and complement-mediated lysis. The purified lymphocytes were incubated in rhIL-2 (100 Cetus U/ml) anti-CD3 (12F6, 0.1 µg/ml), or PHA (1 µg/ml) for 3 days and [³H]thymidine incorporation was determined after a 6-hour pulse.*

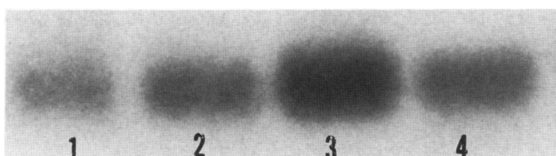

Figure 8. *Slot-blot analysis of CD4 mRNA from lung and blood lymphocytes. Total cellular mRNA was isolated from fresh pulmonary T lymphocytes and homologous blood T lymphocytes by acid guanidium thiocyanate-phenol-chloroform extraction of 5 × 10⁶ cells. The 10 μg of mRNA was applied to nitrocellulose filters in a slot blot apparatus and the filters were baked, prehybridized, and hybridized to ³²p cDNA probes for human CD4 and β-actin. Lane 1, CD4 mRNA from PBL; lane 2, β-actin from PBL; lane 3, CD4 mRNA from pulmonary lymphocytes; lane 4, β-actin from pulmonary digests.*

Co-Ligation of CD3:CD4 with a Bispecific MAb Yields CD4^{dim} Expression

Because >95% of CD4$^+$ lymphocytes isolated from the lung are CD45RO$^+$, indicating their prior sensitization to antigen *in vivo*,[17] we speculated that dim CD4 expression by pulmonary T cells might be related to ligation of Tiαβ-CD3:CD4 by antigen and class II (Ia) MHC molecules on the surface of antigen-presenting cells *in vivo*. CD45RA$^+$ PBL were activated with plastic immobilized anti-CD3 (12F6), anti-CD4 (OKT4), or a bispecific anti-CD3:CD4 (12F6:OKT4) that mimics ligation of the Tiαβ-CD3:CD4 multireceptor by antigen and class II MHC molecules. Substantial mitogenic responses by PBL, as judged by [^{3}H]thymidine incorporation at 72 hours, were achieved with anti-CD3 and anti-CD3:CD4 but not anti-CD4 (data not shown). The surface membrane staining intensities of CD3 and CD4 antigens were measured in parallel, and a ratio of the CD4:CD3 mean fluorescence staining intensities was constructed for days 3 and 13 postactivation. Unstimulated lymphocytes maintained in complete medium showed a progressive decrease in their CD3 and CD4 expression. After stimulation with anti-CD3, the mean fluorescence staining intensities of CD3 and CD4 were increased at day 3 (10 and 79%, respectively) but decreased at day 13 (44 and 54%, respectively) compared with the medium control. By contrast, the CD4:CD3 ratio was increased from medium control at both time points.

After stimulation with anti-CD3:CD4, the CD3 staining intensity decreased slightly (28%). However, CD4 staining was substantially diminished (61%), as was the CD4:CD3 ratio (Table 2, Figure 9). Lymphocytes stimulated with anti-CD4 showed little change with respect to medium controls (data not shown).

Staining with F(ab′)$_2$ goat anti-mouse IgG-FITC revealed 8% residual staining at day 3 and <2% at day 13, effectively excluding the possibility that changes in epitope expression were due to masking of either CD3 or CD4 epitopes by antibodies that might have leached from the plastic surface.

Table 2. *Changes in CD3 and CD4 Expression After Stimulation*

Stimulator	Antigen	Mean Fluorescence Staining Intensity* Day 0	Day 3	Day 13
Medium	CD3	842	717	657
Anti-CD3		842	925	368
Anti-CD3:4		842	778	603
Medium	CD4	739	571	387
Anti-CD3		739	1321	401
Anti-CD3:4		739	534	287

* Data are representative of two experiments and represents the mean fluorescence staining intensities of nylon wool-separated normal human blood lymphocytes after stimulation, as described in the Materials and Methods section. There is a progressive decrease in CD4 expression in medium controls over time.

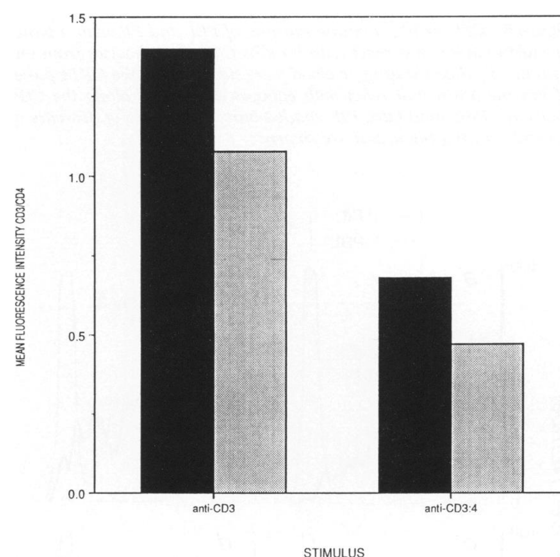

Figure 9. *Ratio of CD4:CD3 expression after stimulation with anti-CD3 or anti-CD3:CD4. Purified human blood lymphocytes were stimulated by immobilized anti-CD3 or anti-CD3:4 for 72 hours* in vitro *and then expanded in culture without IL-2 for up to 13 days. The cells were stained with FITC-conjugated anti-CD3 (Leu4) or anti-CD4 (Leu3a), analyzed in a FACS 440, and the ratio of mean fluorescence intensities was calculated. Day 3, shaded bars; day 13, solid bars.*

When long-term cultured T cell clones and lines established from a variety of benign and malignant tissues in our laboratory and maintained with IL-2 were examined, they did not show decreased CD4 expression. To investigate the role that IL-2 had on CD4 expression by cultured T cells, PBLs were stimulated with anti CD3:CD4 and then cultures were supplemented with IL-2 (10 Cetus U/ml). T cells cultured for 14 days to several months showed a Tiαβ-CD3:CD4 expression that was comparable to that of unstimulated PBL (data not shown).

Discussion

We have previously reported that T lymphocytes isolated from human lungs show the surface immune phenotype of antigen-sensitized T cells.[17] During the course of those studies, we noted that freshly isolated pulmonary T lymphocytes routinely display diminished cell surface membrane expression of the Tiαβ-CD3:CD4 molecular complex. In this report, we have focused primarily on characterizing changes in the expression of CD4 by pulmonary T cells.

Our results, based on cytofluorimetry, show that all extracytoplasmic epitopes of CD4 are decreased on pulmonary T cells compared with PBL. Although the precise alignment of CD4 epitopes remains uncertain,[11, 33] the amino acid residues of CD4 have been putatively assigned to molecular domains D1 to D4 based on sequence homology with Ig variable domains. Merkenshlager et al and others[30, 34, 35] have concluded that the anti-Leu3a and OKT4D epitopes map exclusively to the D1 domain; the OKT4C, OKT4E, and OKT4F map to both the D1 and other CD4 domains, whereas the OKT4B and OKT4 epitopes are located entirely outside the D1 domain. Although the OKT4F epitope was minimally diminished with respect to the other CD4 epitopes in this study, we believe that diminished surface expression of the entire extracytoplasmic portion of the CD4 molecule is likely, and that a molecular conformational change that spares the OKT4F epitope cannot account for the diminished CD4 levels seen on pulmonary T cells. Because a truncated form of CD4 has not been observed on activated T cells, we favor the possibility that dim surface CD4 expression reflects either posttranscriptional shedding and/or internalization of the Tiαβ-CD3:CD4 multireceptor on pulmonary T cells. Our position is based on the observations that: 1) levels of CD4 mRNA in pulmonary lymphocytes are comparable to or greater than those of PBL, 2) both Tiαβ and CD3 are also diminished on pulmonary T cells, and 3) loss of CD4 during T cell activation has been reported by Weyand et al[14] in human T cell clones. However, because CD4 expression is diminished to a greater extent than Tiαβ-CD3, other mechanisms of regulation are likely to be involved in the generation of dim CD4 cells.

A link between diminished CD4 expression and previous T cell activation is suggested by the current experiments. CD4 participates in antigen presentation to T cells by binding MHC class II antigens and modulating effector cell responses.[2, 7, 12] We simulated antigen presentation nonspecifically by activating peripheral blood T cells with the bispecific anti-CD3:CD4 MAb.[20] The parental monospecific OKT4 MAb used to construct this bispecific antibody effectively inhibits antigen presentation and the OKT4 epitope appears to participate directly in native responses to antigen.[3, 8, 36] A role for the simultaneous cross-linking of CD3 to CD4 in yielding diminished CD4 expression is further supported by the marked decrease in CD4 expression observed after stimulation by the bispecific MAb. We have not yet determined how cross-linking directly yields dim CD4 expression, but we favor a role for CD4 shedding and/or internalization. The changes in CD4 expression after activation by anti-CD3:CD4 MAb could not be accounted for by masking CD4 epitopes, because decreased CD4 was not observed after stimulation with monospecific anti-CD4.

Although this report has focused on CD4 expression by pulmonary T cells, it is expected that diminished expression of the Tiαβ-CD3:CD4 multireceptor complex will also be observed in other tissues. In this regard, Smith and Roberts-Thomson[37] reported diminished expression of both CD3 and CD4 antigens on T lymphocytes isolated from the synovial fluid of patients with rheumatoid joint disease and lymphocytes isolated from chronically inflamed human small intestine are also CD4^{dim} (G. Russel personal communication). Zanders et al[38] had previously demonstrated that antigen excess in the absence of appropriate antigen-presenting cells triggers the loss of CD3 from the surface of T cell clones and can lead to a state of relative anergy. It is possible that the pulmonary millieu favors ineffective presentation of antigen leading to diminished Tiαβ-CD3:CD4 expression in lung and other organs.

IL-2 and other T cell mitogens, including lectin and anti-CD3, were able to promote CD4 expression on pulmonary T cells. We hypothesize that CD4^{dim} lymphocytes may be generated preferentially in the setting of limited IL-2 availability, which follows antigen stimulation *in vivo*. This is supported by our finding that high levels of CD4 were maintained in long-term T cell cultures maintained with IL-2. Because the lung is a source of soluble factors that antagonize the activities of IL-2 *in vivo*, including transforming growth factor-β,[39] prostaglandin E-2,[40] and surfactant apoproteins,[41] the normal lung may be a site of limited IL-2 availability favoring the presence of diminished CD4 expression by T cells.

The physiological consequences of diminished CD4 expression by pulmonary T cells are uncertain. CD4 is an adhesion molecule that increases the binding affinity of Tiαβ to the antigen/MHC class II complex.[3, 7] Diminished CD4 expression might decrease antigen-driven T cell activation in the lung or, alternately, favor the activation of T cells with high affinity

Tiαβ receptors for antigen *in vivo*. The finding that a comparable diminution of CD8 antigens does not occur on the surface of pulmonary T cells may indicate a fundamental difference in the pathway of class I and II MHC-restricted cell activation *in vivo*.

The role of CD4 as the critical cellular receptor for HIV-1 makes its diminished surface expression noteworthy with respect to the pathogenesis of AIDS. Schnittman et al[42] have reported that $CD4^+$ memory T cells can be readily infected with HIV-1 yielding increased levels of virus compared with infected naive T cells purified from blood, and in preliminary studies we have shown that unstimulated pulmonary $CD4^+$ T cells show a substantially increased level of HIV-1 yield compared with unstimulated $CD4^+$ blood cells, despite their lesser expression of CD4 (B. Conway, manuscript in preparation). This appears to reflect the altered physiology of CD4 memory cells. We are currently examining differences in HIV-1 binding to $CD4^{dim}$ pulmonary lymphocytes. Because natural CD4 ligands other than HIV-1 and class II MHC have been suggested,[43–45] further studies will be required to establish the role played by $CD4^{dim}$ T cells during inflammation.

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References

1. Meuer SC, Schlossman SF, Reinherz EL: Clonal analysis of human cytotoxic T lymphocytes: $T4^+$ and $T8^+$ effector T cells recognize products of different major histocompatibility complex regions. Proc Natl Acad Sci USA 1982, 79:4395–4399
2. Marrack P, Endres R, Shimokevitz R, Zlotnik A, Dialynas D, Fitch F, Kappler J: The major histocompatibility complex-restricted antigen receptor on T cells. II. Role of the L3T4 product. J Exp Med 1983, 158:1077–1091
3. Janeway CA Jr: The T-cell receptor as a multicomponent signalling machine: CD4/CD8 coreceptors and CD45 in T-cell activation. Annu Rev Immunol 1992, 10:645–674
4. Jameson BA, Rao PE, Kong LI, Hahn BH, Shaw GM, Hood LE, Kent SB: Location and chemical synthesis of a binding site for HIV-1 on the CD4 protein. Science 1988, 240:1335–1339
5. Sattentau QJ: Interactions of HIV gp120 with the CD4 molecule on lymphocytes and in the nervous system. Ann NY Acad Sci 1990, 594:355–361
6. Moore JP, McKeating JA, Norton WA, Sattentau QJ: Direct measurement of soluble CD4 binding to HIV-1 virions: gp 120 dissociation and its implications for virus cell binding and fusion reactions and their neutralization by soluble CD4. J Virol 1991, 65:1133–1140
7. Doyle C, Strominger JL: Interaction between CD4 and class II MHC molecules mediates cell adhesion. Nature 1987, 30:256–259
8. Biddison WE, Rao PE, Talle MA, Goldstein G, Shaw S: Possible involvement of the OKT4 molecule in T cell recognition of class II HLA antigens. J Exp Med 1982, 156: 1065–1076
9. Wilde DB, Marrack P, Kappler J, Dialyns D, Fitch FW: Evidence implicating L3T4 in class II MHC antigen reactivity; monoclonal antibody GK1.5 (anti-L3T4a) blocks class II MHC Ag specific-proliferation, release of lymphokines and binding by cloned murine helper T lymphocyte lines. J Immunol 1983, 131:2178–2183
10. Greenstein JL, Kappler J, Marrack P, Burakoff SJ: The role of L3T4 in recognition of Ia by a cytotoxic, $H\text{-}2^d$-specific T cell hybridoma. J Exp Med 1984, 159:1213–1224
11. Sleckman BP, Peterson A, Jones WK, Foran JA, Greenstein JL, Seed B, Burakoff SJ: Expression and function of CD4 in a murine T cell hybridoma. Nature 1987, 328:351–353
12. Carrera AC, Sanchez-Madrid F, Lopez-Botet M, Bernabeu C, DeLandazuri MO: Involvement of the CD4 molecule in a post activation event on T cell proliferation. Eur J Immunol 1987, 17:179–186
13. Solbach W: Tumor promoting phorbol esters selectively abrogate the expression of the T4 differentiation antigen expressed on normal and malignant (Sezary) T helper lymphocytes. J Exp Med 1982, 156:1250–1251
14. Weyand CM, Goronzy J, Fathman CG: Modulation of CD4 by antigenic activation. J Immunol 1987, 138: 1351–1354
15. Sleckman BP, Bigby M, Greenstein JL, Burakoff SJ, Sy MS: Requirements for modulation of the CD4 molecule in response to phorbol myristate acetate: role of the cytoplasmic domain. J Immunol 1989, 142:1457–1462
16. Fields AP, Bednarik DP, Hess A, May WS: Human immunodeficiency virus induces phosphorylation of its cell surface receptor. Nature 1988, 333:278–280
17. Marathias KP, Preffer FI, Pinto C, Kradin RL: Most human pulmonary infiltrating lymphocytes display the surface immune phenotype and functional responses of sensitized T-cells. Am J Respir Cell Mol Biol 1991, 5:470–476
18. Chomczynski P, Sacchi N: Single step method of RNA isolation by acid guanidium thiocynate-phenol-chloroform extraction. Anal Biochem 1987, 162:156–159
19. Alwine JC, Kemp DJ, Stark GR: Method of detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. Proc Natl Acad Sci USA 1977, 74:5350–5354
20. Wong JT, Colvin RB: Bi-specific monoclonal antibodies: selective binding and complement fixation to cells that express two different surface antigens. J Immunol 1987, 139:1369–1374

21. Mage MG, McHugh LL, Rothstein TL: Mouse lymphocytes with and without surface immunoglobulin: preparative scale separation in polystyrene tissue culture plates coated with specifically purified anti-immunoglobulin. J Immunol Methods 1977, 15:47–56
22. Saltini C, Kirby M, Trapnell BC, Tamura N, Crystal RG: Biased accumulation of T lymphocytes with "memory"-type CD45 leukocyte common antigen gene expression on the epithelial surface of the human lung. J Exp Med 1990, 171:1123–1140
23. Akbar AN, Terry L, Timms A, Beverley PC, Janossy G: Loss of CD45R and gain of UCHL1 reactivity is a feature of primed T-cells. J Immunol 1988, 140:2171–2181
24. Sanders ME, Makgoba MW, Sharrow SO, Stephany D, Springer TA, Young HA, Shaw S: Human memory T lymphocytes express increased levels of three cell adhesion molecules (LFA-3, CD2, and LFA-1) and three other molecules (UCHL-1, CDw29, and Pgp-1) and have enhanced IFN-γ production. J Immunol 1988, 140:1401–1407
25. Serra HM, Krowka F, Ledbetter JA, Pilarski LM: Loss of CD45R(Lp 220) represents a post thymic T-cell differentiation event. J Immunol 1988, 140:1435–1441
26. Streuli MC, Morimoto M, Schrieber M, Schlossman SF, Saito H: Characterization of CD45 and CD45R monoclonal antibodies using transfected mouse lines that express individual human leukocyte common antigens. J Immunol 1988, 141:3910–3914
27. Holt PG, Robinson BW, Reid M, Kees UR, Warton A, Dawson VH, Rose A, Schon-Hegrad M, Papadimitriou JM: Extraction of immune and inflammatory cells from human lung parenchyma: evaluation of an enzymatic digestion procedure. Clin Exp Immunol 1986, 66:188–200
28. Kieber-Emmons T, Jameson BA, Morrow WJ: The gp120-CD4 interface: structural, immunological and pathological considerations. Biochim Biophys Acta 1989, 989:281–300
29. Mizumaki T, Fuerst TR, Berger EA Moss B: Binding region for human immunodeficiency virus (HIV) and epitopes for HIV-blocking monoclonal antibodies of the CD4 molecule defined by site-directed mutagenesis. Proc Natl Acad Sci USA 1988, 85:9273–9277
30. Merkenschlager M, Buck, Beverley DC, Sattentau QJ: Functional epitope analysis of the human CD4 molecule: the MHC class II-dependent activation of resting T-cells is inhibited by monoclonal antibodies to CD4 regardless whether or not they recognize epitopes in the binding of MHC class II or HIV gp120. J Immunol 1990, 145:2839–2845
31. Bach M, Phan-Dinh-Tuy F, Bach JF, Wallach D, Biddison WE, Sharrow SO, Goldstein G, Kung PC: Unusual phenotypes of human inducer T cells as measured by OKT4 and related monoclonal antibodies. J Immunol 1981, 127:980–982
32. Ault KA: Applications in Immunology and Lymphocyte Analysis: Flow Cytometry and Sorting. Edited by Melamed MR, Lindmo T, Mendelsohn ML. New York, Wiley-Liss, 1990, pp 685–696
33. Doyle C, Shin J, Dunbrack RL Jr, Strominger JL: Mutational analysis of the structure and function of the CD4 protein. Immunol Rev 1989, 109:17–37
34. Merkenschlager M, Altmann DM Ikeda H: T-cell allore-sponses against HLA-DQ and -DR products involve multiple epitopes on the CD4 molecule: distinct mechanisms contribute to the inhibition of HLA Class II-dependent and independent T-cell responses by antibodies to CD4. J Immunol 1990, 145:3181–3187
35. Perosa F, Dannecker G, Ferrone S, Dammacco F: Immunochemical and functional characterization of anti-idiotype antibodies to a mouse anti-CD4 monoclonal antibody. Int J Clin Lab Res 1991, 21:179–185
36. Wassmer P, Chan C, Logdberg L, Shevach EM: Role of the L3T4 antigen in T-cell activation. II. Inhibition of T-cell activation by monoclonal anti-L3T4 antibodies in the absence of accessory cells. J Immunol 1985. 135: 2237–2242
37. Smith MD, Roberts-Thomson PJ: Lymphocyte surface marker expression in rheumatic diseases: evidence for prior activation of lymphocytes in vivo. Annu Rheum Dis 1990 49:81–87
38. Zander ED, Lamb JR, Feldmann M, Green N, Beverly PC: Tolerance of T-cell clones is associated with membrane changes. Nature 1983, 303:625–627
39. Ahuja SS, Paliogianni F, Yamada H, Balow JE, Boumpas DT: Effect of transforming growth factor-beta on early and late activation events in human T cells. J Immunol 1993, 150:3109–3118
40. Foley P, Kazazi F, Biti R, Sorrell TC, Cunningham AL: HIV infection of monocytes inhibits T-lymphocyte proliferation response to recall antigens, via production of eicosanoids. Immunology 1992, 75:391–397
41. Paine R 3d, AChevis, Tocws GB: A factor secreted by a human alveolar epithelial like cell line blocks T-cell proliferation between G1 and S phase. Am J Respir Cell Mol Biol 1992, 6:658–666
42. Schnittman SM, Lane HC, Greenhouse J, Justement JS, Baseler M, Fauci AS: Preferential infection of $CD4_+$ memory T cells by human immunodeficiency virus type 1: evidence for a role in the selective T-cell functional defects observed in infected individuals. Proc Natl Acad Sci USA 1990, 87:6058–6062
43. Baixeras E, Huard B, Miossec C, Jitsukawa S, Martin M, Hercend T, Auffray C, Triebel F, Piatier-Tonneau D: Characterization of the lymphocyte activation gene 3-encoded protein: a new ligand for human leukocyte antigen class II antigens. J Exp Med 1992, 176:327–337
44. Koulova L, Clark EA, Shu G, Dupont B: The CD28 ligand B7/BB1 provides costimulatory signal for alloactivation of $CD4_+$ cells. J Exp Med 1991, 173:759–762
45. Rand TH, Cruikshank WW, Center DM, Weller PF: CD4-mediated stimulation of human eosinophils: lymphocyte chemoattractant factor and other CD4-binding ligands elicit eosinophil migration. J Exp Med 1991, 173:1521–1528