Expression of the α1β1 integrin, VLA-1, marks a distinct subset of human CD4+ memory T cells

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The α1β1 integrin, very late antigen-1 (VLA-1), is a collagen receptor expressed in many CD4+ T cells localizing to inflamed tissues. Here we show that the expression of VLA-1 is a stable marker of a distinct subset of CD4+ memory T cells. Thus, in human peripheral blood lymphocytes (PBLs), approximately 1–4% of the CD4+ T cells express VLA-1, and following T cell receptor activation ex vivo, the percentage of VLA-1+ cells increases within the CD45RO+ population. Importantly, the activated VLA-1+ and VLA-1− cells can be isolated and maintained in culture as phenotypically stable subsets. Functionally, CD4+ memory T cells, operationally defined as the cells that divide rapidly following stimulation with a recall antigen, are highly enriched for VLA-1+ cells. Moreover, depletion of the small fraction of VLA-1+ cells present in CD4+ PBLs prior to stimulation significantly abrogated the proliferative response to recall antigens. Notably, the VLA-1+ cells in fresh CD4+ PBLs are composed of resting CD45RO+/RA−, CCR7−, CD62L+, CD25−, and VLA-4hi cells. Interestingly, this VLA-1+ subset is enriched for Th1-type cells, and Th1-polarizing conditions during T cell activation favor the emergence of VLA-1+ cells. Thus, VLA-1 expression is a stable marker of a unique subset of human memory CD4+ T cells that predominantly differentiates into Th1 cells.

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Introduction

The α1β1 integrin is a heterodimeric cell surface receptor composed of a β1 chain shared also by other β1 integrins and an α1 chain that contains a specific "inserted" I-domain in its extracellular portion responsible for collagen recognition (1, 2). The α1β1 integrin uniquely binds to collagen IV but also binds to collagen I and weakly to laminin (3–5). The binding of very late antigen-1 (VLA-1) to these ECMs is thought to facilitate cellular migration through the laminin- and collagen IV–rich basement membranes, as well as to enhance migration within the collagen-rich interstitium (6). Moreover, VLA-1 interaction with collagen activates signaling pathways that promote cell survival and progression through the cell cycle (7–9).

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Nonstandard abbreviations used: very late antigen-1 (VLA-1); T cell receptor (TCR); delayed-type hypersensitivity (DTH); rheumatoid arthritis (RA); purified protein derivative (PPD); peripheral blood lymphocyte (PBL); phycoerythrin-conjugated (PE); synovial fluid lymphocyte (SFL); magnetic cell separation system (MACS); toxic shock syndrome toxin-1 (TSST-1); tetanus toxoid (TT); psoriatic arthritis (PsA).

In T cells, VLA-1 becomes expressed only very late (more than 6 days) after T cell activation (10, 11). Furthermore, VLA-1/collagen interactions augment T cell receptor–mediated (TCR-mediated) proliferation and cytokine secretion (12, 13). Importantly, recent studies in murine models of inflammation show that deleting the α1 integrin gene or blocking the functions of VLA-1 in vivo with mAb's prevents the development of delayed-type hypersensitivity-like (DTH-like) immune responses, including those associated with graft-versus-host disease, adjuvant or Ab-induced arthritis, and hapten-induced colitis (14–18). Notably, in the wild-type untreated animals the majority of T cells (and monocytes) infiltrating the inflamed tissues express VLA-1 (15, 18).

Similarly, human T cells infiltrating inflamed tissues, particularly in Th1-mediated inflammation, are highly enriched for VLA-1+ cells. For example, the T cells infiltrating the synovium of rheumatoid arthritis (RA) patients (12, 19), the lungs of sarcoidosis patients (20), and tuberculin purified protein derivative–induced (PPD-induced) skin blisters (11) are highly enriched in VLA-1–expressing cells. It is of interest that these VLA-1+ T cells in the inflamed tissue usually coexpress CD45RO, a marker of previously activated T cells (21, 22). In the fresh peripheral blood lymphocytes (PBLs) of normal individuals as well as patients, however, the expression of VLA-1 is restricted to only a small fraction of the CD45RO+ T cells (21, 23). The frequent coexpression of VLA-1 with CD45RO in both inflammatory sites and in peripheral blood, taken together

with the observations that VLA-1 is expressed only in a fraction of the CD45RO cells, suggests the possibility that VLA-1 may mark a subset of memory T cells.

In this regard, precise identification of $CD4^+$ memory T cells is problematic, partly because unambiguous markers of memory have not been described (24). Thus, although CD45RO expression has been used to distinguish naive from memory T cells, it is clear that not every T cell that expresses CD45RO is necessarily a memory cell, since virtually all recently activated T cells also express CD45RO. In this regard, CD45RO expression reflects more accurately an imprint of prior activation than a marker of memory cells. In addition, recent studies give rise to the notion that the memory lineage is composed of different subsets based on the expression of other cell surface molecules, including chemokine receptors, selectins, and adhesion molecules (24–27). For example, the memory T cell pool has been subdivided based on the expression of the lymph node–homing chemokine receptor CCR7 into two major subsets. Because $CCR7^+$ cells home predominantly to lymph nodes, they have been termed the central memory subset. Migration of memory T cells from the lymph nodes to the blood and peripheral organs is associated with the loss of CCR7 expression and the functional differentiation of the memory cells into effector T cells capable of secreting cytokines and functioning as inducer and cytotoxic cells. Thus, these $CCR7^-$ cells have been termed effector memory T cells (25).

Here we will present evidence that following $CD4^+$ T cell activation, whereas all the T cells express CD45RO, stable VLA-1 expression is observed only in a subset of the $CD45RO^+$ T cells. To determine whether this VLA-1–expressing subset represented memory cells, we defined the memory $CD4^+$ T cells functionally by their greater capacity to rapidly proliferate upon re-encounter with a recall antigen (e.g., tetanus toxoid, PPD, or mumps antigens). We used the vital dye CFSE dilution technique (28, 29) to identify the memory population of cells as those that have rapidly divided following triggering with recall antigens. This enabled us to quantitate using flow cytometry not only the proportion of memory cells but also their cell surface phenotype with respect to VLA-1 and other surface markers. We found that the small fraction of VLA-1^+ T cells in fresh human PBLs form a major fraction within the memory cells proliferating in response to recall antigen and are predominantly Th1-type memory cells. Moreover, depletion of the $CD4^+$ VLA-1^+ T cells from the PBLs markedly abrogates the recall response to recall antigens. Interestingly, the circulating $CD4^+$ VLA-1^+ memory cells are predominantly $CCR7^-$, $CD25^-$, VLA-4^{hi}, and enriched for $CD62L^+$ cells.

Methods

Ab's and reagents. The hybridoma producing the function-blocking anti-human VLA-1 (1B3.1) that recognizes the α chain I-domain was originally generated in our laboratory at Columbia University (30). Hybridoma cells producing anti-human VLA-1 (TS2/7) mAb were purchased from American Tissue Culture Collection (Rockville, Maryland, USA). Another mAb that also recognizes the I-domain of human VLA-1 (AGF.1.3) was kindly provided by Biogen Inc. (Cambridge, Massachusetts, USA), and the phycoerythrin-conjugated (PE-conjugated) mAb to human VLA-1 (SR84; CD49a) was purchased from PharMingen (San Diego, California, USA). The following fluorochrome-conjugated murine anti-human mAb's were all purchased from PharMingen: CD4, CD8, CD25, CD45RO, CD45RA, CD62L, CD69, VLA-2 (CD49b), VLA-4 (CD49d), anti–IFN-γ, and anti–IL-4. Isotype control mouse IgG1, IgG2a, and IgG2b (pure and fluorochrome-conjugated), biotinylated anti-human CCR7, and streptavidin-PE were also obtained from PharMingen. FITC-conjugated anti-TCR Vβ2 was purchased from Immunotech (Marseille, France). The hybridoma producing mAb's to anti-CD3 (OKT3) was purchased from American Tissue Culture Collection. Fluorescein-conjugated $F(ab')_2$ fragment goat anti-mouse IgG plus IgM were purchased from Jackson ImmunoResearch Laboratories (West Grove, Pennsylvania, USA).

FACS analysis. The immunostaining and subsequent flow-cytometric analysis of human lymphocytes was carried out as described previously (31). Briefly, cells were first treated with human Ig (Sigma-Aldrich, St. Louis, Missouri, USA) to reduce nonspecific Ab binding to Fc receptors and subsequently were incubated with saturating concentrations of the indicated fluorescein-conjugated mAb for 15 minutes at 4°C. In some experiments, when pure mAb were used for the initial step, the cells were washed and then incubated for 15 minutes at 4°C with fluorescein-conjugated $F(ab')_2$ fragments of polyclonal goat anti-mouse IgG plus IgM. Fluorescence intensity was measured on a FACScan cytofluorograph (Becton Dickinson Immunocytometry Systems, San Jose, California, USA) and analyzed using the Cellquest software (Becton Dickinson Immunocytometry Systems). Viable lymphocytes were defined by their forward scatter/side scatter (FSC/SSC) characteristics or propidium iodide (10 μg/ml) in order to more accurately exclude dead cells from analysis. Of note, a very stringent compensation for interdetector spillage of fluorescence was necessary during sample acquisition due to the usually low fluorescence intensity, which is characteristic of VLA-1 staining in most VLA-1^+ human T cells.

T cell cultures and lines. PBLs and synovial fluid lymphocytes (SFLs) were isolated by Histopaque (Sigma-Aldrich) gradient centrifugation. All human studies were approved by the institutional review board of Columbia University. The $CD4^+$ T cells were further isolated from PBLs by negative selection using the magnetic cell separation system (MACS), according to the manufacturer recommendations (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany). Briefly, PBLs were first incubated for 20 minutes at 4°C with a hapten-Ab cocktail to CD8, CD11b, CD16, CD19, CD36, and CD56.

They were subsequently washed and incubated with anti-hapten microbeads and separated over a magnetic column system. The VLA-1$^+$ and VLA-1 fractions were likewise isolated using the MACS method. Briefly, cells were stained with anti–VLA-1 mAb's (AGF.1.3), washed, incubated for 15 minutes with goat anti-mouse microbeads (Miltenyi Biotech GmbH), and then selected over magnetic columns, as above. In some experiments, the VLA-1$^+$ cells were isolated by incubation with TS2/7, a mAb directed to a different epitope of VLA-1 to exclude effects unique to the AGF 1.3 mAb's. Purity of subsets following isolation was determined by immunostaining and FACS and usually was greater than 95%.

Purified T cells were plated at 1×10^6 to 2×10^6 cells/well into 24-well plates containing 2×10^6 cells/well of irradiated autologous PBLs (250 Gy) in 1 ml of medium consisting of X-Vivo 15 (BioWhittaker Inc., Walkersville, Maryland, USA) supplemented with 1% heat-inactivated human AB serum, 100 U/ml penicillin, and 100 μg/ml streptomycin and maintained in a humidified 37°C, 5% CO_2 incubator. The cells were stimulated immediately after plating with either 0.1 μg/ml of toxic shock syndrome toxin-1 (TSST-1) superantigen (Sigma-Aldrich), 2.5 μg/ml anti-CD3 mAb's, 3 flocculation units/ml (Lf/ml) tetanus toxoid (TT; Massachusetts Biological Laboratories, Worcester, Massachusetts, USA), 5 μg/ml PPD (Parke-Davis Division, Warner Lambert Co., Morris Plains, New Jersey, USA), 5 μg/ml mumps viral antigens (BioWhittaker Inc.), or medium alone. To generate long-term T cell lines, the purified fresh CD4$^+$ PBLs were stimulated with 0.1 μg/ml of TSST-1 and irradiated autologous APCs every 10 days. The cells were cultured in a medium consisting of RPMI, 10% FCS (HyClone Laboratories, Logan, Utah, USA), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Three days after the initial stimulation, and every 3 days thereafter, the medium was supplemented with 50 U/ml of recombinant IL-2 (Hoffman-La Roche Inc., Nutley, New Jersey, USA). The VLA-1$^+$ and VLA-1$^-$ fractions were usually purified from the mixed cultures several days after the second stimulation and maintained as above. To further generate VLA-1$^+$ and VLA-1$^-$ CD4$^+$ T cell clones, the cells were plated in 96-well U-bottom plates at a density of 3, 1, or 0.3 cells/well containing either 10^5 irradiated autologous PBLs (250 Gy) or autologous EBV-transformed B cells (500 Gy) and pulsed with 0.1 μg/ml of TSST. Subsequently, proliferating wells were screened for their VLA-1 phenotype, and selected clones were expanded by repeated stimulation with TSST-1–pulsed irradiated APCs every 10 days.

Intracellular cytokine staining and Th polarization assays. T cells were activated with 20 ng/ml of phorbol 12,13-dibutyrate and 0.8 μM of ionomycin (Sigma-Aldrich) in the presence of 2 μg/ml of monensin (GolgiStop; PharMingen) for 5 hours at 37°C. Following this brief activation, cells were harvested, fixed, and permeabilized using the Cytofix/Cytoperm Plus kit and then immunostained for intracellular IFN-γ or IL-4 production, all in accordance with the manufacturer's recommendations (PharMingen). All samples were then analyzed on a FACScan cytofluorograph (Becton Dickinson Immunocytometry Systems).

To induce Th1 polarization, the CD4$^+$ cells were stimulated with 2.5 μg/ml of anti-CD3 mAb in a medium containing 5 ng/ml recombinant human IL-12 (R&D Systems Inc., Minneapolis, Minnesota, USA). To induce Th2 polarization, the CD4$^+$ cells were activated in a medium containing 10 μg/ml of neutralizing anti–INF-γ mAb's and 20 ng/ml of recombinant human IL-4 (both from R&D Systems Inc.). Cytokines and anti–IFN-γ mAb's were thereafter added to the cultures every 3 days, and cells were harvested and used for the different assays at day 8.

Analyzing T cell proliferation using the CFSE dilution method. The flow cytometric–based analysis of T cell proliferation by serial halving of the fluorescence intensity of the vital dye, CFSE, has been described previously (29, 32). Briefly, T cells at 10^7/ml were suspended in 1 ml RPMI and then pulsed with 4 μM CFSE (Molecular Probes Inc., Eugene, Oregon, USA) for 15 minutes at 37°C with constant shaking. Subsequently, the CFSE was quenched with 50% human AB serum for 1 minute, and the cells were washed twice with large volumes of RPMI medium. The desired subsets of cells were purified and then triggered with either a polyclonal stimulus, recall antigens, or medium alone, as described above. The cells were then cultured for 8 days without the addition of IL-2 to minimize the background proliferation. In experiments addressing the proliferative contribution of the VLA-1$^+$ subset to the overall recall response, we first isolated CD4$^+$ PBLs using negative depletion, as above. Subsequently, cells were rested overnight and then immunomagnetically depleted of the rare VLA-1$^+$ CD4$^+$ T cells prior to antigenic triggering. To nullify the effects resulting from the separation procedure itself, we used sham depletion as our control group. The sham depletion consisted of incubating the cells with an isotype-matched mouse IgG (instead of the anti–α1 integrin mAb's) followed by goat anti-mouse immunobeads. This was followed by passing the cells through an identical magnetic column as done for the experimental group. The cells were harvested on day 8 and analyzed for CFSE content (generation number), various surface markers, and, when indicated, for intracellular cytokines. Since some dye is lost from the parental generation and some T cells can slowly proliferate in response to soluble factors in the medium (bystander effect), we considered in the data analysis as antigen-responsive only those CD4$^+$ T cells that have undergone more than cellular divisions.

Statistical analysis. The statistical analysis of the various samples was performed by a two-tailed paired Student's *t* test, Wilcoxon signed rank test, or the repeated measures ANOVA test, as appropriate. In all statistical tests a *P* value less than 0.05 was considered significant.

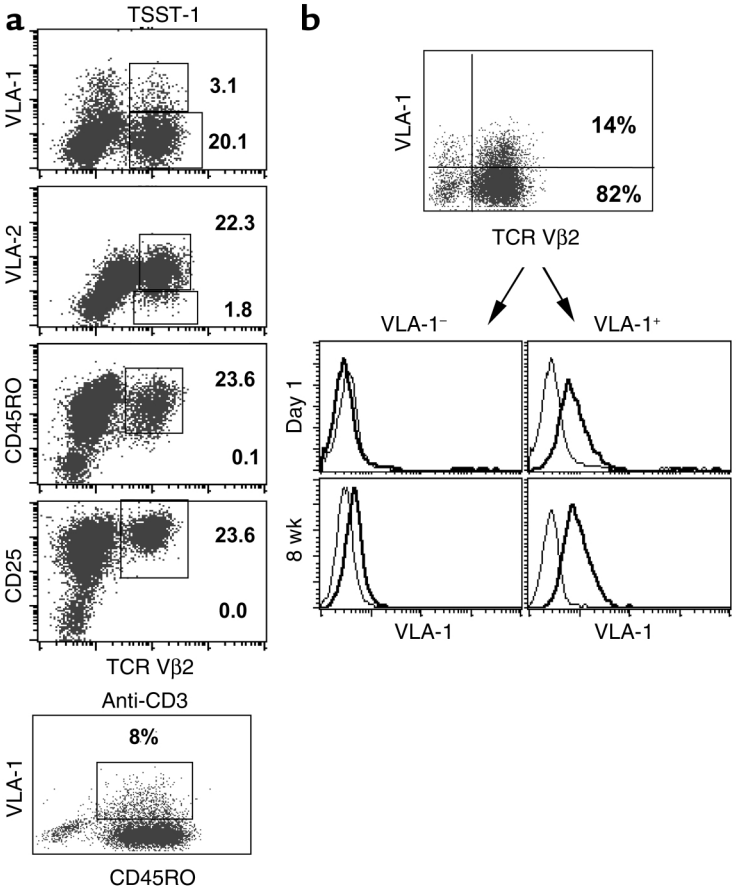

Figure 1
VLA-1 is expressed only in a subset of the activated CD45RO$^+$ CD4$^+$ T cells. (**a**) CD4$^+$ T cells were isolated from PBLs as described in Methods. Subsequently, the cells were stimulated with irradiated APCs and TSST-1 (upper four panels) or anti-CD3 (bottom panel). The cultures were assayed at day 8 for the cell surface coexpression of CD4 and Vβ2, together with VLA-1, CD45RO, CD25, or VLA-2. The data shown are representative of at least ten independent experiments in different normal donors. (**b**) CD4$^+$ PBLs were stimulated twice (every 10 days) with APCs and TSST-1. Subsequently, the cultures were purified into VLA-1$^-$ or VLA-1$^+$ cell fractions. The cells were maintained in recombinant human IL-2–containing medium and stimulated every 10 days. The two cultures were analyzed for surface expression of VLA-1, both at day 1 and 8 weeks later. The data are representative of more than five independent experiments in different normal blood donors.

Results

The VLA-1 receptor is expressed in vitro in a subset of CD45RO$^+$ T cells. VLA-1 is expressed in a small fraction of fresh, resting PBLs (approximately 1–4%), and almost all these VLA-1$^+$ cells also coexpress CD45RO (21, 23). Following TCR activation of unselected human PBLs, an increase in the percentage of VLA-1$^+$ cells becomes evident 1–2 weeks after activation (10). For example, in initial studies we analyzed the expression of VLA-1 in fresh CD4$^+$ T cells induced to expand by direct TCR triggering using the TSST-1 superantigen, which exclusively triggers TCR Vβ2$^+$ cells. We followed the expression of VLA-1 in the Vβ2$^+$ T cells for up to 14 days. By day 8, as expected, most of the Vβ2$^+$ T cells expressed the activation molecules CD45RO and CD25 (Figure 1a, upper four panels). In contrast, however, the VLA-1 molecule is expressed in only a small fraction (range: 6.8–13.5%, n = 10; Figure 1a) of the activated CD45RO$^+$ Vβ2$^+$ cells, and this expression was maximal by day 7 and stable thereafter. Furthermore, in fresh CD4$^+$ T cells stimulated with anti-CD3 and maintained under similar conditions, the expression of VLA-1 was also detected in only a small fraction (5.3–12.1%, n = 5) of the activated CD45RO$^+$ cells (Figure 1a, bottom panel). In contrast to VLA-1, the α2β1 integrin (VLA-2), which is also expressed late after activation, is expressed on more than 90% of the Vβ2$^+$ T cells.

Because VLA-1 is expressed in only a fraction of the CD45RO$^+$ T cells, we next asked whether the expression of VLA-1 is a stable phenotype of this subset of T cells. Thus, fresh CD4$^+$ T cells were stimulated twice (10 days apart) with TSST-1, and then at day 21, a time point when the majority of the cells are Vβ2$^+$ but only a fraction express VLA-1 (approximately 10–20%), we purified the VLA-1$^+$ and VLA-1$^-$ cells from the mixed cultures (Figure 1b). Subsequently, the two different lines were cultured separately in IL-2–containing medium and repeatedly stimulated with TSST-1 and irradiated APCs, every 10 days. We found that the majority of cells in these two different cultures maintained their respective VLA-1$^+$ or VLA-1$^-$ phenotypes for prolonged periods of time (Figure 1b). Moreover, using limiting dilution cloning, we derived highly purified T cell lines/clones that maintained a stable VLA-1$^+$ or VLA-1$^-$ phenotype. Taken together, these data demonstrate that VLA-1 is a stable cell surface marker of a subset of CD45RO$^+$ T cells.

VLA-1 marks a major subset within the memory lineage of CD4$^+$ T cells. As shown above, following activation VLA-1 is expressed only in some CD45RO$^+$ CD4$^+$ T cells. Thus, we hypothesized that these VLA-1$^+$ cells represent memory cells, operationally distinguished by their greater capacity to proliferate upon re-encounter with a recall antigen (e.g., TT, PPD, or mumps antigens). We used the CFSE dye-dilution technique to identify the

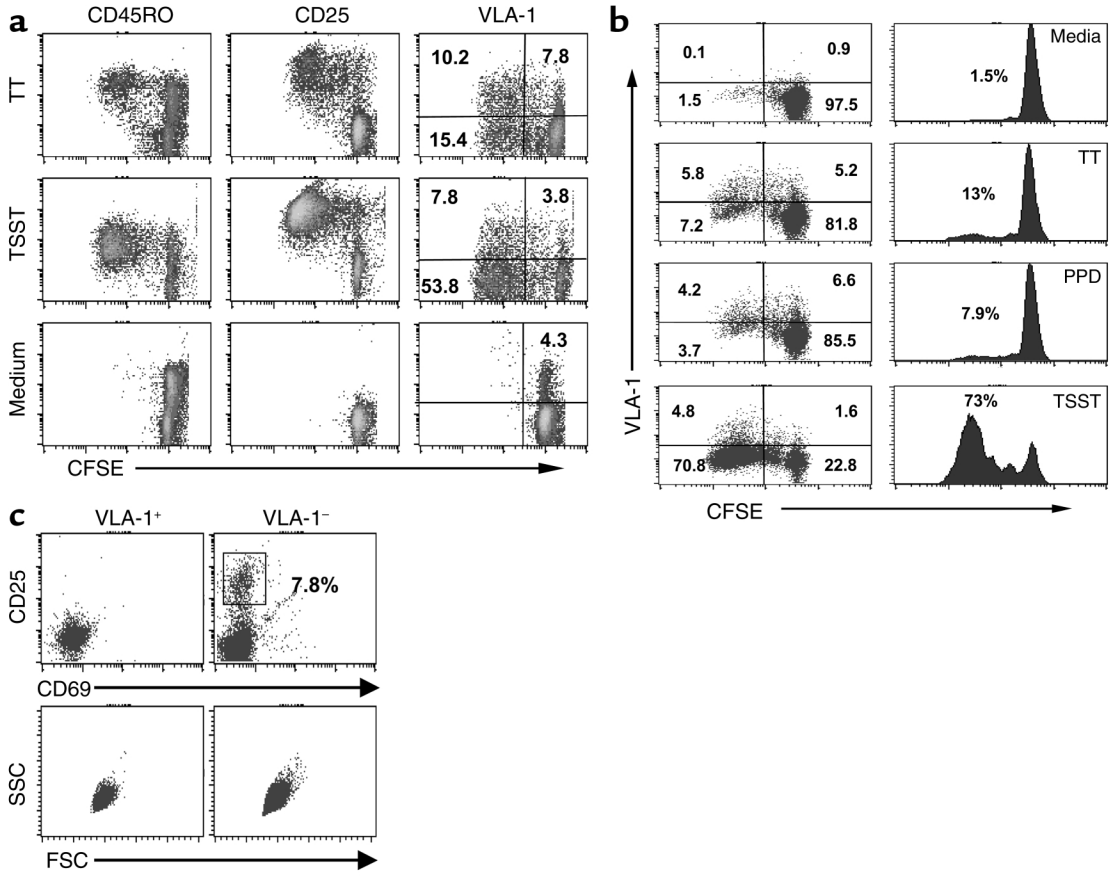

Figure 2

VLA-1 expression preferentially distinguishes a large fraction of the CD4+ T cells responding to recall antigens. (**a**) CD4+ T cells from PBLs were labeled with CFSE and plated in 24-well plates at 10^6 cells/well containing 2×10^6 irradiated APCs and triggered by TT (upper panels), TSST-1 (middle panels), or medium (lower panels). The cells were harvested on day 8 and analyzed for CFSE content and the expression of VLA-1, CD25, or CD45RO. The data shown are representative of more than ten independent experiments in different donors. (**b**) CD4+ PBLs isolated from a PPD+ and TT-vaccinated individual were labeled with CFSE and triggered with TT, PPD, or TSST-1, harvested on day 8, and analyzed for generation number and VLA-1 expression. (**c**) CD4+ T cells from fresh PBLs were purified into VLA-1+ and VLA-1− fractions and analyzed for relative size (FSC/SSC) and coexpression of CD25 and CD69. The data are representative of more than five different blood donors.

memory cells, defined as the population of cells that have undergone at least two to three successive cellular divisions during the first week following recall antigen triggering. This approach enabled us to easily analyze individual cells for their cell generation number together with surface VLA-1 expression, as well as other surface molecules previously associated with memory cell subsets. We compared the response to recall antigen with the polyclonal response to superantigen, reasoning that polyclonal stimulation would not only trigger memory cells but also indiscriminately trigger the large population of antigen-naive cells present in PBLs. We predicted that if VLA-1 marks the memory population, we should observe a significant increase in the percentage of VLA-1–expressing cells in the cells dividing in response to recall antigens as compared with cells responding to polyclonal triggering.

Thus, fresh CD4+ PBLs from normal donors were labeled with CFSE and stimulated with TT (or other nominal antigens) or TSST-1. As shown in a representative experiment (Figure 2a), VLA-1+ is expressed on the surface of more than 40% of the T cells rapidly dividing in response to TT triggering. In contrast, in T cells stimulated by TSST-1, a significantly smaller fraction (approximately 12%) of the rapidly dividing cells express VLA-1. In addition, the combined data from similar experiments in multiple donors ($n = 11$) showed a significant enrichment for VLA-1+ T cells in TT-specific dividing cells (range: 16.8–59.5%) and a considerably lower expression of VLA-1 following polyclonal triggering (range: 3.1–12.7%; $P < 0.01$, Student's t test). Furthermore, as seen in Figure 2b, the recall response to PPD in a sensitized individual was also enriched for VLA-1+ cells (56%), as was the recall response to mumps virus antigens (range: 42–63%, $n = 4$).

Because a single T cell dividing n times will generate 2^n daughter cells, the proportion of CFSE-low cells at day 8 correlates directly with the original precursor frequency of TT-specific T cells in PBLs. Thus, as expected, this proportion was different for each donor, as was

the proportion of VLA-1$^+$ cells within the memory population. Nevertheless, a dramatic enrichment for VLA-1$^+$ cells following TT stimulation compared with TSST-1 stimulation was observed in all donors. Moreover, as shown in Figure 2a, the proportion of VLA-1$^+$ cells that have divided following TT stimulation (upper right panel) was much higher compared with the proportion of dividing CD45RO$^+$ T cells (upper left panel). Thus, these data are compatible with the notion that persistent VLA-1 expression distinguishes a T cell population highly enriched for memory cells.

As noted above, in normal individuals VLA-1 is expressed only in a small fraction of approximately 1–4% of the circulating CD45RO$^+$ CD4$^+$ T cells. Moreover, these VLA-1$^+$ cells are small cells that do not express CD25 or CD69 (Figure 2c), two molecules that usually mark all recently activated T cells. Because these findings suggest that the CD45RO$^+$ CD4$^+$ VLA-1$^+$ PBLs represent a population of resting cells, it was of interest to determine directly their contribution to the overall response to recall antigens. Thus, we depleted these VLA-1$^+$ cells from fresh PBLs and in parallel submitted the control group to a sham depletion procedure, as described in Methods. Subsequently, the VLA-1 and sham-depleted populations were labeled with CFSE, pulsed with TT or with TSST-1, and cultured for 8 days. We found that VLA-1 depletion significantly diminished the proliferative response to TT. In contrast, the depletion procedure had minimal effect on the proliferative response to TSST-1 (Figure 3a), in which a large fraction of dividing cells are likely derived from the naive Vβ2$^+$ cells in PBL. Overall, as depicted in Figure 3b, the combined data from the VLA-1 depletion experiments performed in seven normal individuals demonstrate a significant reduction in the recall response to TT that ranged between 36% and 64% when compared with TSST-1 stimulation (range: 6.7–5.1%; $P = 0.02$, Wilcoxon signed rank test). Likewise, VLA-1 depletion significantly reduced the response to other recall antigens, including PPD (65% reduction; $n = 1$) and mumps antigens (range: 50–67%; $n = 4$, $P < 0.05$).

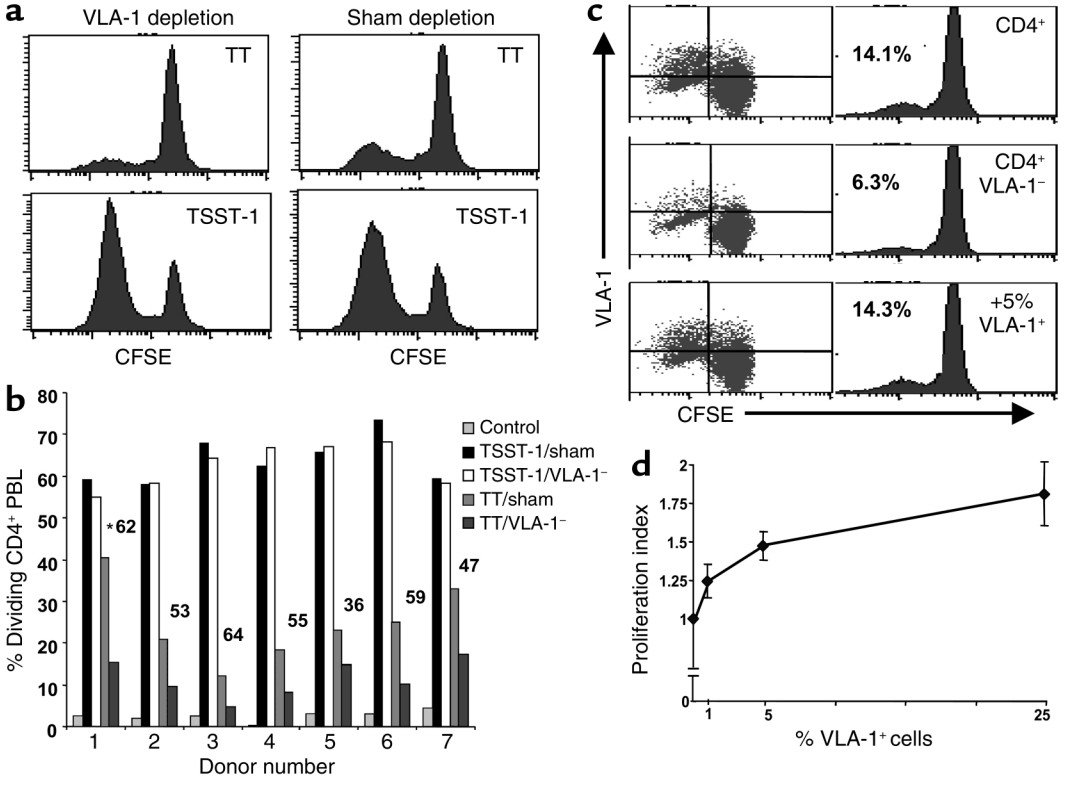

Figure 3

Depleting VLA-1$^+$ cells from the PBLs significantly abrogates the proliferative response to TT. (**a** and **b**) CD4$^+$ T cells from fresh PBLs of normal individuals were depleted of the VLA-1$^+$ cells or sham depleted (see Methods). Subsequently, the two cell groups were labeled with CFSE and stimulated with either TT or TSST-1 and irradiated APCs. The cells were harvested at day 8 and analyzed for CFSE dilution. (**a**) A representative experiment for donor 6. (**b**) The bar graph shows the results from seven different individuals. The *P* value of 0.02 (Wilcoxon signed rank test) was obtained by comparing the relative reduction induced by VLA-1 depletion in the combined TT-stimulation experiments to the relative reduction measured in the TSST-stimulation experiments (asterisk indicates percentage of reduction in the TT response when comparing sham to VLA-1 depletion). (**c** and **d**) The sham-depleted CD4$^+$ PBLs or the VLA-1$^-$ fraction with a increasing numbers of VLA-1$^+$ cells added back (0%, 1%, 5%, and 25%) were stimulated with TT and cultured for 8 days and then assayed for cellular divisions and VLA-1 expression. (**c**) A representative experiment in one donor. (**d**) Graph shows the combined results obtained from five different individuals. The proliferation index was calculated as the percentage of proliferation in a given sample divided by the percentage of proliferation in the pure VLA-1$^-$ sample. The error bars represent ± SEM, and the *P* value was less than 0.01 (repeated measures ANOVA test).

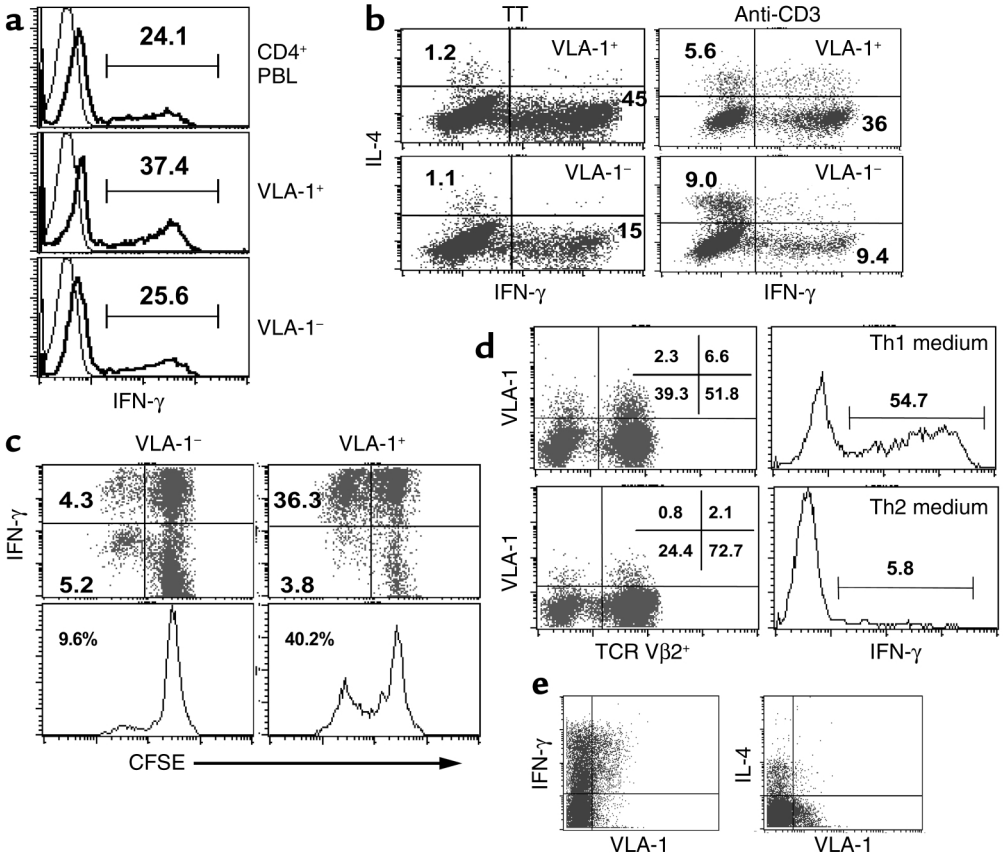

Figure 4

VLA-1 expression is associated with Th1 polarization. (**a**) CD4+ PBLs were purified into VLA-1+ and VLA-1− fractions, activated with PMA/ionomycin for 5 hours, and analyzed for intracellular IFN-γ production. (**b**) CD4+ PBLs were stimulated with TT or anti-CD3; 10 days later the cells were harvested and purified into VLA-1+ and VLA-1− fractions, activated with PMA/ionomycin, and analyzed for intracellular IFN-γ and IL-4. (**c**) CD4+ PBLs were labeled with CFSE, stimulated with TT, and 10 days later purified into VLA-1+ and VLA-1− fractions. Subsequently, the two subsets were activated and analyzed for divisions and intracellular IFN-γ. (**d**) CD4+ PBLs were stimulated with TSST-1 in two different environments: Th1 (IL-12+) or TH2 (IL-4+ and anti–IFN-γ). Ten days later cells were harvested and analyzed for VLA-1 and Vβ2 surface expression (left) or activated and analyzed for intracellular IFN-γ (right). (**e**) Fresh SFLs from RA ($n = 4$) and PsA ($n = 2$) patients were immediately activated and analyzed for surface expression of CD4, VLA-1, and intracellular IFN-γ. The dot plots were obtained by pregating on CD4+ events, and a representative patient sample is shown.

To further address the contribution of the VLA-1+ subset to the recall response, we asked whether adding back the VLA-1+ fraction eluted from the columns to the VLA-1− fraction could restore the proliferative response. We found that adding back the VLA-1+ cells results in the complete reconstitution of the recall response (Figure 3c). Moreover, as shown in Figure 3d, adding back the VLA-1+ cells at increasing numbers into the VLA-1− fraction resulted in a dose-dependent increase ($P < 0.01$, $n = 5$, repeated measures ANOVA test) in the recall response. Interestingly, a near optimal proliferative recall response was reached at the 5% VLA-1+ cell frequency, a number that approximates the upper limit of the physiological frequency of VLA-1+ cells in the PBLs. This is further underscored by the observation that increasing the VLA-1+ cell ratio above 25% had no additional incremental effect on the recall response (data not shown).

Taken together, these results show that VLA-1 expression preferentially marks a subset of memory cells. Moreover, the circulating VLA-1+ CD4+ T cells are resting memory cells that account for a significant fraction of the proliferative response to recall antigens.

VLA-1 expression is associated with the Th1 phenotype. It is known that the development of Th1-mediated inflammatory responses in vivo is markedly abrogated in anti-VLA-1–treated mice and in $\alpha 1^{-/-}$ knockout mice (15, 18). Moreover, as discussed above, a high percentage of the T cells infiltrating the inflammatory lesions in a variety of Th1-mediated diseases, both in mice and humans, express VLA-1. Thus, it was of interest to determine whether VLA-1 is preferentially expressed on Th1 cells. We first addressed this question in fresh CD4+ PBLs purified into VLA-1+ and VLA-1− fractions. Thus, the two cell populations were briefly activated with PMA/ionomycin and subsequently analyzed for intracellular production of IFN-γ and IL-4. We found that the VLA-1+ fraction of CD4+ T cells was somewhat enriched for cells expressing intracellular INF-γ com-

pared with the VLA-1$^-$ fraction (Figure 4a), whereas IL-4–producing cells were equally infrequent (approximately 2%) within the two fractions.

Next we asked whether, in fresh CD4$^+$ T cells induced to expand ex vivo by either a recall antigen (e.g., TT) or anti-CD3, the VLA-1$^+$ progeny is enriched for Th1 cells. Thus, following activation, the cells were cultured for 8 days, purified into VLA-1$^+$ or VLA-1$^-$ fractions, and analyzed for production of intracellular IFN-γ and IL-4. We found that the ratio of IFN-γ$^+$ cells in the VLA-1$^+$ fraction, regardless of the stimulation, was significantly higher (threefold to fourfold) compared with the VLA-1$^-$ fraction (Figure 4b). Moreover, in CD4$^+$ T cells activated by anti-CD3, the VLA-1$^-$ fraction was enriched for Th2 cells. Interestingly, following TT triggering, which induces Th1-biased responses in most normal individuals, we observed that within the dividing memory progeny almost all of the VLA-1$^+$ cells (more than 90%) produce IFN-γ (Figure 4c). Moreover, the VLA-1$^+$ fraction contained a higher frequency of TT-specific memory CD4$^+$ T cells (Figure 4c, lower panel).

These observations suggest that following TCR triggering, VLA-1$^-$ expression is preferentially associated with Th1 polarization. To further address this notion, we stimulated fresh CD4$^+$ PBLs with TSST-1 and conditioned them in either Th1-polarizing (+ IL-12) or Th2-polarizing (+ IL-4 and anti–IFN-γ neutralizing mAb's) environments. We found that culturing the cells in a Th1 environment induced an increased percentage of VLA-1–expressing cells. In contrast, the percentage of VLA-1$^+$ cells was reduced in the Th2 environment (Figure 4d, left panel). In addition, as expected, the frequency of IFN-γ–secreting cells was significantly increased in the Th1 environment (right panel).

Next, it was of interest to ask whether VLA-1 expression is also associated with the Th1 phenotype in vivo in chronically activated T cells. To address this question, we analyzed T cells isolated from fresh synovial fluids of patients with RA (n = 4) or psoriatic arthritis (PsA) (n = 2) for cytokine production, upon brief ex vivo activation. Again, we found that more than 90% of VLA-1–expressing CD4$^+$ T cells had a Th1 phenotype in all samples. Moreover, previous studies show that the synovial CD4$^+$ T cells from patients with RA are highly enriched for VLA-1$^+$ cells and usually contain 20–40% VLA-1$^+$ cells. In nonrheumatoid patients, including those with PsA, this percentage is usually lower, at 10–20% (12, 19). Taken together, these observations demonstrate that VLA-1 preferentially marks a major subset within the memory and effector Th1 population both in vitro and in situ at sites of inflammation. It should be noted, however, that VLA-1 expression does not mark all Th1-polarized cells.

Cell surface molecules coexpressed on VLA-1$^+$ and VLA-1$^-$ subsets of human CD4$^+$ cells. Because in previous studies of both murine and human memory cells, the CCR7, CD62L, and VLA-4 molecules have been used to define subsets of memory and effector cells (25, 33, 34), we next evaluated the expression of these molecules in the VLA-1$^+$ and VLA-1$^-$ subsets present in fresh PBLs and in the cell population that proliferates in response to recall antigens, ex vivo. We first analyzed the VLA-1$^+$ cells present in PBLs (designated R1 in Figure 5a) and found that these cells invariably did not express the CCR7 molecule, but were enriched in CD62L$^+$ and VLA-4^{hi} cells (Figure 5b). In this regard, the CCR7$^-$ VLA-4^{hi} phenotype has been shown previously to be associated with tissue-localizing effector memory cells in mice (35). The high percentage of CD62L$^+$ cells in the VLA-1$^+$ population is of interest because CD62L is crucial for lymphocyte migration to lymphoid tissues (36). In contrast, the VLA-1$^-$ CD45RA$^-$/RO$^+$ subset (designated R2) was more heterogeneous with respect to the expression of CCR7, CD62L, and VLA-4. As expected, the naive CD45RA$^+$ T cell population was highly enriched for cells expressing CCR7 and CD62L and low levels of VLA-4.

In addition, we attempted to analyze the differential coexpression of CCR7 and CD62L in the VLA-1$^+$ and VLA-1$^-$ CD4$^+$ memory T cells dividing in response to TT triggering. We found that the majority of the cells, regardless of their VLA-1 phenotype, downregulate

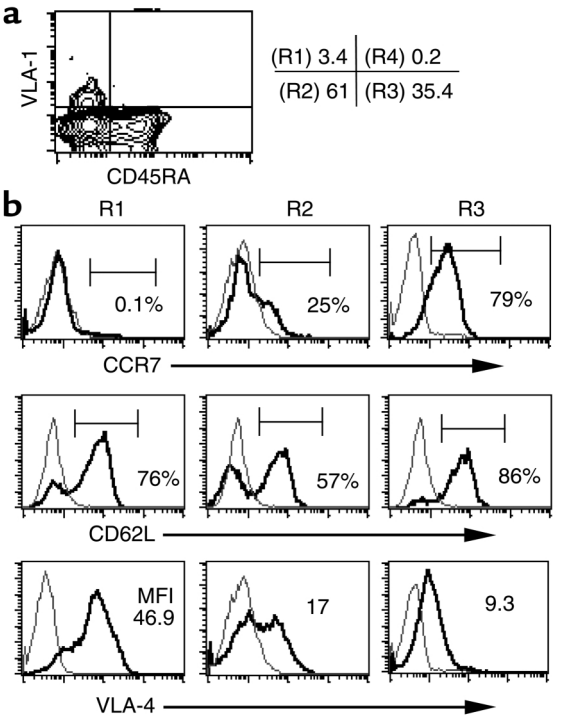

Figure 5
The VLA-1$^+$ CD4$^+$ T cells have a distinct cell surface phenotype. (**a**) Fresh CD4$^+$ PBLs were analyzed for the cell surface coexpression of CD45RA and VLA-1, and the four quadrants were identified as R1 to R4, where R1 = VLA-1$^+$ CD45RA$^-$; R2 = VLA-1$^-$ CD45RA$^-$; R3 = VLA-1$^-$ CD45RA$^+$; and R4 = VLA-1$^+$ CD45RA$^+$. (**b**) Isolated CD4$^+$ T cells were further purified into VLA-1$^+$ and VLA-1$^-$ fractions and then analyzed for the coexpression of CD45RA together with CCR7, CD62L, or VLA-4. The histograms represent the viable CD4$^+$ T cell gate, and the data are representative of more than five PBL samples from different normal individuals. MFI, mean fluorescence intensity.

both CCR7 and CD62L within the first week following antigen activation. This further underscores the previous observations that the expression and downregulation of CCR7 and CD62L in T cells is highly activation dependent.

Discussion

The studies described here provide evidence that in human $CD4^+$ T cells VLA-1 expression is highly restricted to a distinct subset of memory cells with the potential to mediate Th1-type immune responses in peripheral tissues. Several lines of evidence support this conclusion. First, when fresh $CD4^+$ PBLs are activated by a polyclonal trigger, even though all responding $CD4^+$ T cells stably express CD45RO, only a small percentage expresses VLA-1, and the capacity to express VLA-1 is a stable property of this T cell subset. Second, when memory T cells are triggered by a recall antigen, VLA-1 becomes expressed on a large fraction of the rapidly dividing cells that can be readily assayed by CFSE dilution. Third, the very small fraction (approximately 1–4%) of VLA-1^+ $CD4^+$ T cells found in fresh PBLs contribute significantly to the overall recall response. Fourth, the VLA-1^+ fraction of the memory T cells dividing after recall antigen stimulation is highly enriched for Th1-polarized effector cells. Last, VLA-1^+ $CD4^+$ T cells isolated from the peripheral blood are composed of small, nonactivated cells that have a distinct cell surface phenotype of $CCR7^-$, $CD62L^+$, and VLA-4^{hi}. Likewise, VLA-1^+ $CD4^+$ T cells isolated from fresh SFLs of arthritis patients lack CCR7 and CD25 expression and are predominantly Th1 cells; nevertheless, many Th1 cells do not express VLA-1. Thus, VLA-1 expression in a subset of human memory $CD4^+$ T cells is associated with a distinct phenotype, suggesting that their main function in vivo is to mediate DTH-like (immune) responses in peripheral tissues.

The primary evidence that VLA-1 expression is a stable marker of a subset of activated T cells stems from our initial observation that following polyclonal TCR triggering of $CD4^+$ PBLs, VLA-1 does not mark all activated $CD45RO^+$ T cells. Moreover, VLA-1^+ and VLA-1^- $CD45RO^+$ subsets purified from the activated T cell cultures and maintained in culture over time retain their VLA-1^- or VLA-1^+ phenotype. Because the $CD45RO^+$ phenotype is known to contain the memory cells, we asked whether VLA-1 expression defined a subset of memory cells. In this regard, as already noted, precise identification of human $CD4^+$ memory T cells is difficult, mainly because the phenotypic markers (e.g., CD45RO) used to distinguish memory T cells actually mark prior antigen activation rather than the acquisition of memory functions. Operationally, we defined memory cells as those cells that rapidly divide (more than two divisions) in response to recall antigens within the first week following recall antigen triggering ex vivo. We observed a dramatic increase in the percentage of VLA-1^+ cells in this rapidly dividing population. Interestingly, following polyclonal triggering of T cells in which both naive and memory cells are activated, a much smaller percentage of VLA-1^+ $CD45RO^+$ cells is observed. Taken together, these data suggest that VLA-1 is a unique marker because it preferentially distinguishes a subset within the activated $CD45RO^+$ memory T cells. Interestingly, in human peripheral blood the depletion of VLA-1^+ $CD4^+$ T cells, which account for only 1–4% of the cells, markedly reduces (36% to 64%) the proliferative recall response to TT.

Even though VLA-1 expression marks a large subset within the dividing $CD45RO^+$ $CD4^+$ memory T cells, a substantial fraction of the cells within this memory population does not express VLA-1. Thus, it was of interest to study the functions associated with the VLA-1^+ and VLA-1^- $CD4^+$ T cell subsets responding to recall antigens. We chose to study the profile of Th cytokine secretion by the VLA-1^+ and VLA-1^- subsets because in humans T cells localizing to inflamed tissues in Th1 cell–driven immune responses are enriched for VLA-1^+ cells (11, 12, 19, 20). Thus, we studied whether VLA-1 expression in $CD4^+$ T cells is preferentially associated with the Th1 phenotype defined in vitro by the production of IFN-γ but not IL-4. We found that following recall antigen or polyclonal triggering, the purified VLA-1^+ subset was highly enriched for effector Th1 cells. The VLA-1^- fraction was more heterogeneous, although somewhat enriched in Th2 cells, and also contained Th1, Th0, and nonpolarized cells. Moreover, activating $CD4^+$ PBLs under different Th-polarizing conditions demonstrated that a Th1 environment favors the emergence of VLA-1^+ cells. Consistent with these ex vivo findings, we also observed that the great majority of VLA-1^+ $CD4^+$ T cells isolated from inflamed synovial fluids of patients with RA, where they form 10–40% of the population, express IFN-γ but not IL-4 (Figure 4e). It should be noted, however, that many Th1 cells in SFLs do not express VLA-1. Therefore, VLA-1 should not be considered a marker of Th1 polarization, but rather a marker of a distinct subset of antigen-experienced Th1 cells.

The precise mechanism by which a Th1-biased cytokine environment favors the induction of VLA-1^+ cells is unknown. In this context, several experimental observations suggest that the microenvironment in the lymph nodes, including interactions with local dendritic cells, may influence the peripheral homing preference of effector memory T cells (37). The type of antigen, the adjuvant used, and the route of immunization can all affect this environment. Thus, it will be of interest to study the influence of these parameters on the induction of VLA-1^+ memory T cells in vivo in animal models, where the nature of the antigen and the immunization conditions can be readily manipulated.

An additional distinction between the VLA-1^- and VLA-1^+ fraction in PBLs is that the $CD4^+$ T cells expressing CD25 are nearly all contained in the VLA-1^- $CD45RO^+$ fraction, whereas the VLA-1^+ subset in PBLs is virtually devoid of $CD25^+$ (and $CD69^+$) cells. Moreover, these VLA-1^+ cells also do not exhibit the

increased size and granularity usually observed in recently activated T cells (Figure 2c). These observations are consistent with detailed studies in mice, showing that long-lived antigen-experienced T cells are usually small, resting cells that do not express the activation markers CD25 or CD69 (24, 34). It is of interest that upon activation ex vivo both VLA-1$^+$ and VLA-1$^-$ CD4$^+$ PBL T cells re-express CD25 and CD69.

Moreover, Lanzavecchia and colleagues have suggested that human memory T cells can be divided into two functionally distinct subsets based on the expression of CCR7 (25), a lymph node–homing chemokine receptor (38). The CCR7$^+$ subset of memory cells that circulate through the lymphoid system was termed central memory. In contrast, the CCR7$^-$ memory T cells predominantly migrate into peripheral tissues and are enriched for effector-type cells; thus they are termed effector memory cells. We find that VLA-1 expression is associated with the CCR7$^-$ phenotype. It is important to note, however, that CCR7 expression is neither a specific marker of the memory lineage nor a stable marker; many CCR7$^+$ memory and naive T cells differentiate into CCR7$^-$ T cells following antigen activation (25, 27). Because VLA-1 expression is a stable phenotypic marker of a subset of CCR7$^-$ CD45RO$^+$ T cells, we propose that the expression of VLA-1 might more accurately identify the subset of memory T cells that localize to nonlymphoid tissues. This notion is further supported by the in vivo observations showing that VLA-1$^+$ cells are frequent among T cells isolated from inflamed tissues (e.g., SFL and PPD induced skin blisters). In contrast, we find that VLA-1 is only rarely expressed on CD45RO$^+$ CD4$^+$ T cells isolated from lymphoid tissues (e.g., hyperplastic tonsils), even though many of the tonsillar T cells express molecules associated with the postactivation phenotype, including CD45RO and CD25 (data not shown).

In addition to CCR7, the L-selectin molecule (CD62L) has also been suggested to distinguish central memory cells (39). The CD62L molecule mediates T cell trafficking to lymphoid tissues through interactions with its ligand, the mucosal addressin cell-adhesion molecule-1, particularly expressed in the high endothelial venules (40). Therefore, CCR7$^+$ cells that migrate into lymph nodes usually express high levels of CD62L (34). CD62L, however, is also expressed in a significant percentage of CCR7$^-$ memory cells (25). Moreover, similar to CCR7, the CD62L molecule is not a stable surface marker, and usually T cells rapidly shed this receptor following cellular activation (41). Interestingly, we find that the VLA-1$^+$ subset is highly enriched for CD62L$^+$ cells. Our interpretation of these data is that CD62L expression is a feature of resting memory T cells, as exemplified by the VLA-1$^+$ PBLs. In this context, other studies show that CD62L expression identifies the majority of T cells that proliferate to recall antigens in human PBLs (42, 43). Taken together, our results provide evidence that the VLA-1$^+$ CD62L$^+$ phenotype identifies a subset of quiescent memory CD45RO$^+$ CD4$^+$ PBLs.

Another distinction of the circulating VLA-1$^+$ T cells is that most express high levels of VLA-4, whereas the VLA-1$^-$ fraction contains both VLA-4^{lo} and VLA-4^{hi} cells. In this context, in a recent study Swain and colleagues showed that in mice, anti-viral CD4$^+$ Th1 cells found in situ at the site of viral infection show a distinct surface phenotype of CCR7$^-$ and VLA-4^{hi} (35). In addition, earlier studies from the laboratories of Morimoto and Schlossman have indicated that the expression of high levels of CD29 (the β1 integrin chain shared by all VLA molecules) identifies the majority of CD45RA$^-$ PBLs with memory functions (43, 44). Taken together, these data may imply that the VLA-1$^+$ VLA-4^{hi} cells form an important subset within the CD29^{hi} memory population. It should be noted, however, that the CD29^{hi} population also contains cells that do not express VLA-1, of which a small percentage (2–5%) express VLA-2. The stability and distinct functions of this VLA-1$^-$ VLA-2$^+$ population is unknown.

The function of the VLA-1 molecule expressed on the surface of a fraction of the memory T cells remains to be fully elucidated. For instance, because VLA-1 is the primary receptor for collagen IV (3), it is thought to facilitate the migration of lymphocytes through vascular basement membranes into tissues (18). VLA-1 is also a receptor for collagen I (5), which is abundant in interstitial tissues, and thus VLA-1 may also facilitate the migration of lymphocytes in tissues. In addition, it is known that signaling via VLA-1 in T cells augments subthreshold TCR triggering (12, 13). In this context, studies in the $\alpha 1^{-/-}$ mice show that VLA-1 is the sole collagen receptor that can activate the adaptor protein Shc pathway (7). This signaling was shown to promote cell survival and progression through the cell cycle in fibroblasts (8, 9). In addition, Meharra and colleagues (45) observed that in the $\alpha 1^{-/-}$ mice the α1β1 integrin is required for the survival of certain subsets of intestinal T cells within epithelial tissues. Moreover, we found recently that signaling via VLA-1 can induce IFN-γ production in activated Th1-type cell lines, independent of concomitant TCR retriggering (unpublished observation). Thus, VLA-1 functions may include inducing cellular programs that sustain Th1-mediated immunity in collagen-rich environments.

A corollary of our data is that VLA-1 expression may distinguish autoaggressive Th1 cells in human autoimmunity. This conclusion is compatible with the recent findings in mice that blockade of the VLA-1 pathway, either by genetic deletion ($\alpha 1^{-/-}$) or with anti–VLA-1 Ab's, reduces tissue inflammation and the incidence and severity of disease in several murine models of Th1-mediated autoimmunity, including graft-versus-host disease and hapten-induced colitis (14, 18). Because VLA-1 is also highly expressed in other cells, including activated monocytes, it is possible that the in vivo effects of VLA-1 blockade may be due to interference with the functions of a number of different VLA-1–expressing cell types involved in the pathogenesis of autoimmunity.

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