

Quantifying Antigen-Specific CD4 T Cells during a Viral Infection: CD4 T Cell Responses Are Larger Than We Think

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The number of virus-specific CD8 T cells increases substantially during an acute infection. Up to 90% of CD8 T cells are virus specific following lymphocytic choriomeningitis virus (LCMV) infection. In contrast, studies identifying virus-specific CD4 T cell epitopes have indicated that CD4 T cells often recognize a broader array of Ags than CD8 T cells, consequently making it difficult to accurately quantify the total magnitude of pathogen-specific CD4 T cell responses. In this study, we show that CD4 T cells become CD11a^{hi}CD49d⁺ after LCMV infection and retain this expression pattern into memory. During the effector phase, all the LCMV-specific IFN- γ ⁺ CD4 T cells display a CD11a^{hi}CD49d⁺ cell surface expression phenotype. In addition, only memory CD11a^{hi}CD49d⁺ CD4 T cells make IFN- γ after stimulation. Furthermore, upon secondary LCMV challenge, only CD11a^{hi}CD49d⁺ memory CD4 T cells from LCMV-immune mice undergo proliferative expansion, demonstrating that CD11a^{hi}CD49d⁺ CD4 T cells are truly Ag specific. Using the combination of CD11a and CD49d, we demonstrate that up to 50% of the CD4 T cells are virus specific during the peak of the LCMV response. Our results indicate that the magnitude of the virus-specific CD4 T cell response is much greater than previously recognized. *The Journal of Immunology*, 2011, 187: 5568–5576.

CD4 T cells play a vital role in coordinating adaptive immune responses during viral infection. However, a number of factors conspire to make it difficult to quantify virus-specific CD4 T cell responses. During viral infections, CD4 T cells specific for known epitopes accumulate at much lower numbers than their CD8 T cell counterparts. In addition, in contrast to CD8 T cells that maintain a stable memory population, the number of memory CD4 T cells gradually decreases over time (1). Furthermore, activated CD4 T cells can differentiate into one of a number of subsets that have unique cytokine production signatures, making the identification of Ag-specific CD4 T cells based solely on cytokine release more difficult (1, 2). Two common methods used to identify pathogen-specific effector and memory CD4 T cells are in vitro peptide stimulation followed by intracellular cytokine staining (ICS) or MHC class II tetramer staining (3, 4). However, both require prior knowledge regarding the Ag specificity of the CD4 T cells (4). Moreover, recent studies aimed at identifying pathogen-specific CD4 T cell epitopes have indicated that CD4 T cells often recognize a broad array of pathogen-encoded proteins, resulting in a low frequency of CD4 T cells specific to any individual epitope. These obstacles have prevented accurate quantification of the magnitude of pathogen-specific CD4 T cell responses (5).

Recent studies have demonstrated that, following lymphocytic choriomeningitis virus (LCMV) infection, Ag-specific CD8 T cells downregulate the cell surface expression of CD8 α and increase expression of the trafficking molecule CD11a (6, 7). Importantly the magnitude of pathogen-specific CD8 T cell responses can be determined using this technique without prior knowledge of the Ag specificity of the responding CD8 T cells. However, no analogous technique is currently available to estimate the magnitude of the Ag-specific CD4 T cell response following viral infection. Activated CD4 T cells downregulate CD62L and CCR7 expression, allowing for their egress from lymph nodes (8, 9). These activated CD4 T cells subsequently upregulate cell surface expression of integrins such as $\alpha_4\beta_1$ (CD49d and CD29, respectively) and $\alpha_L\beta_2$ (CD11a and CD18, respectively) that mediate their migration to the site of infection (8, 9). Therefore, given the important role of integrins in the migration of effector CD4 T cells, we sought to determine whether modulation of these trafficking molecules would provide a reliable cell surface phenotypic profile to identify Ag-specific CD4 T cells after viral infection.

In this study, we demonstrate that LCMV-specific CD4 T cells increase cell surface expression of CD11a and induce expression of CD49d following infection and that expression of these molecules remains stable into memory. We show that, whereas naive CD4 T cells are CD11a^{lo}CD49d⁻, LCMV-specific effector CD4 T cells identified by LCMV-peptide-driven IFN- γ production are CD11a^{hi}CD49d⁺. In addition, we demonstrate that only CD11a^{hi}CD49d⁺ CD4 T cells from LCMV-immune mice produce IFN- γ after stimulation and that only CD11a^{hi}CD49d⁺ CD4 T cells from LCMV-immune mice respond after a secondary LCMV challenge. Our results demonstrate that the combination of CD11a and CD49d can be used to determine the magnitude of the Ag-specific CD4 T cell response following infection and that all LCMV-specific memory CD4 T cells are CD11a^{hi}CD49d⁺. Importantly, we demonstrate that up to 50% of the CD4 T cells at the peak of the LCMV response are CD11a^{hi}CD49d⁺, indicating that the magnitude of the Ag-specific CD4 T cell response following viral infection is much greater than previously recognized.

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Abbreviations used in this article: ATCC, American Type Culture Collection; BAL, bronchoalveolar lavage; BFA, brefeldin A; ICS, intracellular cytokine staining; i.n., intranasal; LCMV, lymphocytic choriomeningitis virus; PV, Pichinde virus; RSV, respiratory syncytial virus; Tg, transgenic; VACV, vaccinia virus.

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Materials and Methods

Mice and viruses

BALB/cAnNCr, C57BL/6NCr, B6-Ly5.2/Cr, and outbred Swiss Webster mice between 6 and 8 wk of age were purchased from the National Cancer Institute (Frederick, MD). C57BL/6 SMARTA TCR-transgenic (Tg) mice (10) were a gift from Michael J. Bevan (University of Washington, Seattle, WA). Female mice were used in all experiments. The Armstrong strain of LCMV and Pichinde virus (PV) were gifts from Raymond M. Welsh (University of Massachusetts Medical School, Worcester, MA) and were both propagated on BHK-21 cells (American Type Culture Collection [ATCC], Manassas, VA) (11). The Western Reserve strain of vaccinia virus (VACV) was also a gift from Raymond M. Welsh and was propagated on BSC-40 cells (ATCC) (11). The A2 strain of respiratory syncytial virus (RSV) was a gift from Barney S. Graham (National Institutes of Health, Bethesda, MD) and was propagated on HEp-2 cells (ATCC) (12). All experimental procedures using mice were approved by the University of Iowa Animal Care and Use Committee.

Adoptive transfer of SMARTA CD4 T cells

SMARTA CD4 T cells (1×10^4) were adoptively transferred i.v. into naive C57BL/6 mice. Twenty-four hours after transfer, recipient C57BL/6 mice were infected intranasally (i.n.) with 5×10^5 PFU LCMV (13). Spleen and lungs were harvested at days 8 and 30 postinfection. Lung mononuclear cells were isolated as described previously (12). Cells were stained with mAbs specific to CD4 and CD90.2 as well as to CD11a, CD29, CD44, CD49b, CD49d, CD49e, CD54, CD62L, CD90.1, CD103, or PSGL-1 (eBioscience, San Diego, CA) and analyzed on a BD FACSCanto (BD Bioscience, San Jose, CA) as described previously (12). Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

In some experiments, SMARTA CD4 T cells ($2-3 \times 10^6$) were adoptively transferred i.v. into naive C57BL/6 mice, and recipient mice were infected i.p. 24 h later with 5×10^5 PFU LCMV, 1×10^6 PFU PV, or 100 μ g CpG. Spleens were harvested at day 8 postinfection (11) and splenocytes were stained with mAbs specific to CD4, CD11a, CD49d, and CD90.1; they were subsequently analyzed on a BD FACSCanto flow cytometer. To purify CD11a^{hi}CD49d⁺ and CD11a^{lo}CD49d⁻ CD4 T cells, splenocytes from day 60 or older LCMV-immune B6-Ly5.2/Cr (CD45.1⁺) mice were stained with CD4-allophycocyanin-Cy7 (BioLegend, San Diego, CA). Cells were subsequently stained with anti-Cy7 magnetic beads (Miltenyi Biotec, Auburn, CA) and positively selected using an AutoMACS (Miltenyi Biotec). AutoMACS-enriched cells were stained for CD90.2, CD49d, and CD11a and sorted using a BD FACSAria II (BD Biosciences). Sorted populations were labeled with CFSE (Molecular Probes, Carlsbad, CA), and 1.2×10^5 CFSE-labeled cells were adoptively transferred i.v. into C57BL/6 (CD45.2⁺) mice.

RSV infection

BALB/c mice were infected i.n. with 3.1×10^6 PFU RSV. Lung and bronchoalveolar lavage (BAL) mononuclear cells were isolated and stained with mAbs specific to CD4, CD11a, CD49d, and CD90.2 as described previously (14). Cells were analyzed on a BD FACSCanto flow cytometer, and data were analyzed using FlowJo software.

Intracellular cytokine stain

Spleen and lung cells from LCMV-infected mice and lung cells from RSV-infected mice were incubated with either 1 μ M of the LCMV-derived peptide GP₆₆₋₈₀ (Biosynthesis, Lewisville, TX) (5, 15) or the RSV-derived peptides G₁₈₃₋₁₉₅ or F₅₁₋₆₆ (16-18), respectively, in the presence of 10 μ g/ml brefeldin A (BFA; Sigma-Aldrich, St. Louis, MO) for 5 h at 37°C (5, 13). PBL from LCMV-immune mice were stimulated with 50 ng/ml PMA (Sigma-Aldrich), and 500 ng/ml ionomycin (Sigma-Aldrich) in the presence of 10 mg/ml brefeldin A for 3.5 h at 37°C (19). After stimulation, cells were surface-stained with mAbs specific to CD4, CD11a, CD49d, and CD90.2. Intracellular staining for IFN- γ was performed as described previously (13). Cells were analyzed on a BD FACSCanto flow cytometer, and data were analyzed using FlowJo software.

Results

Alterations in CD11a and CD49d cell surface expression on virus-specific Tg-CD4 T cells following acute LCMV infection

The magnitude of pathogen-specific CD8 T cell responses can be determined through the use of the surrogate activation markers CD8^{lo}CD11a^{hi} (6, 7). We sought to develop a similar strategy to identify Ag-experienced CD4 T cells to quantify the magnitude of

pathogen-specific CD4 T cell responses. Previous studies have demonstrated that *Salmonella*-specific CD4 T cells are CD11a^{hi} (20). However, CD11a expression decreases on memory CD4 T cells after viral infection, making it difficult to distinguish naive from memory CD4 T cells based solely on CD11a expression. Furthermore, in contrast to CD8 T cells, CD4 T cells do not modulate CD4 expression. Therefore, we sought to find additional markers to more easily identify Ag-specific CD4 T cells.

Activated CD4 T cells use various integrins to traffic to sites of inflammation following infection (8, 9); therefore, we sought to determine whether modulation of integrins would provide a reliable cell surface phenotypic profile to identify Ag-specific CD4 T cells. We used an adoptive transfer model to examine the cell surface expression of several trafficking molecules after acute LCMV infection. LCMV-specific SMARTA CD4 T cells (1×10^4 ; CD90.1⁺) were adoptively transferred into naive C57BL/6 mice (CD90.2⁻), followed 24 h after transfer by i.n. LCMV infection, mimicking the natural route of LCMV infection. At both acute and memory time points (i.e., days 8 and 30, respectively) we analyzed the cell surface expression of a wide range of integrins and adhesion molecules on gated SMARTA cells from the spleen (Fig. 1A) and lung (Fig. 1B). At both time points, CD4 T cells from the spleen and lung exhibited increased expression of the memory marker CD44 compared with CD4 T cells obtained from the spleen and lung of naive SMARTA mice (Fig. 1). In addition, we observed increased expression of the adhesion molecules CD11a, CD49d, and CD29 on both acute and memory CD4 T cells from the spleen (Fig. 1A) and lung (Fig. 1B) compared with naive SMARTA cells. Several additional markers were examined (i.e., CD62L, CD54, β 7 integrin, CD49b [DX-5], CD49e, CD49f, CD103, PSGL-1, CD45RB, and Ly6C); however, the majority of CD4 T cells did not exhibit altered cell surface expression of these molecules at either acute or memory time points (Fig. 1). These data suggest that following LCMV infection, LCMV-Tg CD4 T cells upregulate the cell surface expression of CD11a, CD49d, CD29, and CD44 and retain this expression pattern into memory.

Changes in CD11a and CD49d cell surface expression on endogenous CD4 T cells following acute LCMV infection

We next asked whether modulation of integrins could be used to distinguish naive from activated CD4 T cells within the endogenous CD4 T cell population following acute LCMV infection. To address this question, we examined cell surface expression of CD11a in combination with CD49d, CD29, or CD44 on endogenous (CD4⁺CD90.1⁻) splenic (Fig. 2A) and lung (Fig. 2B) CD4 T cells after LCMV infection. We found that CD11a in combination with all three markers allowed for the clear discrimination of the activated endogenous CD4 T cells from naive CD4 T cells during both acute and memory time points. However, the fraction of CD11a^{hi}CD49d⁺ CD4 T cells from naive mice was substantially lower than that of either the CD11a^{hi}CD44⁺ or CD11a^{hi}CD29⁺ populations (Fig. 2). Therefore, we focused on the combined expression of CD11a and CD49d for the remainder of our studies. Importantly, at day 8 after LCMV infection ~40% of the CD4 T cells in the spleen and 50% in the lung were CD11a^{hi}CD49d⁺, suggesting that the magnitude of the LCMV-specific CD4 T cell response is much larger than what has been previously demonstrated using in vitro peptide stimulation followed by either ICS or ELISPOT analysis for IFN- γ production (5, 21-23).

Increased CD11a and CD49d cell surface expression requires interaction with cognate Ag

Infections induce both TCR signals and inflammatory cytokines, either of which could alter the expression of integrins. To determine

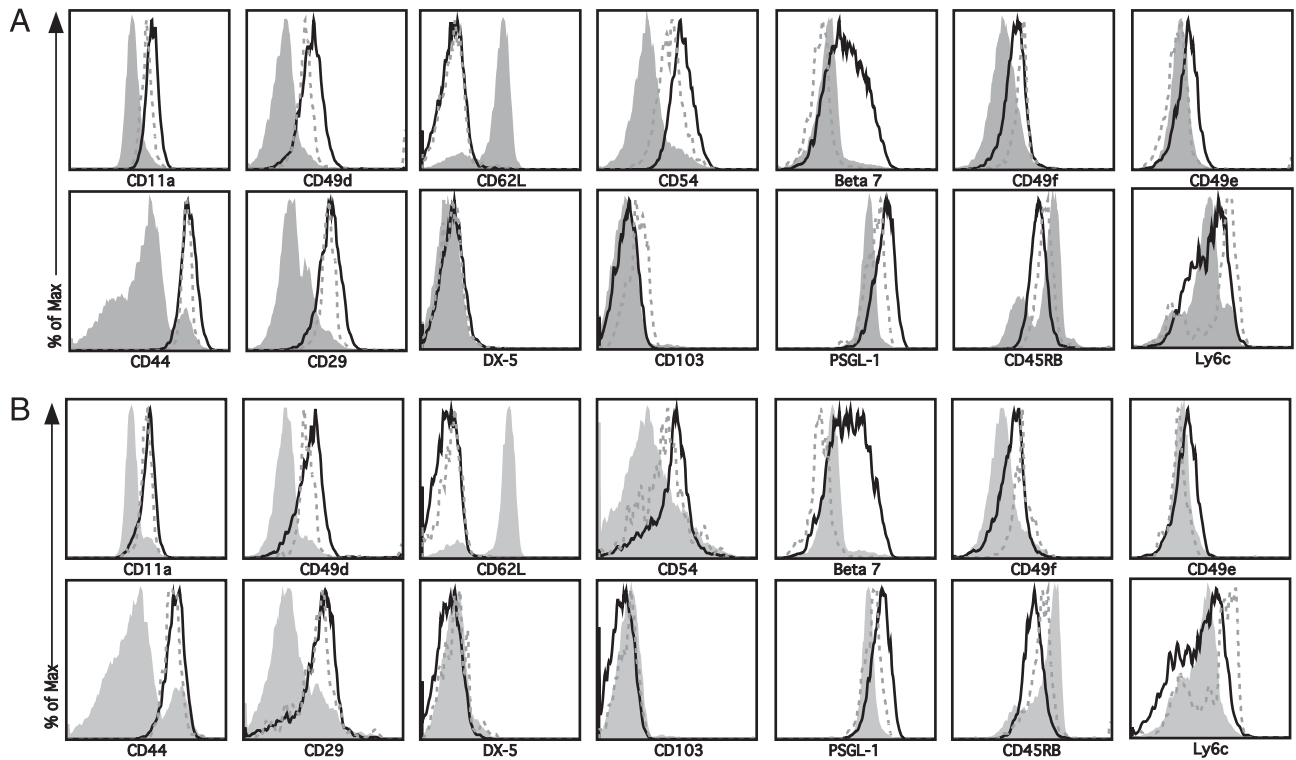


FIGURE 1. Identification of markers that distinguish Ag-specific CD4 T cells. Thy1.1 SMARTA CD4 T cells were transferred into naive Thy1.2 C57BL/6 mice, followed by i.n. infection with LCMV 24 h later. Spleens (A) and lungs (B) were harvested on days 8 (black line) and 30 (dashed gray line) postinfection. Representative plots depict cell surface expression of CD11a, CD49d, CD44, CD29, CD62L, CD54, β_7 integrin, CD49f, CD49e, CD49b (DX-5), CD103, PSGL-1, CD45RB, and Ly6C on SMARTA CD4 T cells ($CD4^+Thy1.1^+$). Surface marker expression on CD4 T cells from the naive SMARTA donor mouse (shaded gray line) is also shown. Similar results were obtained from four independent experiments with three to four mice per group.

whether modulation of CD11a and CD49d cell surface expression requires TCR interaction with cognate Ag, we adoptively transferred naive SMARTA CD4 T cells into naive C57BL/6 mice. Recipient mice were subsequently administered either 100 μ g CpG or 1×10^6 PFU PV to induce a nonspecific inflammatory envi-

ronment. Additional recipients received either PBS or LCMV as a negative and positive control, respectively. Spleens were harvested 8 d later, and we examined CD11a and CD49d expression on the transferred SMARTA CD4 T cells (Fig. 3). SMARTA cells isolated from either CpG-treated or PV-infected animals displayed

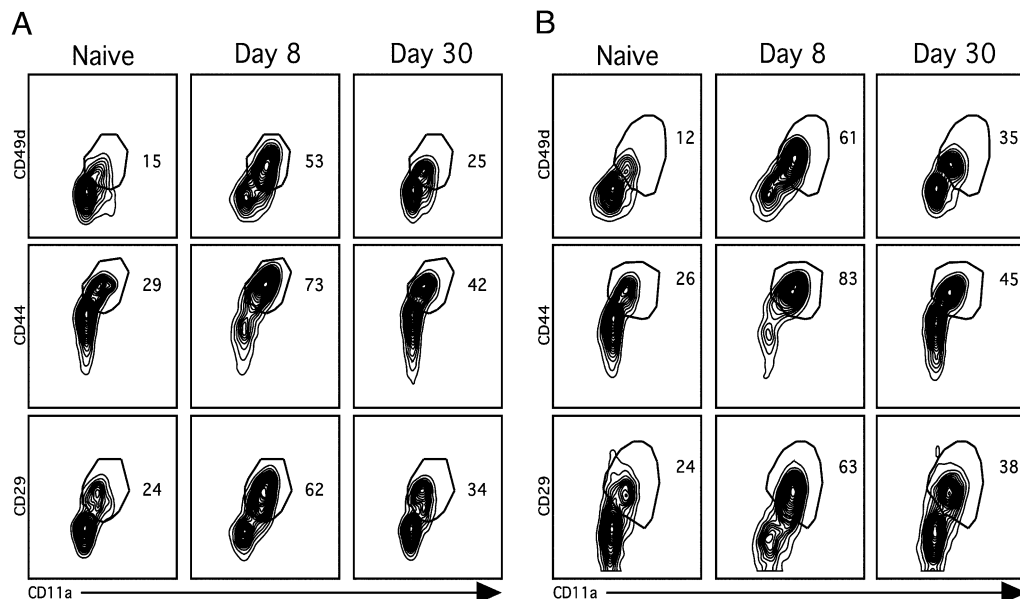


FIGURE 2. Identification of surface markers that distinguish endogenous Ag-specific CD4 T cells. Spleens (A) and lungs (B) were harvested from naive or LCMV-infected mice as described in Fig. 1. Representative plots depict cell surface expression patterns of CD11a in combination with CD49d, CD29, or CD44 on endogenous CD4 T cells ($CD4^+Thy1.1^-$). Similar results were obtained from four independent experiments with three to four mice per group.

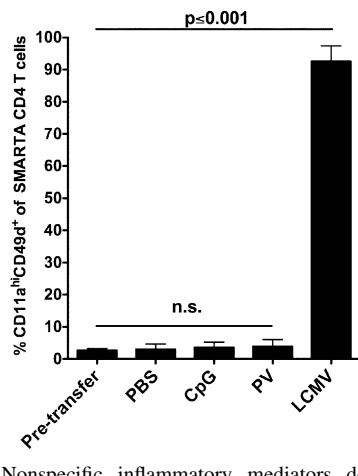


FIGURE 3. Nonspecific inflammatory mediators do not modulate CD11a CD49d cell surface expression. Thy1.1 LCMV-Tg SMARTA CD4 T cells were transferred into Thy1.2 C57BL/6 mice, and 24 h later mice were administered PBS, CpG, PV, or LCMV i.p. Spleens were harvested at day 8 postinfection, and SMARTA CD4 T cells (CD4⁺Thy1.1⁺) were analyzed for cell surface expression pattern of CD11a and CD49d. Combined results are shown from three independent experiments with an $n = 3$ mice per group. Error bars represent \pm SD. Data were analyzed using unpaired t tests.

no increase in the cell surface expression of CD11a and CD49d compared with the PBS control mice (Fig. 3). However, SMARTA cells obtained from LCMV-infected mice demonstrated a significant ($p < 0.001$) upregulation of CD11a and CD49d. These data show that Ag stimulation is required to cause increased cell surface expression of CD11a and induce cell surface expression of CD49d on CD4 T cells and that expression of these molecules is not modulated by nonspecific inflammation generated by either a TLR agonist or a heterologous viral infection.

CD11a^{hi}CD49d⁺ effector CD4 T cells are Ag-specific

We have demonstrated that the cell surface expression of CD11a and CD49d are modulated on CD4 T cells after LCMV infection; however, we next wanted to determine whether these CD11a^{hi}CD49d⁺ CD4 T cells are truly Ag-specific. To confirm that CD11a^{hi}CD49d⁺ CD4 T cells represent Ag-specific cells, spleens and lungs were harvested from LCMV-infected C57BL/6 mice at the peak of infection (i.e., day 8), followed by in vitro peptide stimulation with the LCMV-specific CD4 T cell immunodominant epitope GP₆₆₋₈₀ (5, 15). When IFN- γ production from CD11a^{hi}CD49d⁺ CD4 T cells versus CD11a^{lo}CD49d⁻ CD4 T cells was compared, we observed that essentially all the IFN- γ -producing CD4 T cells in the spleen and lung were CD11a^{hi}CD49d⁺ (98.0 and 98.7%, respectively; Fig. 4A, 4B). These data demonstrate that LCMV-specific effector CD4 T cells are CD11a^{hi}CD49d⁺. Furthermore, the data indicate that 40–50% of the CD4 T cells during acute LCMV infection are virus specific.

We further tested whether CD11a and CD49d expression patterns could distinguish naive from Ag-experienced CD4 T cells following acute RSV infection. Similar to LCMV infection, CD4 T cells in the lung and BAL upregulated CD11a and CD49d cell surface expression following acute RSV infection (Fig. 5A; day 8). Although CD4 T cells in the lung parenchyma remain CD11a^{hi}, at day 15 postinfection, CD4 T cells within the lung airways display downregulated expression of CD11a as described previously (24). However, unlike CD11a, CD49d expression was not decreased on CD4 T cells in the lung airways, suggesting that CD49d is sufficient to distinguish Ag-specific cells within the lung airways following at least day 15 postinfection. To determine whether the

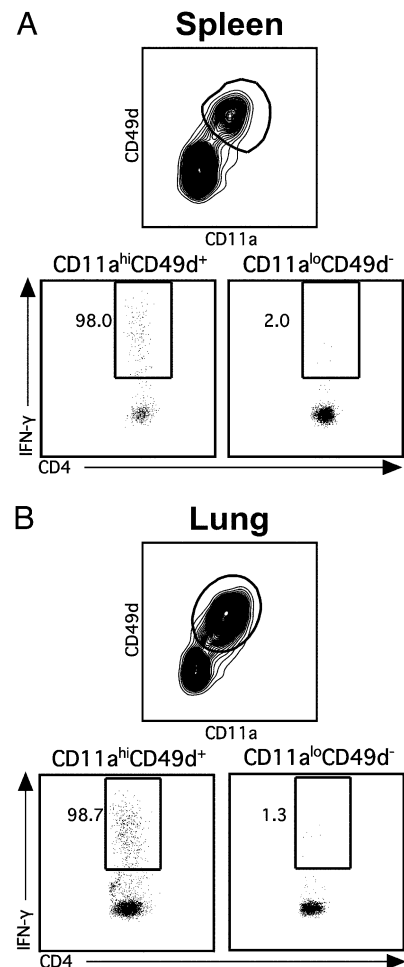


FIGURE 4. Ag-specific effector CD4 T cells are CD11a^{hi}CD49d⁺. C57BL/6 mice were infected i.n. with LCMV, and spleens (A) and lungs (B) were harvested at day 8 postinfection. CD4 T cells were incubated in BFA alone or stimulated with the LCMV-derived peptide GP₆₆₋₈₀. Representative staining depicts IFN- γ production by CD4⁺CD90.2⁺ T cells gated on either CD11a^{hi}CD49d⁺ (left panels) or CD11a^{lo}CD49d⁻ (right panels). Numbers denote the percentage of IFN- γ ⁺ CD4 T cells that are either CD11a^{hi}CD49d⁺ or CD11a^{lo}CD49d⁻. Similar results were obtained from four independent experiments with three to four mice per group.

CD11a^{hi}CD49d⁺ CD4 T cells in the lung and the lung airways are RSV specific, we used in vitro peptide stimulation followed by ICS to identify RSV-specific CD4 T cells and examined IFN- γ production from CD11a^{hi}CD49d⁺ CD4 T cells versus CD11a^{lo}CD49d⁻ CD4 T cells. RSV G₁₈₃₋₁₉₅-specific (16, 17) and RSV F₅₁₋₆₆-specific (18) lung CD4 T cells were CD11a^{hi}CD49d⁺ (Fig. 5B), demonstrating that the cell surface upregulation of CD11a and CD49d on Ag-experienced effector CD4 T cells occurs during other viral infections.

CD11a^{hi}CD49d⁺ memory CD4 T cells are Ag specific

Having established that effector CD4 T cells are CD11a^{hi}CD49d⁺, we next asked whether CD11a and CD49d cell surface expression can be used to identify LCMV-specific memory CD4 T cells. To address this question, we sorted CD11a^{hi}CD49d⁺ and CD11a^{lo}CD49d⁻ CD4 T cells from LCMV-immune (i.e., \geq day 60 after LCMV infection) CD45.1⁺ congenic mice, CFSE-labeled the sorted cells, and subsequently adoptively transferred them into naive CD45.2⁺ recipients. Recipient mice were either infected with LCMV or given PBS as a control, and the CFSE dilution profiles of the transferred cells isolated from the spleen were ex-

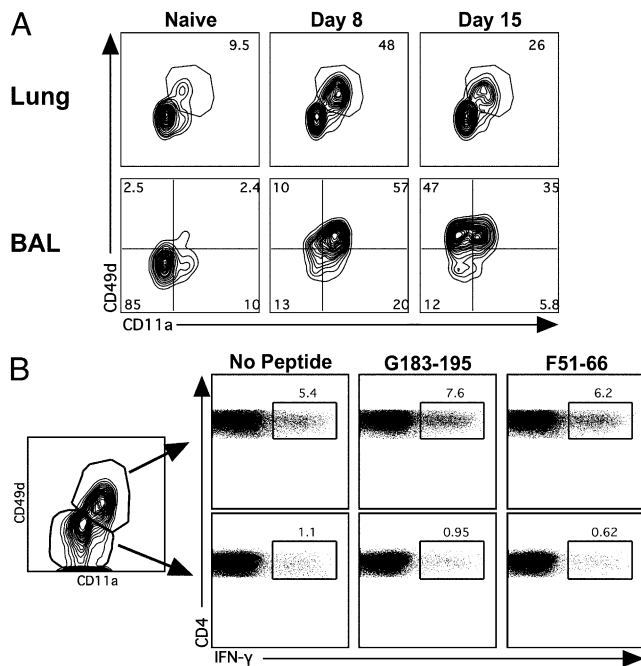


FIGURE 5. CD11a and CD49d identify Ag-specific CD4 T cells following acute RSV infection. BALB/c mice were infected with RSV i.n. *A*, Representative plots depict CD11a and CD49d expression on lung and BAL CD4⁺Thy1.2⁺-gated CD4 T cells at days 8 and 15 postinfection. *B*, Lung CD4 T cells from day 8 RSV-infected BALB/c mice were incubated in BFA alone or stimulated with either the RSV peptide G₁₈₃₋₁₉₆ or F₅₁₋₆₆. Representative plots depict IFN- γ production between CD11a^{hi}CD49d⁺ (*top panels*) and CD11a^{lo}CD49d⁻ (*bottom panels*) CD4 T cells (CD4⁺CD90.2⁺). Similar results were obtained from four independent experiments with three to four mice per group.

amed at day 4 postinfection. CD11a^{lo}CD49d⁻ CD4 T cells did not proliferate in either control PBS or LCMV-infected mice (Fig. 6A). In contrast, CD11a^{hi}CD49d⁺ CD4 T cells proliferated vigorously upon LCMV infection, but did not proliferate in PBS control animals (Fig. 6B). To further confirm that CD11a^{hi}CD49d⁺ CD4 T cells within LCMV-immune mice are truly LCMV-specific cells, we stimulated CD4 T cells from the PBL of LCMV-immune mice and examined IFN- γ production. When IFN- γ production from CD11a^{hi}CD49d⁺ CD4 T cells versus CD11a^{lo}CD49d⁻ CD4 T cells was compared, we observed that essentially all the IFN- γ -producing CD4 T cells in the PBL of LCMV-immune mice were CD11a^{hi}CD49d⁺ (~98%; Fig. 6C). These data indicate that all the Ag-specific memory CD4 T cells are CD11a^{hi}CD49d⁺. These results show that cell surface expression of CD11a and CD49d can be used to identify Ag-specific CD4 T cells over the course of an infection.

Magnitude and kinetics of the Ag-specific CD4 T cell response following infection

Our data indicate that Ag-specific CD4 T cells can be tracked in inbred populations after viral infection using CD11a and CD49d. We next sought to determine whether CD11a and CD49d expression patterns could be used to track Ag-specific CD4 T cells within an outbred population. C57BL/6, BALB/c, and outbred Swiss Webster mice were infected with LCMV, and CD4 T cells were examined in the blood at various time points following infection (Fig. 7). Few CD11a^{hi}CD49d⁺ CD4 T cells were present in the PBL of any of the strains until day 6. LCMV infection of C57BL/6 mice resulted in a large expansion of CD11a^{hi}CD49d⁺ CD4 T cells that peaked uniformly at day 8 postinfection (56.6 \pm

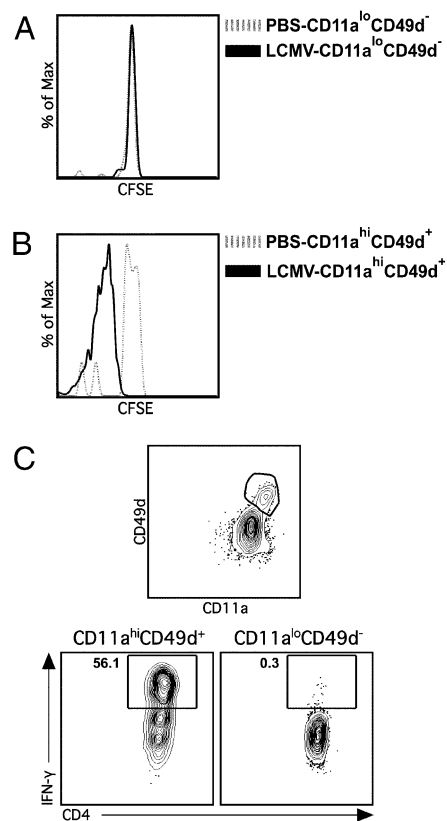


FIGURE 6. Memory CD4 T cells are CD11a^{hi}CD49d⁺. CD11a^{lo}CD49d⁻ (*A*) and CD11a^{hi}CD49d⁺ (*B*) CD4 T cells (CD4⁺CD90.1⁺) were sorted from LCMV-immune (i.e., \geq day 60 after LCMV infection) CD45.1 congenic mice, labeled with CFSE and transferred into naive CD45.2 C57BL/6 mice. Recipient mice were subsequently administered either PBS (dashed gray line) or LCMV (solid black line) i.p., and spleens were harvested at day 4 postinfection. Representative plots show CFSE dilution profiles of transferred CD11a^{hi}CD49d⁺ CD4 T cells (*left*) and CD11a^{lo}CD49d⁻ CD4 T cells (*right*) from mice administered PBS (dashed gray line) or LCMV (solid black line). Similar results were obtained from three independent experiments with three to four mice per group. *C*, PBL were obtained from LCMV-immune mice, and the CD4 T cells were incubated in either BFA alone or stimulated with PMA- γ ionomycin. Representative staining depicts IFN- γ production by CD4⁺CD90.2⁺ T cells gated on either CD11a^{hi}CD49d⁺ (*left*) or CD11a^{lo}CD49d⁻ cells (*right*). Numbers denote the percentage of IFN- γ ⁺ CD4 T cells within each respective population. Similar results were obtained from two independent experiments with three to four mice per group.

4.6%; Fig. 7A). In contrast, LCMV infection of BALB/c mice resulted in a relatively low frequency of CD11a^{hi}CD49d⁺ CD4 T cells that peaked at day 10 postinfection (14.9 \pm 3.3%; Fig. 7B). The peak frequency of CD11a^{hi}CD49d⁺ CD4 T cells in Swiss Webster mice was less uniform than in the inbred strains (43.4 \pm 9.5%; Fig. 7C), similar to what has been reported previously for CD8 T cells during LCMV infection (6). Although the peak frequency of CD11a^{hi}CD49d⁺ CD4 T cells varied, the overall kinetics of the CD4 T cell response in the outbred strain was similar between each individual mouse, with the majority of mice exhibiting a peak at day 10 postinfection (Fig. 7C). Overall these data demonstrate that the genetic background can have a substantial effect on the overall kinetics and magnitude (i.e., C57BL/6 mice versus BALB/c) of the CD4 T cell response following infection with the same virus. Furthermore, these data demonstrate that although outbred hosts display similar overall CD4 T cell expansion and contraction kinetics following infection, there is a large degree of variability in the total magnitude of the CD4 T cell response within an outbred host population.

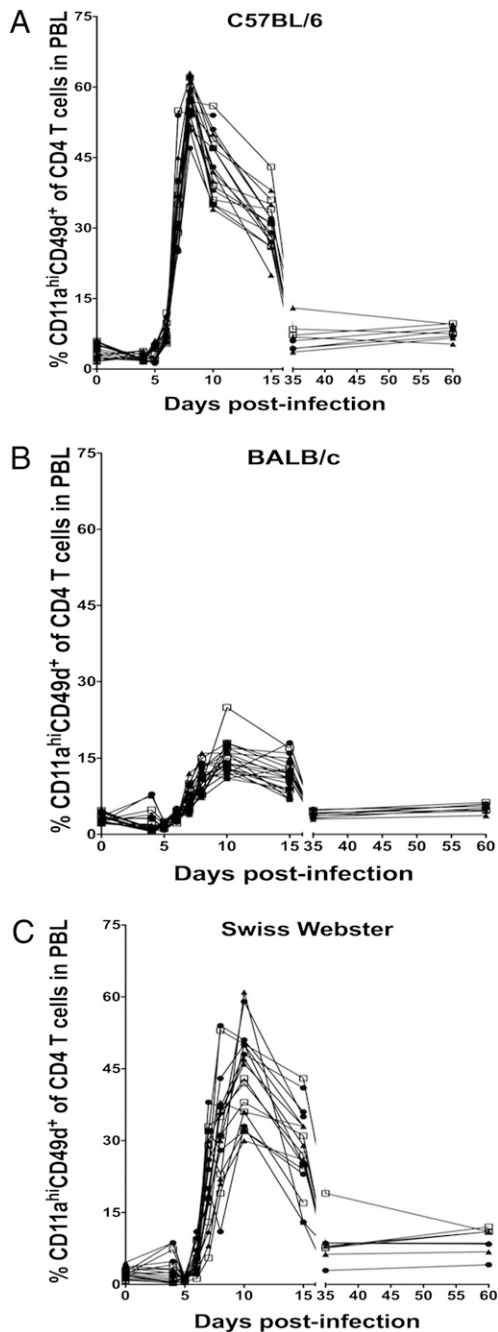


FIGURE 7. Tracking the kinetics and magnitude of the total LCMV-specific CD4 T cell response following infection. C57BL/6, BALB/c and Swiss Webster mice were infected i.p. with LCMV, and PBLs were obtained at 0, 4, 5, 6, 7, 8, 10, 15, 35, and 60 d postinfection. The percentage of CD11a^{hi}CD49d⁺ CD4 T cells (CD4⁺CD90.2⁺) was examined in C57BL/6 (A), BALB/c (B), and Swiss Webster mice (C). Combined results are shown from two independent experiments with 17–18 mice per group.

Kinetics of the CD4 T cell response in immune mice following heterologous infection

Our data indicate that the increase of CD11a cell surface expression combined with the induction of CD49d expression following infection can be used to track Ag-specific CD4 T cells. However, the above experiments examined naive mice infected with a single pathogen in contrast to hosts that would have a previous infection history. Therefore, we sought to determine whether CD11a and CD49d can be used to track newly activated CD4 T cells in an animal that has been previously exposed to other pathogens. We

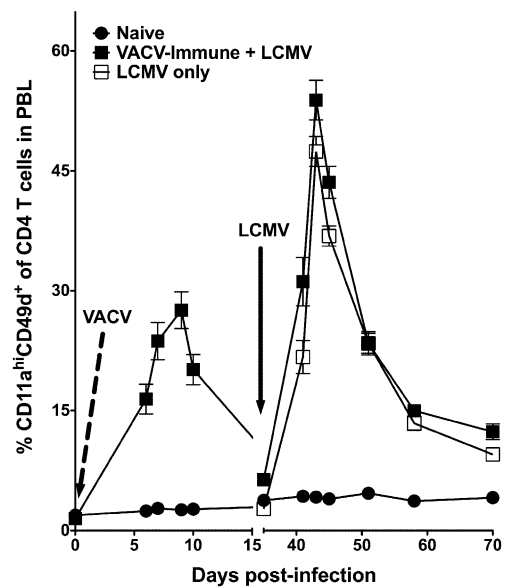


FIGURE 8. Tracking newly generated Ag-specific CD4 T cells following heterologous infection. C57BL/6 mice were infected i.p. on day 0 with VACV (dashed line), PBL was obtained at the indicated days postinfection and the frequency of CD11a^{hi}CD49d⁺ CD4 T cells (CD4⁺CD90.2⁺) was examined. At day 35 postinfection, these mice and naive mice were infected with LCMV i.p. (black arrow), PBL was obtained at the indicated days postinfection, and the percentage of CD11a^{hi}CD49d⁺ CD4 T cells (CD4⁺CD90.2⁺) was examined. Combined results are shown from two independent experiments with seven to eight mice per group. Error bars represent \pm SEM.

infected naive mice with VACV on day 0 and tracked the CD11a^{hi}CD49d⁺ CD4 T cells throughout the acute infection (Fig. 8). At day 35 postinfection, we infected the VACV-immune mice and additional age-matched naive mice (LCMV only) with LCMV. At the time of infection, ~2.5% of the CD4 T cells from the naive mice were CD11a^{hi}CD49d⁺, whereas ~6.5% of the CD4 T cells in the VACV-immune mice were CD11a^{hi}CD49d⁺ because of the presence of virus-specific memory CD4 T cells. Importantly, at the peak of the LCMV infection (i.e., day 43; day 8 postinfection with LCMV), the total magnitude of the primary LCMV-specific CD4 T cell response (i.e., newly generated CD11a^{hi}CD49d⁺ CD4 T cells) in the VACV-immune and LCMV only mice was ~46% (Fig. 8). Furthermore, the frequency of LCMV-specific memory cells (i.e., day 70; day 35 postinfection with LCMV) was ~6.5%, indicating that CD11a and CD49d cell surface expression can be used to track newly generated memory cells after a heterologous infection. These results indicate that, as long as the baseline frequency of the CD11a^{hi}CD49d⁺ memory CD4 T cells within a host is known, the kinetics of a newly generated Ag-specific CD4 T cell response can be accounted for using CD11a and CD49d surface expression.

Discussion

Virus-specific CD8 T cell responses have been examined extensively; however, a number of obstacles hamper the analysis of virus-specific CD4 T cells. Several epitope-mapping studies have indicated that CD4 T cell epitopes are commonly spread throughout the entire viral proteome, and quantitation using immunodominant epitopes often accounts for only a small percentage of the activated CD4 T cells (5, 11, 25, 26). These combined factors make studying the CD4 T cell response using common methods difficult, such as ICS or ELISPOT following in vitro peptide stimulation or MHC Class II tetramers. Therefore, we

worked to identify potential cell surface markers that would reliably identify Ag-specific CD4 T cells after a viral infection.

In this study, we demonstrate that upon acute LCMV infection, LCMV-Tg SMARTA CD4 T cells increase the cell surface expression of CD11a and induce expression of CD49d (Fig. 1). Expression of these integrins remains increased on memory CD4 T cells compared with naive SMARTA CD4 T cells (Fig. 1). To determine whether these markers are modulated in a similar manner on a polyclonal population of CD4 T cells, we examined the cell surface expression pattern of CD11a and CD49d on endogenous CD4 T cells following LCMV infection. We show that after acute LCMV infection, endogenous CD4 T cells from both the spleen and lungs, like SMARTA CD4 T cells, are CD11a^{hi}CD49d⁺ and these cells remain CD11a^{hi}CD49d⁺ into memory (Fig. 2). It is important to note that several previous studies have reported the increased cell surface expression of CD11a or the induction of CD49d expression on acute and memory CD4 T cells following infection. For example, previous studies have noted the induced expression of CD49d (i.e., VLA-4) on CD4 T cells following LCMV infection and that memory LCMV-specific CD4 T cells exhibit increased cell surface CD11a expression (27, 28). In addition, studies examining CMV-specific CD4 T cells following both acute and latent CMV infection reported induced cell surface expression of CD49d and increased cell surface expression of CD11a (29). Furthermore, CD11a cell surface expression is increased on acute and memory CD4 T cells after infection with recombinant VACV and after *Salmonella* infection (20, 25). In addition, Sendai virus-specific memory CD4 T cells express high levels of CD49d (28, 30). These studies further substantiate our data indicating that the combined use of CD11a and CD49d cell surface expression patterns can be used to track Ag-specific CD4 T cells following viral infections.

Although we demonstrate that CD11a and CD49d expression patterns can be used to track Ag-specific CD4 T cells at the peak of the response and into memory, it is important to note that there may be limitations to using these markers during the early stages of infection (i.e., prior to day 4 postinfection). Previous studies have shown that, following influenza virus infection, transferred naive CFSE-labeled influenza-specific TCR-Tg CD4 T cells express high levels of CD49d only after undergoing at least six cell divisions (31). Furthermore, other reports demonstrate that after OVA injection, OVA-specific DO11.10 CD4 T cells from the lymph nodes upregulate CD49d surface expression only after five to six cell divisions (32). Following LCMV infection, LCMV-specific SMARTA CD4 T cells do not undergo proliferation during the first 2 d of infection; however, by day 3 these cells have begun to undergo extensive proliferation, and by day 4 CFSE is completely diluted (33), suggesting that CD11a and CD49d can be used reliably to track Ag-specific CD4 T cells after day 4 postinfection. Consistent with this conclusion, we observed that SMARTA CD4 T cells do not induce CD49d cell surface expression until day 4 post-LCMV infection, whereas we observed increased expression of CD11a by this time (data not shown). The findings in the above studies suggest that the CD11a and CD49d cell surface expression patterns can be used to track Ag-specific CD4 T cells starting at least by day 4 after viral infection.

We demonstrate that modulation of CD11a and CD49d expression requires the presence of cognate Ag and is not altered because of either TLR stimulation or the inflammatory environment created by PV (Fig. 3). Importantly, virtually all of the GP66-80-specific CD4 T cells, as measured by either IFN- γ production following *in vitro* peptide stimulation or tetramer staining, are CD11a^{hi}CD49d⁺ (Fig. 4 and data not shown). Interestingly, following *in vitro* peptide stimulation using GP66-80, which accounts

for >90% of the total LCMV-response to known epitopes (data not shown), we are able to account for only 20–30% of the total CD11a^{hi}CD49d⁺ CD4 T cell population. Similarly, PMA/ionomycin stimulation of LCMV-specific memory CD4 T cells induces IFN- γ production only from ~55% of the CD11a^{hi}CD49d⁺ CD4 T cells (Fig. 6B). In an effort to account for all the CD11a^{hi}CD49d⁺ LCMV-specific CD4 T cells, we stimulated splenic and lung CD4 T cells with LCMV-infected dendritic cells. Interestingly, we observed a similar percentage of IFN- γ -producing splenic CD4 T cells after stimulation with LCMV-infected dendritic cells compared with stimulation with control GP66-80 pulsed dendritic cells (~7.5%; data not shown). A similar discrepancy has been described previously in the VACV model, in which ~20% of the CD4 T cells express CD11a, whereas ~3% of the CD4 T cells produced IFN- γ when stimulated with VACV-infected A20 B cells (25). Furthermore, previous studies have demonstrated that only 50–60% of SMARTA LCMV-Tg CD4 T cells produce IFN- γ following stimulation with the peptide GP61-80, suggesting that a large fraction of the Ag-specific effector CD4 T cells do not produce cytokine upon restimulation with their cognate Ag (33). These studies suggest that the use of CD11a and CD49d may provide a more accurate means to quantify the magnitude of the CD4 T cell response than can be obtained by examination of cytokine responses.

In addition, we demonstrate that effector CD4 T cells from the lung and BAL upregulate cell surface levels of CD11a and CD49d following acute RSV infection (Fig. 5A), indicating the usefulness of this approach in multiple infection models. Furthermore, virtually all the IFN- γ ⁺ CD4 T cells following G183-195 and F51-66 stimulation are CD11a^{hi}CD49d⁺ (Fig. 5B), demonstrating that the RSV-specific CD4 T cells exhibit a CD11a^{hi}CD49d⁺ expression pattern. However, it is important to note that CD4 T cells from the BAL exhibit decreased cell surface expression of CD11a by day 15 after RSV infection, but still retain CD49d expression (Fig. 5A). Downregulation of CD11a expression by memory CD4 T cells in the BAL has been described previously (24); however, the mechanism or biological significance of decreased CD11a expression in the lung airways is currently unclear. In contrast, we demonstrate that CD49d cell surface expression does not decrease on memory CD4 T cells in the lung airway by day 15 (Fig. 5A). Furthermore, preliminary data from our laboratory suggest that the majority of CD4 T cells (~80%) from the BAL at day 30 still exhibit cell surface expression of CD49d (data not shown), thus indicating that CD49d expression, but not CD11a, can be used to track memory CD4 T cells within the lung airways.

Similar to the LCMV-specific effector CD4 T cells, all LCMV-specific memory CD4 T cells are CD11a^{hi}CD49d⁺, because sorted CD11a^{hi}CD49d⁺ but not CD11a^{lo}CD49d⁻ CD4 T cells from LCMV-immune mice proliferate upon secondary LCMV infection (Fig. 6A, 6B). Furthermore, virtually all the IFN- γ producing cells from LCMV-immune mice are CD11a^{hi}CD49d⁺ (Fig. 6C). Additionally, we demonstrate that upon heterologous challenge of VACV-infected mice with LCMV, at the peak of infection the overall magnitude of the primary LCMV-specific CD4 T cell response (i.e., newly activated CD11a^{hi}CD49d⁺ CD4 T cells) is similar to LCMV-infected naive mice at the peak of the response (~45%) and into memory (~6.5%; Fig. 8). These data indicate that CD11a and CD49d can be used to track memory virus-specific CD4 T cells, as well as newly generated virus-specific CD4 T cells in an animal that has been exposed previously to a different pathogen as long as the baseline frequency of CD11a^{hi}CD49d⁺ CD4 T cells is known prior to infection. These data are important in the context of the potential use of CD11a and CD49d expression patterns to track Ag-specific CD4 T cell responses in

human hosts that have likely been exposed to numerous pathogens prior to examination. Interestingly, a recent study has demonstrated that activated, cytokine-producing human CD4 T cells are CD49d⁺ (34). Furthermore, naive human CD4 T cells are CD11a^{lo} and become CD11a^{hi} upon activation and retain elevated CD11a expression into memory (26). These findings suggest that the combination of CD11a and CD49d may be a useful tool to examine the kinetics and magnitude of CD4 T cell responses following either vaccination or infection in humans.

Although several studies have previously noted the expression of CD11a or CD49d on either activated or memory CD4 T cells following infection (20, 24, 28–32), to our knowledge, this study demonstrates for the first time that CD11a and CD49d can be used in combination to track Ag-specific CD4 T cells following viral infection. Previous studies examining the magnitude of the CD4 T cell response in C57BL/6 mice following LCMV infection have shown that ~10% of CD4 T cells at the peak of LCMV infection are virus specific (5, 21, 23). In contrast, our analyses measuring changes in cell surface expression patterns of CD11a and CD49d demonstrates that up to 50% of the CD4 T cells are virus specific at the peak of the LCMV-induced immune response, indicating that the magnitude of the Ag-specific CD4 T cell response is much greater than previously recognized. It is important to note that although more than 50% of the CD4 T cells following LCMV infection of C57BL/6 mice are Ag specific, ~15% of the CD4 T cells within BALB/c mice were Ag-specific (Fig. 7). These data, along with the large degree of variability observed within the outbred Swiss Webster mice following LCMV infection, suggest that the genetic background of the animal greatly controls the magnitude and kinetics of the CD4 T cell response. Interestingly, similar results have been described for CD8 T cells following infection with *Listeria monocytogenes* (6). Much recent work has focused on determining the Ag specificity of CD4 T cells against LCMV and other viruses in different strains of mice. However, until now no technique has been available to accurately quantify the total number of virus-specific CD4 T cells.

We believe that the combined use of CD11a and CD49d will grant, for the first time, to our knowledge, the ability to accurately track the kinetics and magnitude of the entire endogenous CD4 T cell response from the expansion phase into memory. Importantly, we show that CD11a and CD49d cell surface expression patterns can be used to track virus-specific CD4 T cells in outbred populations. These data suggest the potential in using the modulation of CD11a and CD49d expression to verify vaccine-induced responses by determining the magnitude of Ag-specific CD4 T cell responses in humans following immunization.

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Disclosures

The authors have no financial conflicts of interest.

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