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CD31 Is Required on CD4+ T Cells To Promote T Cell Survival during Salmonella Infection

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Hematopoietic cells constitutively express CD31/PECAM1, a signaling adhesion receptor associated with controlling responses to inflammatory stimuli. Although expressed on CD4+ T cells, its function on these cells is unclear. To address this, we have used a model of systemic Salmonella infection that induces high levels of T cell activation and depends on CD4+ T cells for resolution. Infection of CD31-deficient (CD31KO) mice demonstrates that these mice fail to control infection effectively. During infection, CD31KO mice have diminished numbers of total CD4+ T cells and IFN-γ–secreting Th1 cells. This is despite a higher proportion of CD31KO CD4+ T cells exhibiting an activated phenotype and an undiminished capacity to prime normally and polarize to Th1. Reduced numbers of T cells reflected the increased propensity of naive and activated CD31KO T cells to undergo apoptosis postinfection compared with wild-type T cells. Using adoptive transfer experiments, we show that loss of CD31 on CD4+ T cells alone is sufficient to account for the defective CD31KO T cell accumulation. These data are consistent with CD31 helping to control T cell activation, because in its absence, T cells have a greater propensity to become activated, resulting in increased susceptibility to become apoptotic. The impact of CD31 loss on T cell homeostasis becomes most pronounced during severe, inflammatory, and immunological stresses such as those caused by systemic Salmonella infection. This identifies a novel role for CD31 in regulating CD4+ T cell homeostasis. The Journal of Immunology, 2011, 187: 1553–1565.

The generation of effective primary CD4+ T cell-mediated immunity requires the specific recognition of presented Ag. Markers of T cell activation in mice include loss of L-selectin (CD62L) and the acquisition of CD44 expression. Upon priming, CD4+ T cells start to proliferate and acquire the capacity to produce type-specific cytokines. For Th1 responses, which are important for responses to intracellular pathogens such as Salmonella enterica serovar Typhimurium (STm), the key transcription factor that directs differentiation is T-bet and the type-specific cytokine is IFN-γ.

Control of CD4+ T cell homeostasis and survival is complex and requires both soluble mediators such as IL-7 and contact-dependent mechanisms including interaction between the TCR and MHC (1). Using a model of systemic infection with attenuated STm, we identified that CD30 and OX40 expression is required for the survival of Th1 effector (CD62LloCD44hi) but not naive cells (CD62LhiCD44lo) (2). Another molecule reportedly expressed differentially in naive and activated CD4+ T cells is CD31 (3), with high expression on naive cells and lower expression on activated cells. CD31 is an adhesion-dependent signaling molecule, constitutively expressed on many cells throughout the immune system including neutrophils, platelets, and endothelial cells (4). Roles for CD31 on nonlymphocytes have been identified in cellular processes including adhesion, migration, and regulation of the induction of apoptosis in discrete cellular populations (5–7).

What is most striking is that CD31 appears to play a role in restricting activation of immune cells, because its loss is most commonly associated with exaggerated phenotypes in murine models of inflammation. This is reflected in studies that suggest multiple roles for CD31 on CD4+ T cells including downregulating TCR signaling, promoting survival, and inducing integrin-mediated adhesion (8–12). Furthermore, as CD31KO mice age, they can develop a lupus-like autoimmune disease (13), and in some models of immunological stress (e.g., experimental autoimmune encephalomyelitis, LPS-induced toxic shock, or collagen-induced arthritis), they can develop a more severe and aggressive inflammatory response (14–16). This led us to hypothesize that differential CD31 expression on naïve and effector CD4+ T cells may contribute to T cell homeostasis.

A potential confounder to these proposed roles for CD31 has been the finding that resting mice completely deficient for CD31 exhibit a very mild phenotype and, indeed, in most respects appear to be comparable to wild-type (WT) mice (17). Thus, CD31KO
mice have normal numbers of T cells and similar, albeit lower, numbers of B cells. Furthermore, they can mount similar T-dependent and T-independent responses to model nonviable Ags, albeit with modified kinetics (13, 18).

The finding that pronounced CD31-dependent phenotypes are revealed under inflammatory conditions suggests any effects of CD31 loss on CD4+ T cell homeostasis may become more apparent under immunological stress. To test this, we explored the effects of systemic infection with attenuated STm on CD31KO mice. In this model (2), infection in WT mice is self-limiting, with bacterial numbers in the spleen peaking by the first week before falling to very low levels by the fifth week (19). In the first week of primary infection, adaptive immunity does not control bacterial numbers (20–22), but after this time, clearance of intracellular bacteria is dependent upon activated CD4+ T cell survival and T-bet–mediated polarization to Th1, whereas CD8+ T cells or B cells are not required at this stage (2, 21, 23–27).

In this study, we show that CD31 is required for effective control of STm infection and that loss of CD31 on T cells can contribute to this impaired clearance of bacteria. CD31-deficient CD4+ T cells accumulated with delayed kinetics after STm infection despite differentiating to Th1 normally. The reason for the failure of CD31KO T cells to accumulate is related to their increased propensity to undergo apoptosis postinfection. This study thus suggests that CD31 plays a role in promoting T cell survival by helping restrict their activation and that its full role only becomes appreciated during pronounced immunological stresses such as those caused by systemic STm infection.

Materials and Methods

Mice, bacteria, and immunization protocols

C57BL/6 (WT) and CD31KO (6, 17) animals were sourced from in-house colonies in accordance with home office guidelines. OTII and SM1 transgenic mice have been described previously (28, 29). SM1 CD31KO mice were generated by crossing SM1 and CD31KO mice and pairing F1 heterozygous mice. The phenotype of the mice was confirmed by flow cytometry so that all T cells were Vβ2+ (BD Biosciences) and CD31 deficient. T cell-deficient TCRβ−/− mice were obtained from The Jackson Laboratory. Mice were age and sex matched and used between the ages of 6 and 12 wk. Mice were infected i.p. with 5 × 10^7 live-attenuated STm strain SL3261 (30) or OVA-expressing Salmonella (29) in PBS taken from 10^5 live-attenuated STm flagellin protein (31) or with heat-killed bacteria (STm incubated at 60°C for 60 min) in the absence of CD3 and CD28. Stimulated cells were incubated at 37°C for 3 h, followed by 3 h with GolgiStop (as per manufacturer’s protocol; BD Pharmingen). After restimulation, cells were surface stained for CD3, CD4, and CD62L and then fixed and permeabilized with Cytofix/Cytoperm Plus (as per manufacturer’s instructions; BD Pharmingen). Intracellular staining was performed by staining cells with IFN-γ–allophycocyanin or irrelevant allophycocyanin-labeled isotype control (both BD Biosciences).

Purified populations of T cells were isolated by sorting CD3−, CD4−, and CD62L−labeled cells using a MoFlo high-speed cell sorter (DakoCytometry, Ely, U.K.) and sorted to >98% purity. T cell chimeras were generated by i.v. transfer of either purified CD4+ T cells or splenocyte preparations (up to 10^7 cells) in 200 µl PBS. In some experiments, cells were prelabeled with 5 µM CFSE (Molecular Probes, Invitrogen) before transfer to allow determination of subsequent cell proliferation.

Macrophage phagocytosis assay

To assess the capacity of bacteria to infect splenic macrophages, splenocytes from WT or CD31-deficient mice were resuspended in RPMI 1640 medium containing 10% FCS and 164 mM glutamine at 2 × 10^7/ml and allowed to adhere for 90 min in 24-well plates. Nonadherent cells were washed off, and some wells were treated with 100 ng/ml LPS (Alexis Biochemicals) overnight. After overnight incubation, control wells were treated with trypsin to remove adherent cells, and these were then counted using a hemocytometer; the percentage of macrophages was determined using an anti-mouse F4/80-RPE Ab (eBioscience) by flow cytometry. STm was then added to macrophage-containing wells at a multiplicity of infection (MOI) of 10:1 for 4 h. Supernatants were removed, adherent cells were lysed using dH2O containing 0.01% Triton X-100, and total bacterial numbers were quantified as described previously.

Dendritic cell isolation, phenotyping, and in vitro Ag presentation

Splenetic dendritic cells and macrophages were phenotyped by digesting spleens with collagenase D (400 U/ml) for 20 min at 37°C, and cell suspensions were stained for flow cytometry as described above. To assess the capacity of dendritic cells to present Ag, CD11c+ cells were enriched from collagenase D-digested spleens using CD11c-coated magnetic beads (Miltenyi Biotec). Cells were plated in 96-well plates at 3 × 10^5/well and pulsed with either purified STm Ags (5 µg/ml) or OVA (5 µg/ml) for 2 h. After washing, CFSE-labeled FACS-sorted T cells from spleens from WT or CD31KO mice infected for 7 d were added, and the cells cocultured at a ratio of 1 dendritic cell:10 T cells for 3 d before proliferation were assessed by CFSE dilution using flow cytometry.

Measurement of cell proliferation and apoptosis ex vivo after STm infection

T cell proliferation was determined by BrdU uptake. A total of 100 µl BrdU (10 mg/ml) were administered 2 h before sacrifice. The percentage of CD4+ T cells, which had incorporated BrdU, was quantified by flow cytometry using the BrdU Flow Kit (BD Biosciences) in combination with surface staining for CD3, CD4, and CD62L.
Apoptotic CD4+ T cells were detected by flow cytometry using Annexin V-FITC (BD Biosciences) and the DNA-intercalating agent 7-aminactamycin D (7-AAD; Sigma-Aldrich) in combination with surface staining for CD3, CD4, and CD62L.

In vitro apoptosis assay

Splenic T cells from naive WT and CD31KO mice were plated at $5 \times 10^5$ to well in triplicate wells of a 96-well plate. Cells were cultured in either serum-free media (RPMI 1640 medium supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10 mM HEPES) or this same media containing 10% FCS. To assess activation-induced apoptosis, some wells were precoated with 10 µg/ml anti-mouse CD3 Ab, and cells were resuspended with 1 µg/ml anti-mouse CD28 Ab. After 36 h, apoptosis was assessed using Annexin-V-FITC and 7-AAD as described above.

Relative gene expression quantification

CD4+ T cells were FACS sorted and RNA extracted from $10^5$ cells using the RNAqueous (Ambion, Austin, TX) kit as per the manufacturer’s instructions. RNA was reverse transcribed by standard methods using Superscript III (Invitrogen, Paisley, U.K.). Real-time PCR was performed as described using 2× PCR Master Mix (Applied Biosystems). The primer and probe sequences for β-actin, IFN-γ, and T-bet mRNA and its quantification have been previously described (32, 33), and the results are presented as the relative signal per cell.

Statistics

Statistics were calculated using the nonparametric Mann–Whitney sum of ranks test. The $p$ values were calculated using the Analyze-It program (Analyze-It), and $p \leq 0.05$ was accepted as significant.

Results

Mice deficient in CD31 fail to control STm infection

To explore the role of CD31 in immunity to systemic infection with STm, where resolution is dependent on CD4+ T cells (20, 34), WT and CD31KO mice were infected i.p. with $5 \times 10^5$ attenuated STm, and the bacterial burdens were assessed at intervals postinfection (Fig. 1A). Levels of infection in the two groups of mice were comparable 4 d postinfection when innate immunity controlled infection and restriction of bacterial growth is T and B cell independent (19, 21, 23–25, 35). Differences between the groups were apparent at 18 d but were more marked by day 35 when splenic bacterial numbers were clearly higher in the CD31KO mice. Indeed, bacterial numbers were still elevated in CD31KO mice into the third month of infection. The timing of this defective clearance correlates to the time CD4+ T cells are required to control infection. To confirm this, we infected WT and CD31KO mice with and without CD4+ cell depletion for 17 d using a depleting anti-CD4 Ab or rat IgG control (Fig. 1A). As expected (20, 34), CD4 depleted WT mice controlled infection less successfully than WT controls. Nondepleted CD31KO mice controlled infection more poorly than their WT equivalent, and depletion greatly exacerbated this difference, suggesting that the defect in control of infection in CD31KO mice related to CD4+ T cells, although loss of other CD4+ cells or other effects of administering this Ab may also contribute to the differences observed. To test whether defective splenic clearance was also due to a hypersusceptibility of splenic phagocytic cells to STm, LPS-activated adherent splenocytes, of which $\geq55\%$ were F4/80+ (data not shown), were infected in vitro at an MOI of 10 STm/cell (Fig. 1B). These experiments showed that although CD31KO cells had significantly higher intracellular bacterial burdens, this difference was small (<2-fold; Fig. 1B). In addition, there were no differences in the numbers of CD11c+ dendritic cells or F4/80+ macrophages in resting WT or CD31-deficient spleens or infected WT or CD31-deficient spleens or in the ability of these cells to express the markers of activation CD86 and CD40 in response to infection (Fig. 1C and data not shown). Therefore, CD31KO mice fail to resolve STm infection as well as WT mice after the first week of infection.

**CD31 expression falls as CD4+ T cells become activated after STm infection**

Because the timing of defects in bacterial clearance was consistent with there being defective CD4+ T cell-mediated clearance (20, 23,
24, 34), we characterized CD31 expression on WT CD4+ T cells using loss of CD62L and acquisition of CD44 as markers of CD4+ T cell activation. This characterization of CD4+ T cells revealed that expression of CD31 was variable, with expression highest on CD62LhiCD44lo naive cells and lowest on CD62LloCD44hi activated cells (Fig. 2A). However, CD31 expression was never completely lost on these cells. This difference in CD31 expression was independent of the anti-CD31 mAb used (Fig. 2B). Similar differences were found in CD4+CD62LloCD44hi and CD4+CD62LhiCD44hi subsets from OTII and SM1 transgenic CD4+ T cells, where the TCR is specific for peptides from OVA or Salmonella flagellin, respectively (Fig 2C). Therefore, highest expression of CD31 in murine T cells is found on naive CD4+CD62LhiCD44lo T cells and falls as cells become activated.

To assess the expression profile of CD31 on CD4+ T cells postinfection, mice received $5 \times 10^5$ STm i.p., a dose that induces high levels of T cell activation (35, 36). CD31 expression was then assessed on CD4+ T cells, based on CD62L expression, which distinguishes naive from activated CD4+ T cells in mice. This identified three groups: high CD62L expression (hi), intermediate (int), and low (lo) populations (Fig. 2D). The majority of CD4+ T cells had either high or low expression of CD62L, whereas the remaining intermediate population identifies cells that are probably in transit from a naive to an activated effector phenotype. As seen in the absence of infection, reduction in CD31 expression on CD4+ T cells correlated with a loss of CD62L expression, although CD31 was never completely absent on CD4+ T cells (Fig. 2D). When the relationship between the expression of CD31, gain of the activation marker CD44, and loss of the naive cell marker CD62L is correlated, it is clear that loss of CD31 expression is associated with CD4+ T cell activation (Fig. 2E). We also examined the expression of CCR7 and IL-7Ra expression on CD4+ T cells postinfection. This showed that on day 35 >85% of CD44hiCD62LloCD4+ T cells were CCR7hiIL-7Ra+, whereas for CD44loCD62LhiCD4+ T cells, ~20% were CCR7hiIL-7Ra+, and 50% were CCR7loIL-7Ra- (data not shown). These findings further support that CD31 is expressed most strongly by naive CD4+ T cells and expression falls as cells become activated.

CD31KO CD4+ T cells polarize to Th1 and secrete IFN-γ normally but fail to accumulate postinfection

Clearance of primary STm infection is CD4+ T cell dependent, requiring their T-bet–mediated polarization to Th1 and subsequent production of IFN-γ (34). Therefore, we assessed whether loss of CD31 affected the ability of T cells to polarize to Th1. The capacity of splenic WT and CD31KO CD4+ T cells, sorted from spleens 18 and 35 d postinfection, to express mRNA for the key Th1 markers T-bet and IFN-γ was similar on a per cell basis (Fig. 2F).

**FIGURE 2.** CD4+ T cells downregulate CD31 expression during activation. A, Representative FACS staining profiles of CD31 expression on WT splenocytes from a resting, noninfected mouse on naive (CD62LhiCD44lo) and activated (CD62LloCD44hi) CD4+ T cells. B, Graph shows the expression in noninfected WT splenocytes of CD31 on naive (CD62LhiCD44lo) and activated (CD62LloCD44hi) CD4+ T cells using three distinct anti-mouse CD31 mAbs. C, Expression of CD31 on naive (CD62LhiCD44lo) and activated (CD62LloCD44hi) CD4+ T cells from noninfected TCR transgenic mouse strains. B and C, Statistics compares CD31 mean fluorescence intensity (MFI) expression on CD62LhiCD44lo cells to CD62LloCD44hi cells. D, Representative histogram of CD62L expression on WT splenic CD4+ T cells 7 d after STm infection identifying three populations, based on CD62L expression (hi, int, and lo). The graph shows CD31 expression for each of the CD62L subsets for four mice, and bars show significantly reduced expression between CD62Lhi cells and CD62Llo and CD62Lint cells. E, Graph shows the relationship between CD31 and CD44 expression on the three CD62L subsets identified. Graphs show mean and 1 SD for each group, which consisted of four animals per group. MFI values shown have isotype controls subtracted. Data are representative of three individual experiments. *p ≤ 0.05; compares CD31 MFI expression in CD62Llo cells to the other groups.
This was confirmed, using CD3 and CD28 stimulation, for IFN-γ at the protein level 18 d postinfection (Fig. 3B), showing similar levels of IFN-γ production from WT and CD31KO CD4+ T cells. Moreover, >99% of IFN-γ production came from the CD62Llo population, supporting the concept that during responses to STm it is the CD62Llo population that constitutes the effector population. This IFN-γ response was reflected in the capacity of cells after STm infection to respond to STm Ags (23, 25, 34) as shown using splenocytes from day 18-infected animals stimulated with flagellin or heat-killed bacteria (Fig. 3B). Similar results also were seen in CD4+ T cells from WT and CD31KO mice infected for 7 or 35 d (data not shown). In contrast, stimulated CD4+ T cells from noninfected WT and CD31KO mice were largely nonresponsive (Fig. 3B). Therefore, the capacity to differentiate

![Image](https://www.jimmunol.org/content/1557/3/1557.full)
into Th1 CD4+ T cells and produce lineage-specific markers is not impaired in CD31KO CD4+ T cells. However, when total numbers of IFN-γ+CD4+ T cells were quantified in the spleens of infected mice, there was a significantly diminished number of Th1-polarized cells in the CD31KO animals (50% fewer CD31KO compared with WT). Therefore, despite CD31KO CD4+ T cells being able to polarize normally to Th1 after Stm, they are present in reduced numbers compared with WT animals, and this reflects the reduced capacity of the CD31KO to clear the Stm bacterial burden.

Last, we assessed whether there were differences in the capacity of WT and CD31KO dendritic cells to present Ag to WT and CD31KO T cells. Dendritic cells from naive WT and CD31KO mice were pulsed in vitro with purified Stm Ag or OVA before coculturing with CFSE-labeled WT or CD31KO Ag-experienced splenic CD4+ T cells sorted from mice infected for 7 d. These experiments showed that WT and CD31KO T cells could proliferate equally well in response to Stm Ag, independent of whether the dendritic cells were CD31 sufficient or not (representative images shown in Fig. 3C for WT dendritic cells). In contrast, T cells did not proliferate in the absence of Ag or to the irrelevant Ag OVA. This suggests that loss of CD31 on dendritic cells does not adversely influence T cell priming after Stm infection.

CD4+ T cell numbers are reduced in secondary lymphoid tissues in CD31KO mice

Because the capacity of CD31KO CD4+ T cells to polarize to Th1 was unimpaired compared with WT CD4+ T cells, but accumulation of Th1 cells postinfection was reduced, we next assessed whether loss of CD31 affected CD4+ T cell numbers in each tissue. As previously noted (13, 17), there were similar numbers of CD4+ T cells present in the peripheral blood, spleen, and liver of WT and CD31KO mice before infection, although there were always fewer splenic cells with a CD62Lhi naive phenotype. Post-infection, there were fewer CD4+ T cells in the spleens of CD31KO mice compared with WT mice (Fig. 4A), and this was not due to an accumulation of cells in the blood or the liver (Fig. 4A). When the proportions of splenic CD4+ T cells were examined, there were consistently 3- to 4-fold fewer naive CD62Lhi CD4+ T cells postinfection compared with WT cells. Indeed, although infection resulted in an increase in numbers of naive T cells in WT spleens, naive CD31KO T cell numbers were largely unchanged throughout (Fig. 4A). In contrast, CD62Llo CD31KO CD4+ T cell numbers increased postinfection, albeit with delayed kinetics, so that differences between both sets of mice were much less marked (typically <2-fold; Fig. 4A). Therefore, infection results in CD31KO mice having an impaired capacity to maintain CD4+ T cell numbers in the spleen, with naive cell numbers most affected. Although there were highly pronounced, persistent losses in splenic T cells, these differences were not consistently seen in the liver. Thus, T cell numbers in the liver were similar in WT and CD31KO groups before and on days 4 and 35 postinfection but differed on day 18 (Fig. 4A, bottom row). In these experiments, livers were not perfused, and so they probably contain T cells from the blood. Nevertheless, total T cells per 1 ml blood were consistently 1 log or so lower than numbers in the liver, and thus, blood T cells are not likely to account for more than a small fraction of the liver T cells counted.

The difference in splenic CD4+ T cells was unlikely to be due to an inability of the CD31KO spleen to cope with increased cell numbers postinfection because splenomegaly was induced in both WT and CD31KO mice, and after 4 d, CD31KO spleens were consistently larger than WT spleens (Fig. 4B). Furthermore, immunohistological staining of spleen sections with CD3 and IgD to identify white pulp areas showed CD31KO spleens had clearly distinguishable white pulp areas (data not shown). Before infection, these were at 60% of WT levels reflecting the previously reported lower numbers of follicular B cells seen in these mice (Fig. 4B) (13). Postinfection, white pulp area, as a proportion of spleen area, was lower for both groups at each time point, with greater falls observed in CD31KO mice. Nevertheless, although white pulp as a proportion of splenic area was lower in CD31KO mice, the larger spleen size compensated for this difference at later times postinfection (Fig. 4B). Thus, there is no apparent, intrinsic defect in the size of T cell areas to account for the fewer splenic CD4+ T cells in CD31KO mice.

Last, we assessed whether the reason T cells were found in lower numbers in the spleen was because they accumulated in lymph nodes. No significant difference in the size of equivalent WT and CD31KO lymph nodes was detectable (data not shown). Furthermore, when CD4+ T cell numbers were examined in the mesenteric, inguinal, axillary, and cervical lymph nodes, they were found to be either similar or lower than in WT mice (Fig. 4C).

Loss of CD31 on CD4+ T cells is sufficient to account for defective clearance of Stm

Because CD4+ T cells fail to accumulate postinfection, and CD31KO mice fail to clear infection at times when CD4+ T cells are known to be important (2, 20, 23, 24, 34), we next tested whether CD31 on T cells was important to clear infection. First, we tested whether WT and CD31KO T cells could enter the spleen equivalently by mixing WT and CD31KO CD4+ T cells from 7 d infected mice at a 1:1 ratio and transferring these cells into T cell-deficient mice. After 1 h, the number of WT and CD31KO T cells in host spleens after transfer was assessed. Similar numbers of WT and CD31KO CD4+ T cells were found in the spleen (Fig. 5A), suggesting no intrinsic defect in the ability of CD31KO CD4+ T cells to enter this site. To test whether CD31 on T cells was important, we isolated CD4+ T cells from 7 d infected WT and CD31KO mice and i.v. transferred 2.5 × 10^6 of these cells into WT cell-deficient mice to make WT T cell or CD31KO T cell chimeras (Fig. 5B). After 24 h, these chimeras were infected and followed for 7 and 35 d before bacterial burdens and T cell numbers were assessed. Chimeras generated from WT CD4+ T cells had similar bacterial levels to CD31KO chimeras on day 7 when T cells do not contribute to bacterial clearance but substantially lower bacterial burdens on day 35 when T cells are important. This defective clearance correlated with a decrease in the numbers of CD31KO T cells found in the spleen. Therefore, CD31 on CD4+ T cells is required for optimal clearance of infection and accumulation of T cells.

CD31KO CD4+ T cells are more susceptible to apoptosis after Stm infection

As numbers of naive CD4+ T cells were reduced in CD31KO hosts postinfection (Fig. 4A), and the accumulation of Th1 cells in the spleen was reduced (Fig. 3B), we assessed whether CD31 expression contributes to the regulation of apoptosis in WT and CD31KO CD4+ T cells. No differences between WT and CD31KO CD4+ T cells in their expression of CD28 or CD127 (IL-7Rα) were detected (data not shown), suggesting no quantitative defect in responsiveness to B7 or IL-7. Next, levels of apoptosis were assessed by examining surface annexin V binding and 7-AAD uptake (Fig. 6A). In the absence of infection, levels of apoptosis were similar between WT and CD31KO T cells with the exception of CD4+ T cells with the highest expression of CD62L. In
In this group, a higher rate of apoptosis was consistently seen in CD31KO CD4+ T cells. In contrast, postinfection for 4 or 35 d, the rates of apoptosis were consistently higher among the CD31KO CD4+ T cells, and the difference was significantly greater at all levels of CD62L expression (Fig. 6A). The enhanced susceptibility of CD31 T cells to apoptosis was reflected in the responses seen in vitro after culture of cells with and without serum starvation in the presence or absence of stimulation with anti-CD3 and anti-CD28 Ab (Fig. 6A). To examine whether these effects were observed in Ag-specific T cells, we generated CD31KO mice in which all CD4+ T cells are specific for STm flagellin. These were generated by crossing CD31KO mice with SM1-transgenic mice (SM1 CD31KO). T cells from SM1 mice are transgenic for a peptide from STm flagellin. Reflecting our findings in CD31KO mice (Fig. 4), SM1 CD31KO mice had more CD4+ T cells that were CD62Llo before and postinfection than CD31+ SM1 mice (data not shown). We then tested whether CD31KO SM1 cells were more prone to apoptosis. First, we examined responses in vitro by culturing splenocytes from both sets of mice with purified STm flagellin and anti-CD28 or with anti-CD3 and anti-CD28 Ab (Fig. 6B). Under both conditions, the greatest levels of apoptosis were seen in the CD31KO SM1 T cells. To examine whether this was also seen in vivo, we generated chimeras by transferring 10^7 CD31+ or CD31KO SM1 splenocytes into TCRβδKO mice and infected these chimeras the next day. These results showed that there was increased death in CD31KO SM1 mice.
CD4+ T cells with SM1 CD4+ T cells postinfection but not in its absence (Fig. 6C). Thus, loss of CD31 results in increased apoptosis postinfection in CD31KO CD4+ T cells.

Infection promotes apoptosis in CD31-deficient CD4+ T cells

Because CD31 loss on CD4+ T cells alone was sufficient to promote cell loss, we examined whether exposure to infection promoted apoptosis in CD4+ T cells. We i.v. transferred enriched splenocyte preparations containing 1.5 x 10^6 WT or CD31KO CD4+ T cells from either naive, noninfected mice (Fig. 7A) or from mice that had been previously infected for 7 d (Fig. 7B) into TCRβδKO recipients. After 24 h, CD4+ T cell numbers were assessed in the spleen, blood, and liver. WT and CD31KO CD4+ T cells from noninfected donors were found in all sites in recipient mice in similar numbers (Fig. 7A), reflecting what has been described previously (13). In contrast, assessment of CD4+ T cells from infected donors showed a markedly different result (Fig. 7B), as CD31KO T cells were found at 7-, 11-, and 14-fold lower frequencies in the spleen, liver, and blood, respectively, compared with WT T cells. Furthermore, examination of splenic CD31KO CD4+ T cells in these chimeras revealed their increased susceptibility to undergo apoptosis, as measured by ex vivo annexin V staining. This suggests that Ag-experienced CD31KO CD4+ T cells fail to repopulate as efficiently as WT counterparts because of their increased propensity to undergo apoptosis after exposure to STm infection.

Next, we assessed whether there were differences between the capacity of CD62Lhi and CD62Llo subsets to accumulate postinfection. We sorted CD62Lhi and CD62LloCD4+ T cells from 7-d-infected WT or CD31KO spleens and transferred 3.5 x 10^6 WT CD62Lhi or CD62Llo or CD31KO CD62Lhi or CD62LloCD4+ T cells i.v. into naive TCRβδ-deficient recipients. After 24 h, chimeras were challenged with 10^5 STm 24 h later. Bacterial burden in the spleens of these chimeras was assessed (left graph), and the number of T cells present in the spleen 35 d postinfection was quantified (right graph). Graphs show mean and 1 SD for each group, which consisted of at least four mice, and are representative of at least two separate experiments. *p ≤ 0.05.

Discussion

In this article, we show that CD31 expression on CD4+ T cells contributes to efficient control of STm infection, in part by reducing CD4+ T cell loss through apoptosis. In WT CD4+ T cells, there is an inverse correlation between activation status and CD31 expression, with highest expression on naive CD62LloCD44hiCD31KO CD4+ T cells in the blood and liver were higher or similar, respectively. When levels of cell numbers and apoptosis were compared, the difference was less between transferred WT and CD31KO CD62Lhi T cells than for transferred WT and CD31KO CD62Llo T cells.

Loss of CD31 on CD4+ T cells results in dysregulated proliferation after STm infection

Despite increased levels of apoptosis, numbers of activated CD31KO T cells gradually accumulated over time (Fig. 4A). This suggested that the increase in apoptosis (Fig. 6A) may, in part, be balanced by increased proliferation. To assess this, we characterized the proportion of CD4+ T cells actively proliferating by giving a pulse of BrdU 2 h before sacrifice and looking at levels of BrdU uptake in CD4+ T cells with hi, int, and lo expression of CD62L (Fig. 8A). In the absence of infection, no difference was seen between the proportion of CD31KO and WT T cells proliferating, irrespective of the levels of CD62L expression. In contrast, postinfection, the proportion of T cells proliferating increased as CD62L expression decreased. CD31KO CD4+ T cells exhibited the same pattern of proliferation as WT cells, but a higher proportion of cells with the lowest levels of CD62L expression were proliferating. Finally, we examined the capacity of Ag-specific CD31hi or CD31KO CD4+ T cells to proliferate by transferring CFSE-labeled SM1 splenocytes into WT or CD31KO hosts, which were infected 24 h later, and assessing CFSE dilution 3 d later. In the absence of infection, SM1 cells did not proliferate to any appreciable degree, irrespective of CD31 status (data not shown). Postinfection, CD31KO SM1 T cells had proliferated more than CD31hi SM1 T cells, indicating a greater proliferative capacity, and this was not dependent on the host CD31 status (Fig. 8B). Therefore, postinfection, there are more proliferating activated CD31KO CD4+ T cells, which may partially compensate for the increased apoptosis seen in CD62Llo-activated CD4+ T cells, and this was not dependent on host environmental CD31 expression.
T cells. Our findings are consistent with CD31 acting to raise the threshold for CD4+ T cell activation as demonstrated by consistently higher proportions of CD62Llo CD31KO CD4+ T cells being detected before and postinfection (Fig. 4A), and 95% of these cells were also CD44hi. The consequence of infection is to increase the levels of T cell activation and, thus, the susceptibility of CD31KO T cells to undergo apoptosis, which ultimately leads to a reduction in T cells. Therefore, CD31 is necessary to promote controlled activation of T cells and their survival.

As observed previously after STm infection, in WT mice, numbers of splenic naive CD4+ T cells increase (37, 38). This may help amplify the breadth of the antigenic repertoire available and enhance the probability of a productive, cognate T cell interaction. This finding presented with our previous data on T cell survival (2) suggests that during infection there is continuous recruitment of T cells into the response. Nevertheless, the size of the naive pool does not decrease, indicating that the naive CD4+ T cell pool is continuously replenished during infection. Experiments using transgenic SM1 T cells that are specific for flagellin from STm but lack CD31 showed that Ag-specific cells reflected endogenous CD31KO CD4+ T cells in being more proliferative as well as more susceptible to apoptosis. In CD31KO mice, numbers of splenic, naive CD4+ T cells did not change, whereas activated CD31KO CD4+ T cell numbers did increase, albeit with delayed kinetics, so that in CD31KO mice CD62Llo cell numbers were twice as high as noninfected mice by day 18 and four times higher by day 35. This shows that despite increased apoptosis in these mice T cells can slowly accumulate, and this is probably how CD31KO mice are able to eventually clear the infection from the third month onward (Fig. 1A). This contrasts with mice, which lack CD4+ T cells (23, 39, 40) or cannot activate T cells (41, 42) or fail to generate Th1 responses (34, 43), which do not clear infection and can die as a consequence.

CD31KO mice consistently had a higher proportion of T cells with an activated phenotype, irrespective of whether they were infected or not (Fig. 4A); this was also found in CD31KO SM1 mice. Furthermore, infected mice had no defect in their capacity to polarize to Th1 after STm but did have fewer numbers of Th1 cells postinfection (Fig. 3B), because of their increased propensity to die (Fig. 6). This suggests that CD31 can increase the threshold for apoptosis.

**FIGURE 6.** Loss of CD31 results in increased apoptosis of CD4+ T cells after STm infection. A. Levels of apoptosis of splenic CD4+ T cells were compared before and 4 d after STm infection. Left histograms show representative gates for CD62L expression on splenic CD4+ T cells 4 d postinfection. The central panels show representative flow cytometry plots for dual staining of annexin V and 7-AAD on splenic CD62L subsets of CD4+ T cells identified at day 4 post-STm infection (box on plots). Lower graphs show the proportion of splenic T cells that are double positive for both markers in nonimmunized (day 0, left graph) or STm-infected mice (day 4, right graph). The top row, right graph shows levels of apoptosis in WT and CD31KO T cells after in vitro culture for 36 h with and without serum in the presence or absence of anti-CD3- and anti-CD28–stimulating Abs. B, Levels of apoptosis in transgenic SM1 and SM1 CD31KO T cells was assessed after 24 h in vitro culture in the presence or absence of anti-CD3 and anti-CD28 or purified flagellin protein and anti-CD28. C. To test the response of SM1 CD31 T cells in vivo, chimeras were generated by i.v. transfer of up to 10^7 SM1 or SM1 CD31KO-enriched splenocytes (allowing transfer of 10^6 CD4+ T cells) into T cell-deficient hosts. Chimeras were challenged with 10^6 STm and numbers of apoptotic CD4+ T cells quantified after 72 h. Figures are representative of three separate experiments and show mean and SD for four animals per group. *p ≤ 0.05, **p ≤ 0.01.
T cell activation. In the absence of CD31-mediated inhibitory signals, there appears to be continuous, inappropriate activation. This results in increased apoptosis at the very earliest stages of activation, but this is not sufficient to prevent a slow accumulation of activated T cells, and this may in part be due to the increased proliferative capacity observed in activated CD31KO cells post-infection (Fig. 8). A link between CD31, proliferation, and apoptosis has been found in studies on human T cells, which showed CD31lo T cells can proliferate more readily (44). These important roles for CD31 may at first seem contradictory because CD31 expression falls after activation. What is likely to be important in this study is that CD31 expression falls after activation but is not absent, and it is this low level of expression that is likely to be important in helping regulate T cell survival and proliferation. Furthermore, as supported by the original assessment of the CD31-deficient mouse (17), CD31KO mice have a near-normal phenotype in the absence of immunological challenge, and this highlights the importance of STm challenge in identifying these roles for CD31. The findings presented in this article in the context of infection may have important implications for CD4+ T cell survival in other diseases such as autoimmunity or cancer. Low CD31 expression is found on a subpopulation of human, thymic emigrants (45–47), which accumulate as individuals age. This subpopulation may also have an enhanced proliferative capacity, and it has been proposed that this T cell population may enhance susceptibility to autoimmune disease (48). Furthermore, some CD31-expressing tumor cells are less apoptotic and associated with a poorer prognosis for the patient (49, 50).

The time at which this failure to clear STm appears is consistent with this defect being T cell-mediated, because control of infection in the first days of postinfection is T independent (20, 21, 24). The reasons for this impaired T cell-mediated clearance of infection may relate to one or more of 1) deficient T cell numbers impairing clearance of infection; 2) a defective capacity for CD31KO T cells to interact with CD31KO macrophages; 3) a failure of CD31KO macrophages to kill after T cell-mediated activation; 4) an inability of T cells to reach sites of infection; or 5) the 2- to 4-fold reduced density of Th1 cells in CD31KO spleens resulting from differences in splenomegaly, reducing the effectiveness of the response. Although all of these need to be tested extensively, it appears that there is no intrinsic hypersusceptibility of CD31KO macrophages to infection, at least in vitro (Fig. 1B), although our data may also indicate a minor inherent increased susceptibility to infection. Nevertheless, although T cells are demonstrated to be important in this study, our work does not rule out the possibility that there may be additional roles for CD31 on other nonlympho-
cyte populations in controlling this infection that only become apparent at these times. Our data show that transferred CD31KO T cells fail to persist in CD31-sufficient recipients postinfection and that, when these T cells cannot persist, then the infection is not cleared as efficiently (Figs. 5, 7). This, in parallel with data from our earlier studies, indicate that Th1 differentiation alone is not sufficient to control STm infection but that Th1 must be coupled in parallel with survival.

How might CD31 achieve these functions on T cells? It is not likely to function through restricting early entry of T cells into secondary lymphoid tissues from the circulation. This is supported by our data (Fig. 5A) showing there were similar numbers of T cells in the spleen 1 h after transfer. Furthermore, migration of T cells has not been found to be affected by CD31 loss in models of tumor and allograft rejection (51). We suggest that the key activities are mediated through the two ITIMs within the cytoplasmic tail of CD31 (7, 52), which are required for mediating CD31-dependent migration (53), prosurvival signaling (54), and attenuation of signaling through the T cell and other ITIM-containing receptors (8, 55). These ITIMs can bind many signaling proteins, but perhaps most relevant in this study is that the Src homology 2 domain-containing tyrosine phosphatase SHP-2 can be bound by CD31 (56). SHP-2 regulates immune responses including T cell development and function and modulates other inhibitory receptors and intracellular adaptor proteins (57). Therefore, loss of CD31 may result in changes in phosphatase localization intracellularly. If so, targeting of CD31 may offer potential novel targets for intervening in T cell-mediated diseases.

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Disclosures
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