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*J Immunol* 2010; 184:3841-3849; Prepublished online 3 March 2010;
doi: 10.4049/jimmunol.0902281
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Identification of a Unique Population of Tissue-Memory CD4+ T Cells in the Airways after Influenza Infection That Is Dependent on the Integrin VLA-1

Timothy J. Chapman and David J. Topham

During the immune response to influenza infection, activated T cells are distributed to both lymphoid and extralymphoid tissues, including the infected airways where direct recognition of viral Ag-bearing cells takes place. The collagen-binding α4β1 integrin VLA-1 is essential for the development of memory CD8+ T cells in the airways, and although expressed by some CD4+ T cells, its significance has not been demonstrated. We investigated the role of VLA-1 on virus-specific CD4+ T cells during and after primary or secondary influenza infection of mice. The proportion of CD4+ cells expressing CD49a (α4 integrin) was low in all tissues sampled during primary infection but increased in the airways after viral clearance. Furthermore, during the first 24 h of a secondary influenza challenge, the majority of IFN-γ-secreting effector CD4+ T cells from the airways was in the CD49a+ population. Airway CD49a+CD4+ cells also expressed reduced markers of apoptosis compared with CD49a- cells, and fewer memory or effector CD4+ cells could be recovered from airways of α4-/- mice, although lymphoid tissues appeared unaffected. These data suggest VLA-1 expression defines a population of tissue memory CD4+ T cells that act as rapid effectors upon reinfection, and VLA-1 expression is integral to their accumulation in the airways. The Journal of Immunology, 2010, 184: 3841–3849.

Influenza virus poses a major health threat for humans worldwide. This is in large part because of the antigenic shift that occurs in the surface hemagglutinin and neuraminidase proteins, resulting in new virus variants that escape circulating neutralizing Abs. The absence of immunity to variant influenza viruses greatly impacts human health, as highlighted by the emergence of several pandemic strains in the past century (1). Although the current flu vaccine is engineered to primarily elicit neutralizing Ab, a growing body of literature suggests that memory T cells, particularly in the mucosa, may be an effective means of limiting the morbidity and mortality resulting from infection with serologically distinct flu strains, also termed heterosubtypic immunity. T cell responses to conserved epitopes within internal viral proteins have been shown to be protective in mouse models of lethal heterosubtypic influenza infection (2, 3). More recent data suggest that memory T cells residing in extralymphoid tissues such as the lung seem especially capable of aiding in protection against serologically distinct flu variants in mice (4–7).

In cases of heterosubtypic flu infection, T cells are vital to viral clearance and host survival (8). CD4+ cells are important for the generation of neutralizing Ab and the use of memory CD8+ cells, which are effective in future heterosubtypic challenges (9–13). In addition, CD4+ cells aid in the effector response by recruiting immune cells to the site of infection and reducing viral burden through secretion of cytokines and potential cognate interactions with infected targets (14). Although there is obvious benefit to the host in retaining populations of memory CD4+ cells in both the lymphoid tissues and extralymphoid tissues such as the lung, little is known about the signals that select or retain memory cells in the extralymphoid compartment.

One T cell function that becomes increasingly important as lymphocytes move from the circulation and lymphoid tissues to extralymphoid sites is their interaction with extracellular matrix (ECM). Much of the ECM in the lung environment is available to infiltrating T cells, and T cell integrin-ECM interactions can promote T cell motility, survival, and activation (15–17). Previous work has shown that the α4β1 integrin VLA-1 (detected by staining with Ab to the α4 integrin chain/CD49a) is expressed on primed CD8+ T cells in the lung and other extralymphoid tissues, and its interaction with type IV collagen may be important for CD8+ T cell residence in the airways (18). In addition, VLA-1 contributes to the survival of memory CD8+ cells in the lung airways (16). When reduction in the number of flu-specific memory CD8+ cells in the airways is accelerated by Ab blockade or genetic deficiency of VLA-1, mice are more susceptible to mortality upon lethal heterosubtypic challenge (18). This is possibly because of a lack of effector-memory cells localized to the lung airways at the time of challenge, where the flu infection occurs (8, 19, 20). These data suggest VLA-1 is an important mediator of memory T cell retention and survival in extralymphoid tissues and may play a prominent role in the survival and extralymphoid tissue localization of T cells in inflammatory conditions.

Less is known concerning the role of VLA-1 on memory CD4+ T cells. A small proportion of CD4+ cells expresses CD49a during the acute viral response, whereas nearly half of airway CD4+ cells expresses CD49a1 mo postinfection (21), suggesting either enrichment or development of the CD49a+ phenotype after viral infection. This population may be important in promoting
inflammation, because blockade of CD49a resulted in less severe inflammation and resulting disease in several CD4+ T cell-dependent disease models in animals (22–24). However, these studies did not distinguish the effects of CD49a blockade on T cells versus other cell types such as monocytes, which express CD49a, and can play a vital role in the inflammatory response (25, 26). CD49a+CD4+ cells can be recovered from human PBL and airways (recovered by bronchoalveolar lavage [BAL]) and express a highly activated phenotype in both of these sites (27, 28). Although CD49a+CD4+ cells have been identified and characterized in mouse and human, it is largely unknown whether expression of VLA-1 is of any consequence to CD4+ T cells, specifically cells responding to a viral infection localized to extralymphoid tissue like the lung. Given the mixed phenotypes associated with CD4+ cells in the lung (29), an understanding of when and how populations of CD49a+ and CD49a-CD4+ cells contribute to the immune response could be valuable in targeting cells important for protection.

In this article, we studied CD49a+CD4+ T cells in both lymphoid and extralymphoid tissues following infection with influenza virus. CD49a is expressed on a subset of CD4+ T cells during infection that increase in their relative proportion following viral clearance. These cells can be distinguished from other CD4 memory T cells by both cell surface phenotype and function during secondary infection. Integrin α5β1−/− mice have a defect in the accumulation of this subset of primed CD4+ cells in the airway late postinfection, suggesting a dependence on VLA-1 expression. Airway CD49a+CD44hiCD62LloCD49a+CD4+ T cells also have low expression of apoptotic markers compared with their CD49a− counterparts. We have termed these CD44hiCD62LloCD49a+CD4+ T cells as tissue memory because of their distinct recirculation and effector capacity. These findings support the hypothesis that a subset of effector-memory cells selectively accumulate in extralymphoid tissues, with unique features that allow them to survive and persist in extralymphoid tissues postinfection. This knowledge has important implications to understanding secondary influenza immunity and the design of optimized vaccination strategies targeting influenza viruses whose emergence is unpredictable.

Materials and Methods

Mice

C57BL/6 and congenic CD45.1+ B6.SJL mice were purchased from the National Cancer Institute (Bethesda, MD). Integrin α5β1−/− mice (a gift from Biogen Idec, Cambridge, MA) (30), TCR CD4−/− mice on the C57BL/6 background (a gift from Dr. D. Fowell, University of Rochester, Rochester, NY) (31), and TCR transgenic OT-II mice on the B6.PL (CD90.1) background (a gift from Dr. L. Bradley, Sanford-Burnham Medical Research Institute, La Jolla, CA) (32) were bred and maintained at the University of Rochester Animal Housing Facility (Rochester, NY) in specific pathogen-free conditions.

Viral infection and cell transfer

Stocks of influenza A/WSN-OVA11 (H1N1) (33) and A/HKx31/OVA1 (H3N2) (a gift from R. Webb, St. Jude Children’s Research Hospital, Memphis, TN) (34) viruses in allantoic fluid were thawed from −80°C. Virus was diluted in cold DPBS so that a 30-μl volume would contain 500–1000 PFU (for WSN) or ~2.8 × 104 PFU (for ×31) of virus. Recipient mice were first sedated with 2,2,2-tribromoethanol i.p., then given 105–106 PFU (for WSN) or 2.8 × 103 PFU (for ×31) of virus. In all cases, the frequency of OT-IIs in the cell preparation was determined by flow cytometry prior to transfer to take into account day-to-day variations in the enrichment procedure.

Organ harvest

Mice were sacrificed via lethal dose of avertin and exanguinated via brachial artery. BAL samples were collected by three intratracheal lung washes (with C-mem) using a Teflon cannula attached to a 1-ml syringe. Lung tissue, mediastinal lymph node (MLN), and spleen were removed separately. BAL samples were subjected to 45 min of plastic adherence prior to use. Lung tissue was ground through a tea strainer, and lymphocytes were isolated via centrifugation with a Histopaque underlay (Sigma-Aldrich, St. Louis, MO). MLN and spleen were homogenized and filtered through nylon mesh. All organ cells were maintained in C-mem prior to use.

Flow cytometry and apoptosis

A total of 1–2 × 10^6 cells from each organ were placed in individual wells of a 96-well round-bottom plate for staining. FeRs were blocked with anti-CD16/32 clone 2.4G2; from BD Biosciences, San Jose, CA) for 15 min prior to Ab staining. Cells were washed and surface stained with Ab panels containing combinations of CD4-APC/Cy5.5 (eBioscience, San Diego, CA), CD90.1-Biotin (eBioscience, San Diego, CA)/Pacific Orange 2′ (Invitrogen, Carlsbad, CA), CD4-Pacific Blue (BioLegend, San Diego, CA), CD62L-APC/Cy7 (BioLegend), CD11a-PE/Cy7 (BD Biosciences), CD49a-Alexa 488 (BD Biosciences), CD49b-FITC (BD Biosciences), and CD49d-FITC (BD Biosciences); cells were incubated for 30 min on ice in the dark. For apoptotic marker analysis, stained cells were washed in Annexin buffer (10× buffer of 0.1 M HEPES, 1.4 M NaCl, and 25 mM CaCl2 diluted to 1× in dH2O) and resuspended in 100 μl Annexin buffer with 5 μl/well Annexin V-APC (BD Biosciences). After 5-min incubation of cells at room temperature in the dark, 5 μl/well 7-aminoactinomycin D (7-AAD; BD Biosciences) was added for an additional 10 min of incubation time. Cells were washed and resuspended in 100 μl Annexin buffer for FACS. Samples were run on an LSRII (BD Biosciences) cytometer and analyzed with FlowJo (Tree Star, Ashland, OR) software.

Intracellular staining

Spleen cells from naive B6.SJL (CD45.1+) mice were used as APCs and pulsed with either OVA323–339, control peptide, or nothing for 90 min at 37°C. To study the response to whole virus, APCs were infected with influenza (multiplicity of infection = 1) in 1 ml serum-free media for 60 min. Infected cells were then washed and resuspended in C-mem. A total of 1 × 10^6 APCs were added to 1 × 10^6 responders (prepared as described above) for a total volume of 100 μl. GolgiPlug (BD Biosciences) was then diluted 1 μl/ml in C-mem, and 100 μl was added to each well. Cells were incubated for 5–6 h at 37°C. Samples were then surface stained as described above. Samples were washed and resuspended in 100 μl/well Cytofix/Cytoperm Buffer (BD Biosciences) for 15 min. After one Perm/Wash (BD Biosciences), PE/Ab was added in Perm/Wash, and cells were incubated for 30 min on ice in the dark. Samples were resuspended in PBS/BSA for FACS. Staining of BrdU+ cells, after surface staining and permeabilization, the APC anti-BrdU Kit (BD Biosciences) was used to detect BrdU+ DNA in cells. In short, fixed and permeabilized cells were incubated with Cytoperm Plus buffer to permeabilize nuclei and then treated a second time with Cytoperm for detection of BrdU. Cells were then treated with anti-BrdU antibody to Ab staining. Gating of BrdU− cells was determined by parallel staining of cells that did not receive BrdU in the experiment as a negative staining control.

Statistical analysis

Groups of data were compared using two-tailed Student t test or Wilcoxon signed-rank test; resulting values of p < 0.05 were considered significant.

Results

CD49a is expressed on a population of effector CD4+ cells following infection

To follow a population of virus-primed T cells, as well as the CD4+ population as a whole, we studied the influenza response from both endemic CD4+ T cells and adaptively transferred OT-II cells following infection with A/WSN-OVA11 (33). Very few CD49a-expressing OT-II cells were detectable in the early stages of infection (Fig. 1), which is consistent with the time course observed for endemic CD4+ cells during X-31 infection (21). However, the proportion of CD49a+CD4+ cells increased gradually through the peak immune infiltrate and more substantially after viral clearance, most strikingly on those recovered from BAL where >50% of the CD4 T cells were positive, with a smaller proportion (<10%) of primed cells expressing CD49a in the lymphoid tissues (Fig. 1). The enrichment of CD49a+CD4+ cells in the airways after viral clearance suggested to us this population of cells may be uniquely regulated. Therefore, we compared the phenotypes of CD49a+ and
CD90.1 B6.PL congenic background were transferred to C57BL/6 mice prior to infection and CD4+Thy1.1+ cells detected by flow cytometry. The CD49a+ (gray histograms) and CD49a+ cells were analyzed by flow cytometry for expression of CD44 within the CD49a+ subset from the BAL (mean fluorescence intensity [MFI] of CD49a+IFN-γ+ OT-IIs: 17,185 ± 1,768; CD49a+IFN-γ− OT-IIs: 9,917 ± 1,348; p < 0.02). In contrast, at 1 mo postinfection, CD49a+ cells accounted for ~55–60% of the total IFN-γ-secreting endogenous CD4+ or OT-II populations in the BAL upon ex vivo restimulation while in contrast making a minor contribution to the response from lymphoid tissues (Fig. 3A, 3B).

CD90.1+CD4+ cells were further analyzed for effector potential by comparison with the classic CD44hiCD62Llo effector-memory phenotype. We first gated CD4+ cells for CD44hiCD62Llo; then the CD44hiCD62Llo were fractionated by CD49a expression and studied for phenotypic differences one month postinfection.

Influenza-primed CD49a+CD4+ cells rapidly secrete IFN-γ during a recall response

Effector-memory T cells rapidly secrete cytokine in response to Ag. We questioned whether CD49a+CD4+ T cells were functionally different from the effector-memory population as a whole. The effector function of CD49a+ and CD49a−CD4+ T cells was first studied by intracellular IFN-γ staining of mouse BAL, MLN, and spleen cells taken at several time points following primary infection. To compare endogenous CD4+ and transgenic OT-II cell responses, cells were restimulated with whole virus or OVA323–339 peptide, respectively. During the peak (day 8) of the acute primary response, CD49a+ OT-II cells composed <10% of the total OT-II cells (identified as Thy1.1+ CD4+ cells) secreting IFN-γ (Fig. 3D). However, the majority (70 ± 2%) of CD49a+ OT-II cells made IFN-γ, and there was more cytokine production per cell in the CD49a+ subset from the BAL (mean fluorescence intensity [MFI] of CD49a+IFN-γ+ OT-IIs: 17,185 ± 1,768; CD49a+IFN-γ− OT-IIs: 9,917 ± 1,348; p < 0.02). In contrast, at 1 mo postinfection, CD49a+ cells accounted for ~55–60% of the total IFN-γ-secreting endogenous CD4+ or OT-II populations in the BAL upon ex vivo restimulation while in contrast making a minor contribution to the response from lymphoid tissues (Fig. 3A, 3B).

CD90.1+CD4+ cells were further analyzed for effector potential by comparison with the classic CD44hiCD62Llo effector-memory phenotype. We first gated CD4+ cells for CD44hiCD62Llo; then the CD44hiCD62Llo were fractionated by CD49a expression and measured the proportion of cells that made IFN-γ in response to whole virus ex vivo. Among the CD44hiCD62Llo cells, we found the CD49a+ subset was relatively enriched for IFN-γ potential in all organs studied (Fig. 3C). Although the number of these highly Ag-reactive cells was greatest in the airways, their presence in the lymphoid organs suggests that the lung environment is not strictly required to maintain this phenotype. Furthermore, these observations suggest that this is a recirculating population with a program of differentiation that results in preferential though not exclusive localization to the lung.

Contribution of CD49a+CD4+ cells during secondary infection

Because CD49a+CD4+ cells were enriched in the BAL postinfection and capable of rapid effector cytokine secretion ex vivo, we postulated that they play a central role during secondary infection of the lung. To test this, OT-II cells were transferred to mice that were subsequently primed by infection with H1N1 A/WSN-OVAH. One month postinfection, mice were given a secondary heterosubtypic challenge with H3N2 A/X31/OVAH (34). CD49a+ OT-II cells in the BAL accounted for ~80% of the cytokine-positive CD4+ cells 24 h after secondary challenge (Fig. 3D). This is in contrast to the primary response, where activated CD49a+ T cells are not even detectable in the BAL until day 5 (Fig. 1: Ref. 18 and data not shown). The relative contribution of primary day 8, day 30, and day 1 secondary CD49a+ cells show an increasingly dominant role in the IFN-γ response detected by ex vivo restimulation (Fig. 3D). These data show that CD49a+CD4+ cells are reactivated in the first day of secondary challenge in the BAL, and there is a dominant early contribution of CD49a+ cells to the secondary IFN-γ response.

Defect in the accumulation of primed CD4+ T cells to the BAL in α1,4-linked mice

VLA-1 is the major T cell binding partner for collagen IV (30, 36–39), which is abundant in basement membrane. Previous data...
tracellular cytokine staining was performed on BAL samples from days 8 and 30 postinfection by restimulation of primed OT-II cells with OVA323–339-pulsed APCs. 

ex vivo. The same analysis was also performed on CD49a+ and CD49a− peptide. 

response found in the upper right quadrant. Upper plots show results with APCs pulsed with specific Ag, lower plots show results with APCs pulsed with control peptide. 

At day 30, a cohort of micewas given a heterosubtypic challenge with A/x31-OVA II. Intracellular staining was performed days 1 and 7 postsecondary infection. Data are shown as the proportion of the total OT-II IFN-γ response in the BAL contributed by CD49a+ OT-II cells. Data are ± SEM of an n ≥ 3 for each time point.

showed a strong correlation between CD49a expression on BAL CD4+ T cells and lung localization to collagen IV (21). Together, these data suggest a dependence on VLA-1 in CD4+ T cell localization in the BAL. To test this, we infected integrin α1−/− mice with influenza and compared CD4+ T cell recoveries from various organs to wild-type mice. We found comparable cell recoveries from BAL, draining MLN, and spleen during the acute response (Fig. 4A–C). However, 1 mo postinfection, fewer CD4+ T cells were recovered from the BAL of α1−/− mice, suggesting a defect in retention of cells at that site (Fig. 4A), which was not reflected in the lymphoid tissues (Fig. 4B, 4C). Upon restimulation ex vivo, a lower proportion of BAL CD4+ T cells from α1−/− mice were capable of secreting IFN-γ (Fig. 4D), suggesting the loss of CD44hiCD4+ T cells from the BAL of α1−/− mice primarily impacted the tissue-memory population in that organ.

VLA-1 is expressed on a variety of hematopoietic and nonhematopoietic cells (25). Therefore, it was unknown whether the defect in CD4+ T cell recovery in α1−/− mice was a direct effect of α1 deficiency on CD4+ T cells or indirect through another cell type. Adoptive transfer experiments were performed to determine the importance of VLA-1 on CD4+ T cells. CD3+CD4+CD44hi T cells were FACS sorted from naive wild-type or α1−/− mice. To limit competition from endogenous T cells, these donor cells were adoptively transferred into separate cohorts of TCR Cα−/− host mice, along with equal numbers of sorted wild-type CD3+CD8+CD44hi cells (Fig. 5A). The recipients contain no endogenous TCRα-expressing T cells (31) but do have some TCRβ+ cells (40). Donor cells were from congenic B6.PL animals, so endogenous and adoptively transferred cells were distinguished posttransfer by CD90.

Mice were challenged with influenza and sampled 1 mo later. At this time, TCR Cα−/− mice that received α1−/−CD4+ T cells had fewer CD4+ T cells in the BAL and similar cell recovery from the spleen (Fig. 5B, 5C), suggesting VLA-1 expression on the CD4+ T cell itself is important for accumulation to the BAL 1 mo postinfection.

Comparable expansion between wild-type and α1−/− CD4 T cells following infection

One possibility for reduced cell recovery in CD49a-deficient mice is a defect in the ability of the T cells to proliferate. To test this, we fed infected mice BrdU starting at day 2 postinfection and Consider the following table:

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All groups gated on CD44hiCD4+ cells prior to segregation by CD49a expression. Paired data comparing CD49a+ and CD49a− populations were subjected to Wilcoxon signed-rank test.
Differences in apoptotic marker expression between CD49a+ determined by Student a portion from the BAL of CD49b integrin-positive CD4+ T cells were actually higher in pro-BAL expresses markers of apoptosis compared with CD49a+CD4+ cells are reduced in be explained by other mechanisms. numbers of CD11a+ or CD49b+ cells comparable to wild-type mice reduced cell recovery in the deficient mice (Fig. 6n). Surprisingly, this was not observed in the spleen, traffic to the lung (Fig. 4), we also fed mice before (Fig. 4a), fewer CD4+ cells were recovered from α1−/− BAL (Fig. 6c). However, the proportions of BrdU'CD4+ T cells from α1−/− mice were similar if not greater (Fig. 6d), resulting in the same number of BrdU'CD4+ cells being recovered in both groups (Fig. 6e). This suggests the reduced number of CD4+ T cells in α1−/− BAL is not due to a defect in expansion and must be explained by other mechanisms.

CD49a+CD4+ cells are reduced in α1−/− mice The integrins LFA-1 and VLA-4 are integral in the extravasation of primed lymphocytes out of circulation. Although acute cell recoveries suggest α1−/− CD4+ cells are not defective in their ability to traffic to the lung (Fig. 4a), we wondered whether differences existed after viral clearance. Primed CD44hi cells from wild-type and α1−/− mice were stained for the integrins CD11a, CD49b, and CD49d. Although the frequency of CD49d+ cells was the same, CD11a and CD49b integrin-positive CD4+ T cells were actually higher in proportion from the BAL of α1−/− mice (data not shown). However, the reduced cell recovery in the deficient mice (Fig. 6c) resulted in numbers of CD11a+ or CD49b+ cells comparable to wild-type mice (Fig. 7a). In contrast, the frequency and CD49d+CD4+ cells were lower in α1−/− spleen, and the numbers of CD49d+CD4+ cells were lower in both the BAL and spleen (Fig. 7a and data not shown). We also found the MFI of CD49d reduced among CD4+ spleen cells in α1−/− mice, which was not found with CD11a or CD49b (data not shown). These data show a CD49d-expressing population of CD4+ cells is reduced in both the BAL and spleen of α1−/− mice.

Differences in apoptotic marker expression between CD49a+ and CD49a+CD4+ cells Another possible explanation for the reduced recovery after BAL in α1−/− mice was the inability of a specific cell population to survive and/or persist in the lung. To address this, we stained primed CD4+ cells with apoptotic markers both during and postinfection. Fig. 8 shows that a lower proportion of CD49a+CD4+ cells in the BAL expresses markers of apoptosis compared with CD49a+ cells postinfection. Surprisingly, this was not observed in the spleen, where a higher proportion of CD49a+CD4+ cells express apoptotic markers (Fig. 8a). This phenotype was also consistent in the OT-II cell population (data not shown). These populations were further gated on CD62L+ or CD62L− cells. The results show that CD49a+CD4+CD62L+CD49a+CD4+ cells are lowest for apoptotic markers in the BAL (Fig. 8b, 8c).

A reduced proportion of apoptotic CD49a+ cells in the airways may be related to expression of cytokine receptors or antiapoptotic proteins by these cells. To further describe the survival phenotype of CD49a+ cells, we stained BAL, MLN, and spleen cells 2 wk postinfection with IL-2Rα (CD25), IL-7Rα (CD127), and Bcl-2. CD127 was comparably expressed on all populations studied, and there was a trend toward increased CD25 expression on CD49a+CD4+ cells (data not shown). Interestingly, although Bcl-2 levels were the same in BAL CD49a+, CD49a−, and CD49a+CD62L+ populations postinfection, CD49a+CD4+ cells in the spleen expressed high levels of Bcl-2 (Fig. 8d, 8e). The difference in apoptotic phenotype and Bcl-2 expression between airway and spleen suggests that, although CD49a+ cells in both locations are of tissue-memory phenotype, they may be regulated by different

![Image](http://www.jimmunol.org/Downloaded)
mechanisms. Taken together, these results show that highly activated, CD49a+CD4+ cells in the BAL are comparatively low in the expression of markers of apoptosis, which may be one explanation for the enrichment of these cells in the lung postinfection. However, reduced apoptotic marker expression does not appear to correlate with differences in expression of Bcl-2, CD25, or CD127 and must be explained by other mechanisms.

Discussion

In this article, we have demonstrated an important role for the α1β1 integrin VLA-1 in the accumulation of tissue memory CD4+ cells in the airways following influenza infection. Although CD49a+CD4+ cells are enriched for effector potential and in the airway are the dominant rapid effectors within the first 24 h of secondary infection. In the absence of VLA-1, there is a deficit in tissue-memory CD4+ T cells and reduced cytokine effectors in the airways upon restimulation. The presence of a low proportion of CD49a+CD4+ T cells in the lymphoid organs suggests this population is recirculating through the body, although selectively accumulates in the airways. Taken together, this article shows that a population of effector CD4+ T cells accumulates in the airway in a α1-dependent fashion and mediates the rapid secondary effector response in this location. As noted previously (18), we have no evidence showing that VLA-1 is important for recruitment or trafficking into the lung or airways during priming, rather that its role becomes apparent after the virus is cleared. Similar to flu-specific tissue memory CD8+ T cells, there is a population of tissue memory CD4+ T cells in the airways that depend on VLA-1 for recruitment.

Although the precise mechanism of VLA-1–mediated CD4+ T cell accumulation in the airways and other peripheral tissues remains unclear, our hypothesis is that VLA-1 expression increases the half-life of the cells in extralymphoid sites, and this is most evident in the airways and other mucosal sites (43). This is supported by the selective enrichment of CD49a+ cells in the airways after the contraction phase of the immune response (Fig. 1), as well as the specific loss of effector cells from the airways in α1−/− mice after viral clearance (Fig. 4). The simplest explanation is that adhesion to ECM slows migration or loss from the airway environment, and there is evidence of a close association of T cells with areas of basement membrane in the lung (21). However, it is also possible that matrix interactions act to increase the survival of T cells in these environments. This hypothesis is supported by reduced expression of apoptotic markers on CD49a+expressing T cell populations postinfection (Fig. 8). In many extralymphoid sites, matrix interactions are essential for preventing apoptosis (16, 44).

We found that α1−/− mice have reduced numbers of CD49d+CD4+ cells in both the airways and spleen (Fig. 7). There was not a general defect in β1 or other integrins in α1−/− mice, because the expression of CD49b and CD11a was the same compared with wild-type mice (Fig 7). Although differences in CD49d may be a factor in reduced α1−/− CD4+ cell recovery by BAL, it does not by itself explain the defect. First, recruitment of primed α1−/− CD4+ cells to the BAL is normal during acute infection (Fig. 4). Second, the same number of BrdU+CD4+ cells was recovered from BAL of wild-type and α1−/− after viral clearance (Fig. 6). Both pieces of evidence suggest entry of new cells into the airways is not defective, consistent with recent data that BAL T cells are continually replaced after viral infection by newly recruited cells (45). If VLA-4 expression was responsible for reduced

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We found that α1−/− mice have reduced numbers of CD49d+CD4+ cells in both the airways and spleen (Fig. 7). There was not a general defect in β1 or other integrins in α1−/− mice, because the expression of CD49b and CD11a was the same compared with wild-type mice (Fig 7). Although differences in CD49d may be a factor in reduced α1−/− CD4+ cell recovery by BAL, it does not by itself explain the defect. First, recruitment of primed α1−/− CD4+ cells to the BAL is normal during acute infection (Fig. 4). Second, the same number of BrdU+CD4+ cells was recovered from BAL of wild-type and α1−/− after viral clearance (Fig. 6). Both pieces of evidence suggest entry of new cells into the airways is not defective, consistent with recent data that BAL T cells are continually replaced after viral infection by newly recruited cells (45). If VLA-4 expression was responsible for reduced

![FIGURE 6.](Image)

**FIGURE 6.** Turnover of primed CD4+ cells is comparable between wild-type and α1−/− mice. A and B, Wild-type (●) and α1−/− (○) mice were infected with influenza. Two days postinfection, mice were administered BrdU by i.p. injection of 1 mg per animal, followed by continuous feeding of 0.8 mg/ml BrdU in drinking water. At day 5, 7, and 9 postinfection, mice were sacrificed, and BAL (A) and SPL (B) were analyzed by flow cytometry for BrdU+CD44hiCD4+ cells. Data are shown as ± SEM of n ≥ 3/group. C–E, Mice that recovered from influenza infection were administered BrdU as above from day 20 to 25 postinfection. At day 25, BAL samples from wild-type (○) and α1−/− (■) mice were analyzed for recovery of total CD44hiCD4+ cells (C) and the frequency (D) and number (E) of BrdU+CD44hiCD4+ cells. Data are shown as ± SEM of n = 5/group. Values from t test are above the corresponding data.

![FIGURE 7.](Image)

**FIGURE 7.** α1−/− mice have reduced numbers of CD49d+CD4+ cells postinfection. Wild-type (●) and α1−/− mice (○) were infected with influenza and 25 d later analyzed by flow cytometry for expression of integrins on CD4+ cells. BAL (A) and SPL (B) data are represented as the number of CD4+ cells containing positive for the indicated integrin Ab; ± SEM for n = 5/group. Significant values from t test are shown above corresponding data.
extravasation and thus recovery of primed BAL CD4+ cells after viral clearance, we would expect to see this reflected in the recovery of BrdU+ cells. Instead, we favor the view that VLA-1 functions after the cells have entered the airways, and the reduced CD49d population and expression reflects the fact that high levels of CD49a and CD49d are coexpressed by the same cells. Each integrin then functions at distinct steps in the process of getting to and staying in the airways.

One important point is that only about half of the primed CD4+ cells in the BAL express CD49a after influenza infection, whereas nearly all of the BAL CD8+ cells express CD49a (18, 21). This seems to be a general feature of BAL CD4+ cells, because the proportions of CD49a+CD4 T cells is similar postinfection with vaccinia virus, systemic versus respiratory infection, priming with peptide plus LPS, or sensitization and challenge with OVA aerosol (as a model for airway hyper reactivity), even though BAL cell recoveries varied between treatments (data not shown and Ref. 21). This may be the result of homeostatic differences between primed CD4+ and CD8+ cells, because the turnover of primed CD4+ and CD8+ cells and their rate of lung accumulation have been shown to be different (45, 46). Alternatively, VLA-1 expression on both CD4+ and CD8+ cells may be a consequence of strong versus weak TCR stimulation at priming, such that a subset of T cells expressing the “fittest” TCRs are programmed for VLA-1 expression. A recent report by the Bank laboratory showed that the TCR repertoires of VLA-1+ CD4 cells in human inflamed synovium were largely distinct from VLA-1− synovial cells or those in peripheral blood (47). This suggests the enrichment of VLA-1+ cells to extralymphoid tissues may represent a distinct subpopulation within the memory pool. However, it is still unknown whether any potential unique specificities found in the CD49a+ fraction have high-affinity TCRs compared with other specificities. Further work must be done to address this interesting hypothesis.

An important feature of the CD49a+ population in the BAL is the ability to respond to secondary infection. Although at most approximately half of CD4+ cells express CD49a, they are dominant contributors to the rapid effector cytokine response in a 5-h restimulation ex vivo (Fig. 3). Furthermore, the relative contribution of the CD49a+ subset to the cytokine responders 24 h after secondary infection is even greater than at 1 mo postinfection. Rapid recruitment of new cells from lung parenchyma or circulation, induction of CD49a expression on resident cells, or rapid loss of CD49a+ cells could all potentially account for this phenotype in the secondary. Additionally, if CD49a+ cells are localized to regions of the lung where infection is prevalent, they may receive increased stimulation in vivo from the second infection compared with their CD49a− counterparts. Our current experiments cannot distinguish among these possibilities. In either event, the relative abundance of CD49a+ cells and their activity in the BAL during acute secondary infection suggests a central role for these cells in the rapid recall response in the lung. A detailed analysis of the secondary response in the airways will give additional insight into the nature of the rapid recall response.

In general, the high proportion of extralymphoid T cells that have an effector-memory phenotype has led to the hypothesis that unique populations of primed T cells are poised for rapid response to secondary antigenic encounter in the tissue (6). Although this hypothesis is an attractive model, there is little published evidence demonstrating such a role for extralymphoid cells. Data in this report support the “sentinel” hypothesis and suggest that specific memory cell populations are uniquely able to accumulate in extralymphoid tissues for rapid response in case of future infectious challenge. Although CD4+ cells are not strictly required for protection from influenza (8, 48, 49), they may directly engage infected targets in certain conditions (50, 51). Alternatively, they may play a dominant role in orchestrating the early innate response in the tissue through local secretion of chemokines that...
modulate the inflammatory environment. To this end, primed CD4+ cells have been shown to alter the lung innate inflammatory response to respiratory challenge with Sendai virus (52). Further study into the functions of extralymphoid CD4+ cells will be helpful in assessing their utility in protective tissue responses.

The identification of a population of tissue memory T cells that become rapid effectors upon secondary encounter with virus is an important correlate of protection from influenza disease. It may be useful to monitor the CD49a+ populations postinfection or vaccination or to devise strategies to increase the prevalence of this memory population. The potential therapeutic impact of targeting VLA-1 expression on monocytes for sorting of T cell populations.

Acknowledgments

We thank Nathan Laniecki for his skillful assistance with flow cytometric sorting of T cell populations.

Disclosures

The authors have no financial conflicts of interest.

References


