CD62L (L-Selectin) Down-Regulation Does Not Affect Memory T Cell Distribution but Failure to Shed Compromises Anti-Viral Immunity

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CD62L (L-Selectin) Down-Regulation Does Not Affect Memory T Cell Distribution but Failure to Shed Compromises Anti-Viral Immunity

Hannah Richards,* M. Paula Longhi,* Kate Wright,* Awen Gallimore,2* and Ann Ager2,3,*†

The down-regulation of CD62L that accompanies T lymphocyte activation is thought to redirect cells away from lymph nodes to sites of infection. In this study, CD62L was maintained on Ag-activated T cells and their distribution, and ability to clear pathogen from peripheral sites determined. CD62L was down-regulated on Ag-specific CD8 T cells in lungs of C57BL/6 mice but maintained in CD62L transgenic mice at day 8 after influenza infection. However, the numbers of influenza-specific CD8 T cells recruited were similar in CD62L transgenic and C57BL/6 mice. Memory CD8 T cell numbers in the lungs and noninvolved organs 100 days after primary infection were similar in CD62L transgenic and C57BL/6 mice, despite differing CD62L expression. Transgenic mice expressing wild-type CD62L cleared a recombinant vaccinia virus expressing an influenza-derived CD8 T cell epitope as efficiently as C57BL/6 mice. However, transgenic mice expressing a protease resistant mutant of CD62L showed significantly delayed viral clearance, despite normal CTL generation and the presence of CD107a and IFN-γ expressing influenza-specific CD8 T cells. These results demonstrate that CD62L down-regulation is not required for CD8 memory cells to home to sites of infection. However, their ability to clear virus is significantly compromised if CD62L shedding is abrogated. The Journal of Immunology, 2008, 180: 198–206.

Memory T cells are widely distributed throughout secondary lymphoid organs such that recall responses, like primary responses, can be initiated to pathogens irrespective of their route of entry (1, 2). The recent discovery of the widespread distribution and persistence of memory T cells in nonlymphoid organs, including those not involved in the primary infection, suggests that many nonlymphoid, as well as lymphoid organs, may be armed to mount a rapid defense to invading pathogens (3).

In humans, CD45RO T cells have been subdivided according to differential expression of lymph node (LN)4 homing molecules into central memory cells, which coexpress CD62L and CCR7, and effector memory cells, which do not (4). Studies in mice using CD62L (L-selectin) and/or CCR7 support the existence of functionally distinct central and effector memory T cell populations and demonstrate preferential distribution of CD62L+ and/or CCR7+ memory cells in nonlymphoid organs, although CD62L and CCR7 expressing memory cells can also be found in nonlymphoid organs (3, 5, 6). Rapid down-regulation of CD62L on activated T cells has been shown to correlate with loss of LN entry (7) and we, and others, have shown that Ag-activated T cells which maintain CD62L expression retain the ability to enter LN (8, 9). These results, together with the observation that T cells isolated from inflammatory sites express low levels of CD62L (10–12), have suggested that down-regulation of CD62L may be required to redirect activated T cells away from LN, however this hypothesis has not been tested directly. Although the primary function of CD62L is in LN homing, expression levels correlate directly with effector function of CD8 T cells (13) and signaling via CD62L has been shown to costimulate TCR proliferation (14). It is, therefore, possible that the level of CD62L expression may also regulate the proliferation, differentiation and/or survival of Ag-activated T cells.

In this study the relationship between CD62L expression and the migration and function of memory T cells has been explored by maintaining CD62L levels following TCR engagement. TCR-induced changes in CD62L expression result from altered gene transcription as well as altered rates of ectodomain proteolysis (shedding) from the cell surface (15) but their relative contributions to the activity of memory T cells are not known. We have used transgenic mice expressing either wild-type (L8) or a shedding-resistant mutant (LΔP98) of mouse CD62L and the heterologous promoter hcd2 to direct and maintain expression on T cells (8). Using a model of protective immunity to viral infection we have specifically addressed the following questions: Is CD62L down-regulation required for Ag-specific T cells to migrate to sites of infection; Is CD62L down-regulation required for memory T cells to distribute to nonlymphoid organs; Does CD62L expression regulate the differentiation or function of anti-viral CD8 T cells.

Materials and Methods

Mice

C57BL/6 (B6) were bred and maintained at Biomedical Services (Cardiff University, U.K.). Transgenic mice expressing either wild-type murine...
CD62L (L<sup>cd</sup>; previously called WT (8)) or a noncleavable mutant (L<sup>np</sup>) on a B6 CD62L<sup>hi</sup> background (8) were bred at the MRC National Institute for Medical Research (NIMR, London) and maintained at Biomedical Services (Cardiff, U.K.). All experiments were conducted according to institutional guidelines and U.K. Home Office regulations.

**Viruses and virus infection**

Influenza A virus strain E61-13-H17 (H17; H3N2) (16), amplified in embryonated chicken eggs, was obtained from NIMR. The virus was titrated from allantoic fluid by hemagglutination assay. Groups of 3–5 mice were infected intranasally (i.n.) with 20 hemagglutination units (HAU) of influenza virus in 20 μl of PBS. After 8 and 100 days mice were killed, the lung vasculature perfused with ice-cold PBS/2% EDTA, and lungs and draining lymph nodes (LiDLN), ovary and draining LN (OdDLN), and spleens collected for immunostaining. Recombinant vaccinia virus (rVV) expressing either an immunodominant CTL epitope derived from influenza nucleoprotein (rVVNP) (17) or the melanocyte Ag Trp2 (rVVTrp2) (18) have previously been described. For detection of protective immunity to rVV, influenza immune mice were injected i.p. with 5 × 10<sup>6</sup> PFU. Ovaries were removed 5 or 8 days later, and a single ovary from each mouse analyzed for rVV titers, as described previously (19). The remaining ovaries, as well as ODLN and spleen were harvested for immunostaining and in vitro CTL assays. In some experiments influenza-immune, rVVNP challenged mice were transfused with NP68 peptide-loaded splenocytes to measure CTL activity in vivo.

**Cell staining and flow cytometry**

Spleen, LNs, lungs, and ovaries from individual mice or pooled from up to 5 mice were disrupted and passed through 70-μm cell strainers (Falcon; BD Biosciences) to obtain single cell suspensions. In some experiments equal aliquots of pooled lungs and ovaries were incubated with and without 0.1% collagenase D (Clostridium histolyticum; Roche) in RPMI 1640 for 30 min at 37°C before obtaining cell suspension. To analyze influenza-specific CD8 T cells in the organs of mice infected with influenza virus and/or rVV, cells were incubated with fluorescently labeled soluble H2-Db tetramers containing the immunodominant NP68-specific MHC class I tetramer-PE comprising H2-Db and H17 derived nucleoprotein peptide epitope NP<sub>360–374</sub> (NP68) peptide. CD62L transgenic mice have been backcrossed to C57BL/6 (B6) CD62L<sup>hi</sup> to eliminate endogenous CD62L and transgenic lines expressing levels of either wild type (L<sup>cd</sup>; previously called WT (8)) or shedding resistant CD62L (ΔL<sup>np</sup>) comparable with endogenous CD62L in B6 were used for this study (8). As reported previously, the total numbers of CD8 T cells in secondary lymphoid organs of CD62L transgenic mice were similar; however, L<sup>np</sup> mice showed slightly more CD62L<sup>hi</sup> cells because constitutive shedding is abrogated (Fig. 1A) (8). Therefore, CD62L<sup>hi</sup> expressing cells were routinely analyzed according to staining of CD8 T cells in L<sup>np</sup> mice (Fig. 1). Since CD62L expression is restricted to T cells in the transgenic mice (8) we have included B6 mice for control responses to influenza.

**CTL assays**

**In vitro.** Spleen cells harvested from influenza virus infected mice at day 8, or from rVVNP challenged influenza-immune mice at day 8, were plated at 4 × 10<sup>6</sup> cells/well in RPMI 1640 supplemented with 10% FCS (RPMI 10) and cocultured with 1 × 10<sup>6</sup> M NP68 peptide (ASNENMDAM/H<sub>11003</sub> (tet) and Cy5-CD8 (5H10; Caltag Laboratories) and counterstained using FITC-CD62L (MEL-14; BD Pharmingen), Alexa Fluor 610-IFN-γ (XMG1.2; Caltag) and/or FITC-CD107a (ID4B; BD Pharmingen) Ab. After washing and fixation, fluorescence was analyzed by flow cytometry (FACSCalibur), and tetramer-positive CD8 T (CD8<sup>+</sup> tet) cells were subsequently identified using CellQuest Pro Software (BD Biosciences). Numbers of CD8<sup>+</sup> tet per organ were calculated from the total number of events in a lymphocyte/lymphoblast gate.

**In vivo.** Spleen cells harvested from influenza virus infected mice at day 8, or from rVVNP challenged influenza-immune mice at day 8, were plated at 4 × 10<sup>6</sup> cells/well in RPMI 1640 supplemented with 10% FCS (RPMI 10) and cocultured with 1 × 10<sup>6</sup> M NP68 peptide (ASNENMDAM/H<sub>11003</sub>; Invitrogen) pulsed irradiated splenocytes in 24-well plates, as described previously (19). On day 5, cells from 4 wells were collected, washed, and resuspended in 600 μl of RPMI 10. Cell suspensions were serially diluted 3-fold, plated in duplicate in 96-well plates and incubated with 1 × 10<sup>4</sup> Cr-labeled target cells (B16) labeled either with NP68 or with an irrelevant H-2D<sup>b</sup> restricted peptide from LCMV, gp33, for 4 h at 37°C. For 30 min on ice. After further washing, cells were fixed and permeabilized using BD Cytofix/cytoperm (BD Biosciences) according to manufacturer’s instructions and stained with anti-mouse IFN-γ APC (BD Pharmingen) and anti-mouse TNF-α Alexa Fluor 488 (MP6-XT22; BD Pharmingen). Data were collected and analyzed as described above.

**Statistics**

Results are means ± SEM. The Mann-Whitney U test for statistical significance was evaluated using GraphPad Prism 3.0 (*, p < 0.05; ***, p < 0.001).

**Results**

CD62L<sup>hi</sup> Ag-specific T cells distribute normally to sites of inflammation

To determine the effect of maintained CD62L expression on T cell infiltration of infected tissues, CD62L transgenic mice were infected intranasally (i.n.) with E61-13-H17 (H17; H3N2) influenza A virus. CD8 T cells in lung, draining LN (dLN) and spleen were monitored on day 8 postinfection when influenza-specific CD8 T cells are readily seen in the spleens of H17 virally infected mice (20). Influenza-specific CD8 T cells were detected using soluble H2-D<sup>b</sup> tetramers containing the immunodominant nucleoprotein epitope NP<sub>360–374</sub> (NP68) peptide. CD62L transgenic mice have been backcrossed to C57BL/6 (B6) CD62L<sup>hi</sup> to eliminate endogenous CD62L and transgenic lines expressing levels of either wild type (L<sup>cd</sup>; previously called WT (8)) or shedding resistant CD62L (ΔL<sup>np</sup>) comparable with endogenous CD62L in B6 were used for this study (8). As reported previously, the total numbers of CD8 T cells in secondary lymphoid organs of CD62L transgenic mice were similar; however, L<sup>np</sup> mice showed slightly more CD62L<sup>hi</sup> cells because constitutive shedding is abrogated (Fig. 1A) (8). Therefore, CD62L<sup>hi</sup> expressing cells were routinely analyzed according to staining of CD8 T cells in L<sup>np</sup> mice (Fig. 1). Since CD62L expression is restricted to T cells in the transgenic mice (8) we have included B6 mice for control responses to influenza.

CD62L expression on H2-D<sup>b</sup> NP68-specific (tet<sup>+</sup>) CD8 T cells infiltrating the lungs of influenza-infected B6 mice was markedly reduced, as expected (Fig. 1B). In contrast, all CD8<sup>+</sup> tet<sup>+</sup> cells in lungs of ΔL<sup>np</sup> were CD62L<sup>hi</sup>, as were the majority of influenza-specific cells in lungs of L<sup>cd</sup> mice (Fig. 1B). Influenza-specific CD8 T cells in secondary lymphoid organs of B6 mice also showed down-regulation of CD62L. The effects were greater in the spleen where <10% tet<sup>+</sup> cells expressed high levels of CD62L, and expression in draining LN ranged from 25 to 80% CD62L<sup>hi</sup> (Fig. 1C). CD62L expression was maintained in lymphoid organs of CD62L transgenic mice, particularly in ΔL<sup>np</sup> mice, where all CD8<sup>+</sup> tet<sup>+</sup> cells were CD62L<sup>hi</sup> (Fig. 1C).

These results demonstrate that down-regulation of CD62L expression in B6 mice following TCR engagement in vivo is largely controlled by gene silencing and that it is overridden in CD62L transgenic mice by the heterologous hcd2 promoter, as has been shown for other transgenes (21, 22). A comparison of L<sup>cd</sup> and ΔL<sup>np</sup> mice revealed a lesser, but significant, role for shedding since CD62L was down-regulated on ∼20% of influenza-specific CD8 T cells in lymphoid organs of L<sup>cd</sup> mice, which undergo normal TCR-induced shedding (8), but not in ΔL<sup>np</sup> mice (Fig. 1C). Despite maintained expression of CD62L on CD8 T cells in both L<sup>cd</sup> and ΔL<sup>np</sup> mice, similar numbers of influenza-specific cells were harvested from infected lungs as from B6 mice (Fig. 1D). Furthermore, these cells had penetrated the lung parenchyma since...
the vasculature had been perfused before collection. The numbers of influenza-specific CD8 T cells in spleens of CD62L transgenic mice did not differ significantly from B6 mice, however there was a 3-fold increase in the draining LN of L/H9004Ptg mice. At day 8 the frequency of CD8 T cells responding to NP68 was highest in the lungs but did not differ significantly between CD62L transgenic and B6 mice (Table I). Together these results suggest that the inability to down-regulate CD62L does not prevent migration of Ag-activated CD8 T cells to sites of infection, such as influenza-infected lungs. In addition, maintained CD62L expression did not affect the total numbers of influenza-specific CD8 T cells present at this time point. Further analysis of CD8 T cells in lungs and spleen showed that, upon restimulation, the numbers of IFN-γ secreting cells were comparable and CTLs could be generated from spleens of B6, Ltg and L/H9004Ptg mice following 5-day stimulation with NP68-pulsed APCs (data not shown).

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Maintained expression of CD62L on influenza-specific CD8 T cells does not affect recruitment to the site of infection. B6, Ltg, and L/H9004Ptg mice were infected i.n. with 20 HAU of influenza virus. Eight days after infection, cells isolated from the lungs draining LN (LdLN) and spleens were stained with Abs to CD8, CD62L, and with NP68 tetramers and compared with splenocytes from uninfected mice. A, Representative FACS plots showing CD62L staining of CD8 cells in spleens of uninfected B6, Ltg, and L/H9004Ptg. B, Representative FACS plots showing CD62L and tetramer staining of CD8 cells in lungs of infected B6, Ltg, and L/H9004Ptg mice. Numbers are mean percentages of CD62Lhigh CD8 cells (A) and CD8 tet cells (B) in three to five mice. The percentage of CD62Lhigh (C) and total numbers (D) of CD8 tet cells in lungs, LdLN and spleens of B6 (squares), Ltg (triangles), and L/H9004Ptg (circles) mice are also shown. Each symbol represents an individual mouse and data are from one representative experiment (C) or a summary of two independent experiments (D) using groups of at least five mice. Solid lines represent the means within each group. *, p < 0.05.

<table>
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<tr>
<th>Days after Infection</th>
<th>Mouse</th>
<th>Lungs</th>
<th>Spleen</th>
<th>Lung dLN</th>
<th>Ovary</th>
<th>Ovary dLN</th>
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<tr>
<td>Flu day 8</td>
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<td>4.8 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.3 ± 0.2</td>
<td>0.9 ± 0.4</td>
<td>NT&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>0.7 ± 0.1</td>
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<td>NT</td>
<td>NT</td>
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<tr>
<td></td>
<td>L/H9004Ptg</td>
<td>4.4 ± 1.4</td>
<td>1.4 ± 0.2</td>
<td>2.9 ± 0.9</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>Flu day 100</td>
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<td>0.6 ± 0.1</td>
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<td>1.3 ± 0.5</td>
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<tr>
<td></td>
<td>Ltg</td>
<td>0.8 ± 0.4</td>
<td>0.7 ± 0.1</td>
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<td></td>
<td>L/H9004Ptg</td>
<td>2.4 ± 1.4</td>
<td>0.8 ± 0.1</td>
<td>1.0 ± 0.3</td>
<td>1.0 ± 0.6</td>
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<tr>
<td>rVVNP day 5</td>
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<td>NT</td>
<td>23.3 ± 4.9</td>
<td>3.7 ± 1.7</td>
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<tr>
<td></td>
<td>Ltg</td>
<td>NT</td>
<td>4.3 ± 0.9</td>
<td>NT</td>
<td>27.1 ± 2.7</td>
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<td>L/H9004Ptg</td>
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<td>5.4 ± 0.7</td>
<td>NT</td>
<td>27.4 ± 3.5</td>
<td>1.6 ± 0.7</td>
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<sup>a</sup> Results are mean percent of CD8 tet ± SEM (n = 5–17).
<sup>b</sup> NT, Not tested.
CD62L<sup>high</sup> memory CD8 T cells distribute normally to nonlymphoid organs

Next, we determined whether CD62L expression controlled the distribution of influenza-specific memory cells following clearance of the primary infection. Mice were sacrificed ~100 days post infection and organs analyzed for the presence of influenza-specific CD8 T cells using NP68 tetramer. CD8<sup>Tet</sup> cells were found in the lungs, LN draining the site of infection, spleens, as well as in distant LN of B6 and CD62L transgenic mice. Although numbers of CD8<sup>Tet+</sup> cells detected were low, NP68 tetramer did not bind to CD8 T cells in naive B6 mice whereas it bound to the majority of CD8 T cells in F5 mice expressing a transgenic TCR for NP68. In addition, an irrelevant tetramer comprising H2-D<sup>β</sup> and LCMV derived peptide gp33 peptide did not bind to CD8 T cells in F5, naive or influenza-infected mice (Fig. 2A). These results indicate that the binding of NP68 tetramer can be used to identify and enumerate low numbers of Ag-specific memory CD8 T cells in influenza-immune mice.

The total numbers of CD8<sup>Tet+</sup> T cells in lymphoid and nonlymphoid organs were not significantly different between B6 and CD62L transgenic mice, apart from LN which tended toward higher numbers, particularly in L<sub>ΔP<sup>pp</sup></sub> (Fig. 2B). The frequency of NP68 specific CD8 T cells in the lungs was markedly reduced at day 100 but, as found during the infection phase, did not differ between CD62L transgenic and B6 mice (Table I). Influenza-immune CD8 T cells were also found at sites not involved in the primary infection, as has been reported for other virus-specific memory cells (3), such as the ovaries (Fig. 2B; Table I). The total number and frequency of memory CD8 cells in ovaries were similar between B6 and CD62L transgenic mice (Fig. 2B, Table I) and the frequency in ovaries was similar to those found in the lungs and secondary lymphoid organs (Table I). The low numbers of CD8<sup>Tet+</sup> T cells at 100 days after primary infection precluded a definitive analysis of CD62L expression in all organs; however, the majority of NP68-specific CD8 T cells in ovaries of CD62L transgenic mice were CD62L<sup>high</sup> and in B6 mice were CD62L<sup>low</sup> (Fig. 2C). The numbers of NP68-specific CD8 T cells detectable during primary infection were lower than those reported using HKx31 strain of influenza, particularly in the lungs (23), although the total number of CD8 T cells and the frequency of NP68 tetramer binding CD8 cells were similar to those previously reported by us for H17 (19, 20) and for the HKx31 strain (24). To increase the yield of memory CD8 T cells from solid organs we employed collagenase digestion before cell isolation. Although the yield of CD8<sup>Tet</sup> cells from lungs of influenza immune mice was increased ~4-fold, collagenase digestion did not increase the yield of CD8 memory T cells from ovaries (data not shown). However, the frequency of CD8<sup>Tet+</sup> cells in the lungs was not altered following collagenase digestion; thus the number of CD8<sup>Tet+</sup> cells in the lungs reported herein is an underestimate.

These results demonstrate clearly that, in addition to the site of primary infection, influenza-specific memory CD8 T cells are distributed widely to noninvolved organs and that CD62L down-regulation is not required for the widespread distribution of CD8 memory T cells either to nonlymphoid tissues or to sites of infection.

CD62L shedding promotes viral clearance by memory CD8 T cells

To assess directly the potential role of CD62L in regulating T cell function we measured the ability of influenza-specific CD8 memory T cells to clear a secondary infection. To avoid the potential
complications of studying recruitment to the primary site of challenge, we chose to study the ability of influenza-immune mice to clear recombinant vaccinia virus (rVV) expressing an influenza CTL epitope. After systemic infection, vaccinia virus replicates to high titers in ovaries and clearance of rVV expressing CTL epitopes has been used to measure CD8 T cell memory in mice (19, 25).

One hundred days after infection with influenza, mice were challenged with vaccinia virus expressing either NP68 peptide (rVVNPP) or an irrelevant melanocyte Ag (rVVTrp2) and tissues harvested at the peak of infection, day 5. Viral titers in influenza-immune B6 mice receiving rVVNPP were significantly lower than in either nonimmune B6 mice or influenza-immune rVVTrp2 challenged mice, reflecting clearance by NP68 specific CTL (Fig. 3 and data not shown). Viral titers in ovaries of Ltg mice were slightly, but not significantly, lower than those of B6 indicating similar efficiency at vaccinia clearance. In marked contrast to rapid clearance of virus in Ltg mice, rVVNPP clearance was significantly reduced in L/H9004Ptg mice. Some NP68 specific T cell memory was evident however, since viral titers in rVVNPP challenged L/H9004Ptg mice were significantly lower than in rVVTrp2 challenged or in naive L/H9004Ptg mice (Fig. 3 and data not shown). However by day 8, viral titers were undetectable in ovaries of rVVNPP challenged, influenza-immune L/H9004Ptg mice, which indicated delayed kinetics.

FIGURE 3. CD62L shedding promotes efficient viral clearance by memory CD8 T cells. Influenza-immune B6, Ltg, and L/H9004Ptg mice were generated by i.n. infection with 20 HAU of influenza virus, and 100 days later, mice were challenged with recombinant vaccinia virus expressing a MHC class I-restricted peptide epitope derived from the influenza nucleoprotein (rVVNPP) or an irrelevant Ag (rVVTrp2). Mice were sacrificed 5 days after infection and ovaries collected. Ovaries were homogenized and viral titers determined by plaque assay. Symbols represent the number of PFU per ovary in individual B6 (squares), Ltg (triangles), and L/H9004Ptg (circles) immune mice challenged with rVVNPP (closed symbols) and rVVTrp2 (open symbols) from two independent experiments using groups of five mice. ***. p < 0.001; *. p = 0.02.

FIGURE 4. Memory CD8 T cells are present in ovaries of rVVNPP challenged CD62L shedding resistant mice. Influenza-immune B6, Ltg and L/H9004Ptg mice were generated by i.n. infection with 20 HAU of influenza virus, and 100 days later, mice were either challenged with recombinant vaccinia virus expressing a MHC class I-restricted peptide epitope derived from the influenza nucleoprotein (rVVNPP) or remained unchallenged. Mice were sacrificed 5 days later and OdLNs and spleens were harvested for cell counts and were stained for CD8, CD62L, and with NP68-tetramers. A, Total numbers of CD8<sup>+</sup> tet<sup>+</sup> cells in organs of rVVNPP challenged B6 (squares), Ltg (triangles), and L/H9004Ptg (circles) mice. Each symbol represents an individual mouse and bars show means. ***. p < 0.001 (B) Representative FACS plots of CD62L vs tetramer staining on CD8<sup>+</sup> cells isolated from each organ are shown.
rather than an inability to clear virus. Primary responses to vaccinia infection in LΔP^pe mice were not defective since titers in rVVTrp2 challenged flu-immune and rVVNP challenged naive mice were similar to those in L^ge and B6 mice at day 5 and day 8 (data not shown). Since the only difference between L^ge and LΔP^pe mice is the ability of the T cells to down-regulate CD62L by shedding, these results suggest a significant role for CD62L shedding in the control of infection by memory CD8 T cells.

Recruitment and activation of Ag-specific CD8 T cells in ovaries of LΔP^pe mice is normal

The numbers of memory CD8 T cells present in ovaries before infection with rVVNP did not differ between LΔP^pe and L^ge mice; we determined whether the delay in viral clearance in LΔP^pe mice was simply due to lack of recruitment of NP68-specific CD8 T cells to the ovary. As found during primary influenza infection, the lack of CD62L down-regulation on Ag-specific CD8 T cells in LΔP^pe mice did not prevent their recruitment to the site of inflammation, in this case the ovary. At day 5, there was a trend toward enhanced numbers of CD8^+ tet^+ in LΔP^pe mice and, to a lesser extent, in L^ge mice in comparison with B6 mice. This trend was also evident in the spleens of CD62L transgenic mice (Fig. 4A). The trend for enhanced numbers in LΔP^pe mice could be explained by the ongoing struggle to limit infection, as viral titers are high at this time point. CD62L^high and CD62L^low influenza-specific CD8 T cells were detectable in the ovary, draining LN and spleens of L^ge mice at day 5 (Fig. 4B), indicating that CD62L expression on activated memory CD8 T cells is regulated by proteolytic shedding. Since the recruitment of memory CD8 T cells to ovaries during ongoing infection was not reduced in LΔP^pe mice, we measured the cytotoxic capacity of these cells.

The numbers of lymphocytes that can be harvested from ovaries precluded their analysis in direct ex vivo CTL assays. However, there were no obvious differences in the generation of NP68-specific CTLs from spleen cells of LΔP^pe mice in comparison with L^ge mice, although B6 mice were slightly more efficient (Fig. 5A). To determine whether the delayed clearance of rVVNP was attributable to lack of differentiation of CTL effectors in vivo we measured killing of NP68 peptide-loaded target cells in these mice. As shown in Fig. 5B, LΔP^pe mice showed 98% killing of NP68-loaded target cells in the spleen at day 5 following rVVNP challenge, similar to that of L^ge and B6, demonstrating clearly that CTL can differentiate normally in LΔP^pe mice. It was not possible to determine whether CTL effectors were present in the infected ovary at day 5 since CFSE-labeled target and control cells did not enter the ovary in significant numbers even up to 24 h following administration (data not shown). Cell surface expression of CD107a correlates directly with degranulation and release of cytolytic molecules, which indicates potential killing capacity of CD8 T cells; we therefore used this marker to assess the functional capacity of memory CD8 T cells in the ovary. In vitro stimulation of CD8^+ tet^+ cells with specific peptide resulted in down-regulation of the TCR and loss of tetramer binding. However, direct staining with anti-CD107a ex vivo showed that influenza-specific memory
of rVVNPP infection we stained ovary infiltrates for cell surface influenza-immune, rVVNPP challenged mice following re-stimulation in vitro with 1 μM NP68 peptide or irrelevant Ag, Trp2. Results are from ovaries pooled from two mice. B. Cells were stained directly ex vivo with fluorescently labeled soluble MHC class I tetramer, anti-CD8 and anti-IFN-γ, and evaluated by FACS. The geometric mean fluorescence intensity (MFI) of IFN-γ on CD8 T cell populations is given for rVVNPP challenged (closed symbols) and unchallenged (open symbols) mice. Each symbol represents a single mouse and the solid lines indicate means within the group. Data are from one to four independent experiments using groups of at least three mice. *, p < 0.05.

FIGURE 6. Cytokine expression by memory CD8 T cells in rVVNPP challenged CD62L shedding resistant mice. Influenza-immune B6, Ltg, and LΔP mice were generated by i.n. infection with 20 HAU of influenza virus, and 35–100 days later, mice were either challenged with recombinant vaccinia virus expressing a MHC class I-restricted peptide epitope derived from the influenza nucleoprotein (rVVNPP) or remained unchallenged. Mice were sacrificed 5 days later, and ovaries, draining LN, and spleens were harvested. A. Intracellular IFN-γ and TNF-α expression by CD8 T cells from ovaries of influenza-immune, rVVNPP challenged mice following re-stimulation in vitro with 1 μM NP68 peptide or irrelevant Ag, Trp2. Results are from ovaries pooled from two mice. B. Cells were stained directly ex vivo with fluorescently labeled soluble MHC class I tetramer, anti-CD8 and anti-IFN-γ, and evaluated by FACS. The geometric mean fluorescence intensity (MFI) of IFN-γ on CD8 T cell populations is given for rVVNPP challenged (closed symbols) and unchallenged (open symbols) mice. Each symbol represents a single mouse and the solid lines indicate means within the group. Data are from one to four independent experiments using groups of at least three mice. *, p < 0.05.

CD8 T cells in ovary, draining LN and spleens of B6 mice expressed readily detectable levels of CD107a which were ~3-fold higher than on influenza-specific memory cells before rVVNPP challenge (Fig. 5C). CD107a was also up-regulated in all organs of CD62L transgenic mice but the extent of up-regulation was greatest in ovaries of LΔP mice.

Although CTL differentiation is normal in LΔP mice, the delayed clearance of rVVNPP could result from differences in functional capacity of CTLs. The mechanism of vaccinia clearance is cytokine dependent, and IFN-γ has been shown to be particularly important (26). We therefore studied the expression of cytokines by influenza-specific memory CD8 T cells. The fixation and permeabilization required for intracellular cytokine staining significantly reduced tetramer staining, so we used peptide restimulation to detect intracellular cytokines in influenza-specific cells. As shown in Fig. 6A, NP68-specific CD8 T cells with the ability to rapidly secrete IFN-γ and/or TNF-α upon Ag recognition were detectable in the ovaries of CD62L transgenic and B6 mice but the frequency was at least 3-fold higher in LΔP mice. This difference was largely accounted for by increases in the frequencies of both IFN-γ/TNF-α double positives and TNF-α single positives. Interestingly, the frequency of NP68 reactive CD8 T cells detectable by intracellular cytokine staining is higher than by tetramer binding in ovaries of LΔP mice at day 5, which may simply reflect the extent of TCR down-regulation due to the higher Ag load. In an attempt to study the activation status of CD8 T cells at the site of rVVNPP infection we stained ovary infiltrates for cell surface IFN-γ. Direct ex vivo staining for IFN-γ showed detectable levels of cytokine at the surface of in vivo stimulated influenza-specific cells in ovaries of B6 and Ltg mice which were ~3-fold higher than on unstimulated memory cells (Fig. 6B). Cell surface IFN-γ was up-regulated in CD62L transgenic mice and, as found for CD107a, to a greater extent in ovaries of LΔP mice than Ltg mice. Together, these results demonstrate clearly that NP68-specific CD8 T can differentiate into CTL in vivo, that activated NP68-specific CD8 T can differentiate into CTL in vivo, that activated NP68-specific CD8 T cells at the site of viral infection in greater numbers in LΔP mice than Ltg mice. This difference between LΔP and Ltg mice is the inability to shed CD62L, these results identify an important role for CD62L shedding in promoting efficient viral clearance.

Discussion

The results presented here using CD62L transgenic mice provide new information relating to different aspects of CD62L biology which warrant separate discussion. We predicted that the retention of CD62L by activated T cells would allow continued recirculation through LN and that it would impact on the ability of Ag-activated T cells to migrate to nonlymphoid organs. The results presented here do not support this hypothesis, at least for effector CD8 T cell distribution to virally infected tissues, and for memory CD8 T cell distribution to non-inflamed tissues. A primary role for CD62L in the recruitment of activated CD8 T cells to lungs was not indicated, since equivalent numbers of CD62Lhigh and CD62Llow.
Ag-specific CD8 T cells were found in LΔP8 and B6 mice, respectively. The increased recruitment of influenza-specific CD8 T cells to infected ovaries of CD62L transgenic mice raises the possibility that CD62L ligands induced in the challenged ovary may control recruitment from the bloodstream. Trafficking experiments will be required to determine whether CD62L plays a direct role in recruitment of CD8 T cells into infected tissues. However, the results reported here using LΔP8, in which cell surface CD62L resists proteolysis and expression is maintained by the heterologous hcd2 promoter, allow us to conclude that CD62L down-regulation either by ectodomain shedding or by gene silencing is not required to redirect Ag-activated CD8 T cells away from lymphoid organs to sites of infection.

Studies of CD62L down-regulation after TCR engagement in vitro using superantigens or anti-CD3/CD28 have shown clearly that accelerated proteolytic shedding and gene silencing both contribute to the reduction in CD62L expression, with shedding predominating within the first few hours after activation (15). By comparing CD62L expression in Ltg mice with ΔP8 mice we are able to confirm that CD62L expression on influenza-specific effector and memory CD8 T cells in vivo is also controlled by proteolytic shedding as well as transcriptional regulation.

The lack of effect on CD8 T cell distribution contrasts sharply with our demonstration that CD62L down-regulation promotes efficient viral clearance. The primary target of vaccinia infection is the ovarian stroma (25), so the efficiency of viral clearance will depend on the migration of effector T cells from their point of entry or residence in the organ to the infected stroma itself. This will be driven by altered expression of inflammatory chemokines and/or adhesion molecules within the organ and controlled by the infected stromal cells. A possible scenario is that in the complete absence of CD62L down-regulation, influenza-specific cells in ΔP8 mice are not optimally activated to express the correct panoply of adhesion and chemokine receptors required to target infected cells within the ovary. Although the clearance of virus was abrogated in ΔP8 mice, it was not completely absent and by day 8 viral titers in ΔP8 mice were undetectable. It is possible that, as virus replication proceeds relatively unchecked in ΔP8 mice, the accompanying tissue destruction decompartmentalizes the organ and NP68-specific T cells are able to interact with and kill target cells more readily. The fact that viral clearance was normal in CD62L transgenic mice expressing the wild-type molecule indicates a critical role for CD62L down-regulation by ectodomain proteolysis, rather than by gene silencing, in this process.

Several mechanisms could underlie the delayed viral clearance in ΔP8 mice. CD62L ligand engagement on recently activated CD8 T cells, which normally would have shed CD62L, may activate integrin- or chemokine-mediated adhesion and migration (27–30) to alter the kinetics of migration within either the involved lymphoid organs or the infected ovary itself, both of which could affect the efficiency of viral clearance. Although not demonstrated for T lymphocytes, it has been reported that neutrophils expressing a shedding resistant mutant of CD62L show defective chemokine-directed migration (8, 9). It is also possible that, by analogy with other cell surface molecules that undergo ectodomain proteolysis such as CD44 and Notch (31, 32), intramembrane proteolysis of the cleaved CD62L cytoplasmic tail may be required for optimal activation of memory CD8 T cells, and that this signaling pathway is absent in ΔP8 mice. It is generally assumed that shedding of CD62L occurs at the site of T cell priming within lymphoid organs. Classical CD62L ligands, such as those which stain with MEC79 mAb, are expressed on vascular endothelium such as HEV, the site of CD62L-dependent recruitment to LN. However, MEC79 reactive ligands have been reported within the LN parenchyma and nonclassical interstitial ligands have been identified in nonlymphoid organs (33). From the results presented here, we cannot exclude the possibility that TCR induced CD62L shedding occurs within the infected ovary since we have demonstrated clearly that CD62L expressing memory cells can reside there. Another possibility is that engagement of CD62L by the leukocyte ligand PSGL-1 expressed on effector or myeloid cells (33), could negatively regulate memory CD8 T cell activation and/or function in ΔP8 mice. A striking feature of LΔP8 mice is the dramatically reduced levels of circulating soluble CD62L which, at 25 ng/ml, are <5% of those in either Ltg or B6 mice (8). It is possible that the absence of soluble ligands, which could compete for signaling via cell surface CD62L or directly stimulate ligand expressing stromal cells, may also interfere with the efficiency of viral clearance.

In conclusion, we have addressed several important questions relating to the potential role of CD62L in regulating the distribution and function of memory T cells using transgenic mice in which CD62L expression is maintained, rather than down-regulated, following T cell activation. The results presented do not support the hypothesis that CD62L down-regulation is required to redirect activated T cells away from LN to nonlymphoid organs or sites of infection. However, these studies have revealed an important and novel role for CD62L ectodomain proteolysis in promoting rapid and efficient viral clearance. Further studies are required to determine whether the effect is on memory T cell migration within infected organs or target cell recognition per se and which domain in cleaved CD62L is responsible. Transgenic mice expressing ectodomain and cytoplasmic domain mutant forms of CD62L that resist shedding and are defective in signaling will be useful in this analysis (34).

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Disclosures
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