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Dynamic Regulation of T Cell Immunity by CD43¹

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and Rafi Ahmed^{2*}

During a viral response, Ag-specific effector T cells show dramatically increased binding by the mAb 1B11 and the lectin peanut agglutinin (PNA). We investigated the contribution of CD43 expression to 1B11 and PNA binding as well as its role in generation and maintenance of a CD8 T cell response. Analysis of CD43^{-/-} mice revealed no increased 1B11 binding and reduced PNA binding on virus-specific CD8 T cells from -/- mice compared with +/+ mice. Furthermore, we examined the role of CD43 in the kinetics of an immune response. We show that CD43 expression modestly effects generation of a primary virus-specific CD8 T cell response *in vivo* but plays a more significant role in trafficking of CD8 T cells to tissues such as the brain. More interestingly, CD43 plays a role in the contraction of the immune response, with CD43^{-/-} mice showing increased numbers of Ag-specific CD8 T cells following initial expansion. Following the peak of expansion, Ag-specific CD8 T cells from -/- mice show similar proliferation but demonstrate increased Bcl-2 levels and decreased apoptosis of Ag-specific effector CD8 T cells *in vitro*. Consistent with a delay in the down-modulation of the immune response, following chronic viral infection CD43^{-/-} mice show increased morbidity. These data suggest a dynamic role of CD43 during an immune response: a positive regulatory role in costimulation and trafficking of T cells to the CNS and a negative regulatory role in the down-modulation of an immune response. *The Journal of Immunology*, 2002, 168: 6022–6031.

T cells can be classified into three separate classes: naive, effector, and memory. During an immune response, T cells are dramatically modified by both protein expression and protein modification. We can generate and directly visualize Ag-specific CD8 T cells at various times after lymphocytic choriomeningitis virus (LCMV)³ infection using MHC class I tetramers (1). Using this model, we have demonstrated that there are changes in the glycosylation patterns of T cells *in vivo* following viral infection based on binding to peanut agglutinin (PNA), distinguishing naive from activated T cells (2). Additionally, we have recently shown that one surface marker, 1B11, can be used to distinguish between memory and effector T cells (3). 1B11 binding is low on CD8 naive cells and high on Ag-specific CD8 effector T cells, and becomes reduced again on Ag-specific CD8 memory T cells (3).

The abundant T cell surface glycoprotein CD43 (sialophorin, gp115, leukosialin) is a transmembrane protein consisting of a highly *O*-glycosylated extracellular domain of 235 amino acids, a transmembrane domain of 23 amino acids, and an intracytoplasmic

domain of 123 amino acids (4). Posttranslational modifications result in two glycoforms of CD43. The mAb S7 recognizes the lower-molecular mass (115 kDa) glycoform (5), while the mAb 1B11 was initially characterized as recognizing the activation-associated, high-molecular mass (130 kDa) glycoform of CD43 (5). This high-molecular mass glycoform bears core 2 *O*-glycans, an oligosaccharide structure that can be created by the action of the core 2 β -1-6-*N*-acetylglucosaminyltransferase (6). Recently, one report has shown that 1B11 recognizes a novel CD8 T cell-restricted CD45RB epitope that is independent of core 2 *O*-glycans on naive CD8 T cells (7). Thus, it was unclear which glycoprotein(s) 1B11 was binding on activated Ag-specific CD8 T cells.

Although CD43 is one of the most abundant T cell surface glycoproteins, its functions remain controversial. It has been purported to have antiadhesive as well as proadhesive functions in T cell trafficking (8–11). Additionally, CD43 has been shown to play a role in T cell activation, although this, too, remains unclear. Ab cross-linking experiments suggested a costimulatory role *in vitro* (12); however, other groups suggested a negative regulatory role in T cell activation (8, 13). Most recently, it has been shown that CD43 is excluded from the immunological synapse during T cell activation, and this exclusion appears to be mediated by an ezrin-radixin-moesin-dependent mechanism(s) (14–17).

To understand the role of CD43 on effector CD8 T cells during an immune response, we generated Ag-specific effector CD8 T cells in CD43-deficient mice. Surprisingly, there was a complete absence of the up-regulation of 1B11 binding on these cells, firmly establishing that the up-regulated binding of 1B11 observed on effector CD8 T cells *in vivo* requires the expression of CD43. We examined the course of a viral immune response in CD43^{-/-} mice and found that while CD43 plays a positive regulatory role in the costimulation and trafficking of naive T cells *in vivo*, it appears to play a negative regulatory role in the waning/down-modulation of effector T cell responses. These results suggest a dynamic role of CD43 during an immune response that is reflective of its dynamic

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³ Abbreviations used in this paper: LCMV, lymphocytic choriomeningitis virus; PNA, peanut agglutinin; BrdU, bromodeoxyuridine; 7-AAD, 7-amino actinomycin D; i.c., intracranial(ly); WASp, Wiskott-Aldrich syndrome protein.

regulation and modification during T cell activation, death, and homeostasis.

Materials and Methods

Mice

CD43^{-/-} mice and B6.129 control mice (CD43^{+/+}) were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in-house under specific pathogen-free conditions. For acute LCMV responses, naive adult mice (>5 wk) were infected with 2×10^5 PFU of LCMV-Armstrong i.p. For LCMV disease studies, adult mice (4–6 wk old) were infected with 1×10^2 or 1×10^5 PFU LCMV-Armstrong intracranially (i.c.). For chronic studies, adult mice (>5 wk old) were infected with 2×10^6 PFU LCMV clone 13 i.v.

Surface/tetramer staining, flow cytometry, and direct ex vivo CTL assay

Spleens were harvested from mice and passed through a screen in 10% FCS in RPMI with antibiotics and 2-ME. RBCs were lysed and cells were washed and counted for total cell yields. For immunostaining, 1×10^6 cells were stained in 96-well U-bottom plates. All mAbs and apoptosis reagents (anti-CD8 α -PE, anti-CD62L-FITC, anti-CD44-FITC, S7-FITC, S7-PE, 1B11-FITC, anti-Bcl-2-FITC, anti-bromodeoxyuridine (BrdU)-FITC, Annexin V-FITC, and 7-amino actinomycin D (7-AAD)) were purchased from BD PharMingen (San Diego, CA.) Production of MHC class I tetramers D^bNP_{396–404} and D^bgp33–41-allophycocyanin were done as previously described (1). PNA-FITC was purchased from Sigma-Aldrich (St. Louis, MO). Flow cytometry was performed on a FACSCalibur (BD Biosciences, Mountain View, CA). LCMV-specific CTL activity was determined by a 5-h ⁵¹Cr release assay as previously described (1).

Immunohistochemistry

CD8 T cell infiltrates in the brain were assessed by immunohistochemistry. Brains were harvested from mice and cut in half. One half was used to isolate CNS mononuclear cells and the other half was quick-frozen and stored at -70°C until sectioned on a cryostat. Sagittal brain sections were prepared from frozen tissue, ethanol-fixed for 20 min, transferred to PBS, incubated with DAKO block (DAKO, Carpinteria, CA) for 45 min to block endogenous peroxidase, followed by a 15-min block with 5% rabbit serum in PBS, and stained with anti-CD8 α and anti-CD8 β Abs (BD PharMingen) for 90 min. Sections were washed with PBS, incubated for 45 min with biotinylated rabbit anti-rat Ab (Jackson ImmunoResearch Laboratories, West Grove, PA), and developed with the ABC Elite kit (Vector Laboratories, Burlingame, CA) and DAB. Sections were counterstained with Mayer's hematoxylin. Images were analyzed using $\times 4$ and $\times 20$ objectives and captured by digital photomicroscopy for final output.

Isolation of CNS mononuclear cells

Brain tissue was harvested from mice in 1% FCS in RPMI, transferred to an ice-cold 7-ml Tenebroeck glass homogenizer in 5 ml medium, and homogenized to a smooth consistency. Brain homogenates were transferred to conical tubes on ice and adjusted to a 7-ml volume, and 3 ml Percoll (Sigma-Aldrich) was added to yield a final 30% Percoll concentration. This was underlayered with 70% Percoll and centrifuged at 2500 rpm. The interface was removed and washed, RBCs were lysed, and cells were stained with anti-CD8 and MHC class I tetramers D^bNP_{396–404} and D^bgp33–41.

IFN- γ ELISPOT assays

Allospecific T cell responses were measured by IFN- γ ELISPOT assay using splenocytes or Histopaque separated (Sigma-Aldrich) peripheral blood leukocytes obtained from skin-grafted CD43^{+/+} or CD43^{-/-} mice. The capture Ab for this assay, rat anti-mouse IFN- γ (clone R4-6A2; BD PharMingen), was incubated at $4 \mu\text{g/ml}$ in PBS ($100 \mu\text{l/well}$) at 4°C overnight in ester cellulose-bottom 96-well plates (Millipore, Bedford, MA). After washing with PBS, 3-fold serial dilutions of responding cells were added. A total of 5×10^5 irradiated donor splenocytes (BALB/c) were added to each well and plates were incubated overnight at 37°C . After the culture period, cells were removed by washing the plates with PBS-Tween (0.05%). Biotinylated anti-mouse IFN- γ (clone XMG1.2; BD PharMingen) was added at $4 \mu\text{g/ml}$ ($100 \mu\text{l/well}$) for 2 h, plates were washed, and HRP-avidin D (Vector Laboratories) was added for 1 h. Following a final wash, spots were developed with the substrate 3-amino-9-ethyl-carbazole (Sigma-Aldrich) with 0.015% H₂O₂, allowed to air dry, and quantitated.

BrdU incorporation

Mice were fed BrdU (Sigma-Aldrich) at 1 mg/ml in their drinking water, changed daily, for days 8–15 following LCMV-Armstrong challenge i.p. On day 15 mice were sacrificed and peripheral blood and spleen were harvested. After perfusion with 5 ml cold PBS, liver was harvested. PBMCs were isolated as previously described (1). Lymphocytes from the liver were obtained after passage through a mesh screen. Cell suspensions were incubated at 37°C for 1 h with a $10\times$ stock of 2.5 mg/ml collagenase B and 0.29 mg/ml DNase I in PBS. Large tissue debris was discarded and cells were resuspended in 44% Percoll, underlayered with 56% Percoll, and spun for 20 min at 2000 rpm in an IEC centrifuge (IEC, Needham Heights, MA). After isolation from the Percoll gradient, RBCs were lysed and cell yield was determined. Cell surface staining with MHC class I tetramers specific for D^bNP_{396–404} and anti-CD8-PE followed by intracellular staining for BrdU incorporation as per the manufacturer's instructions (BrdU Flow kit; BD PharMingen). Briefly, cells were permeabilized with Cytofix/Cytoperm and treated with DNase to expose incorporated BrdU, followed by staining with anti-BrdU-FITC.

In vitro apoptosis assay

Splenocytes were harvested from infected mice on days 8, 10, or 12 and 1×10^6 cells were cultured for 16 h in 96-well flat-bottom plates at 37°C . Cells were transferred to a 96-well U-bottom plate for staining. Following surface staining with MHC class I tetramers and anti-CD8 Abs, cells were washed and incubated with annexin V staining buffer (10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM CaCl₂) with a 1/20 dilution of annexin V-FITC and a 1/5 dilution of 7-AAD (BD PharMingen) for 15 min at room temperature. Cells were washed and immediately assayed by flow cytometry.

Results

CD43-deficient mice generate Ag-specific effector CD8 T cells

CD43-deficient mice show normal T and B cell development and have similar numbers of T lymphocytes in the periphery (8, 18). Wild-type or CD43-deficient mice were infected i.p. with 2×10^5 PFU of the Armstrong strain of LCMV, and mice were sacrificed 8 or 65 days postinfection. Splenocytes and lymph node T cells were stained with Abs to CD8, S7, or 1B11 and MHC class I tetramers specific for the dominant epitopes D^bgp33–41 or D^bNP_{396–404} (1). Data from spleen cells are shown, but similar results were obtained from lymph node cells. Similar percentages of Ag-specific CD8 T cells were obtained from both genotypes (Fig. 1A).

To analyze binding of mAbs S7 and 1B11, effector and memory cells were gated for expression of CD8 and D^bNP_{396–404} while naive cells were gated on total CD8 T cells from uninfected mice. As previously reported, CD43^{-/-} CD8 T cells do not express the low molecular glycoform of CD43 as measured by the mAb S7 (8), while CD43^{+/+} CD8 T cells express CD43 constitutively on naive, effector, and memory CD8 T cells (Fig. 1B). However, binding by 1B11 changes dramatically in CD43^{+/+} mice; 1B11 binding is low on naive CD8 T cells, dramatically increased on virus-specific effector CD8 T cells, and reduced on virus-specific memory CD8 T cells (Fig. 1B). Double staining of $+/+$ effector CD8 T cells with 1B11 and S7 reveals binding of both Abs on the same cells. Binding by mAbs S7 and 1B11 on T cells from wild-type mice is summarized in Table I.

We saw no evidence of increased 1B11 binding on $-/-$ virus-specific effector CD8 T cells, in marked contrast to $+/+$ cells (Fig. 1B). Other surface markers such as CD44 and L-selectin were unaffected and similar surface expression was observed between $+/+$ and $-/-$ effector CD8 T cells (Ref. 9 and data not shown). These data demonstrate that the increased 1B11 binding on CD8 effector T cells requires the expression of CD43.

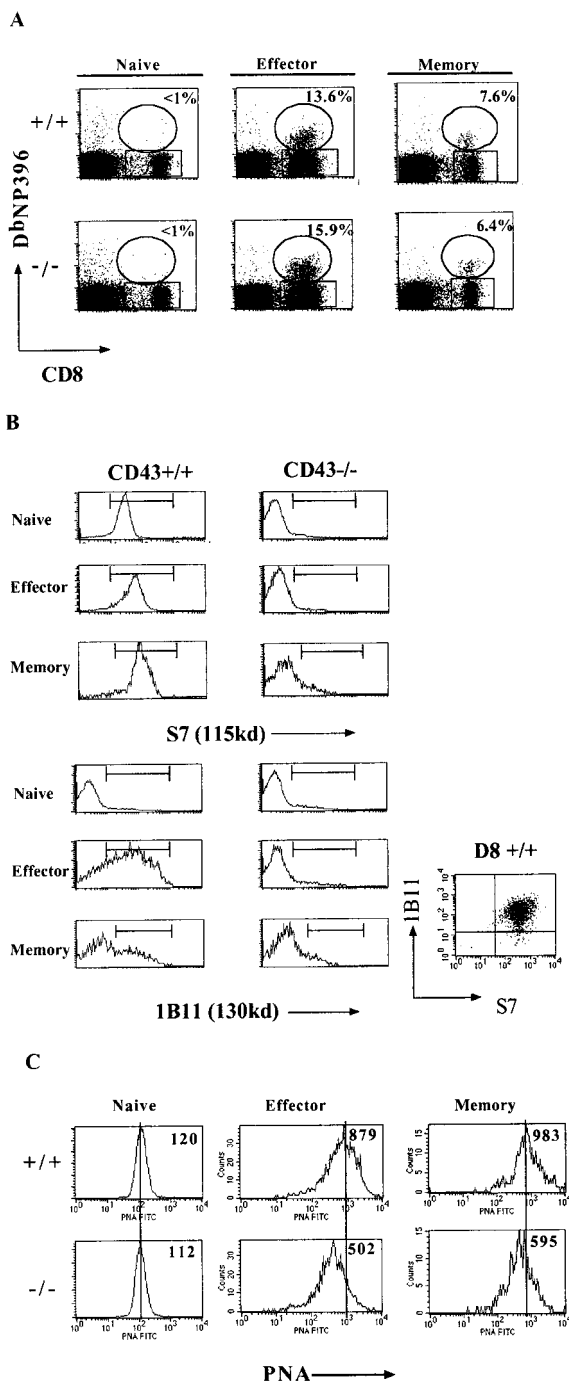


FIGURE 1. Generation of Ag-specific CD8 T cells in CD43^{-/-} mice. **A**, CD43^{+/+} and CD43^{-/-} mice were infected with 2×10^5 PFU LCMV-Armstrong i.p., and the LCMV-specific CD8 T cell response was analyzed on day 0 (naive), day 8 (effector), and day 65 (memory) postinfection. Spleen cells were analyzed with D^bNP₃₉₆₋₄₀₄ MHC class I tetramer and anti-CD8. **B**, Gating on CD8 T cells from uninfected mice (naive) or D^bNP₃₉₆-specific CD8 T cells day 8 (effector) or day 65 (memory) postinfection. Binding by mAbs S7 and IB11 was analyzed for both CD43^{+/+} and CD43^{-/-} mice. CD43^{+/+} CD8 T cells show constitutive binding by S7 and CD43^{-/-} T cells show an absence of S7 binding. D^bNP₃₉₆-specific effector CD8 T cells show increased binding of IB11 in CD43^{+/+} mice, while this binding remains low on CD43^{-/-} CD8 T cells, demonstrating that CD43 expression is required for IB11 binding on effector T cells. Double staining and gating on day 8 effector D^bNP₃₉₆-specific CD8 T cells reveals binding of both S7 and IB11 on the same cells in CD43^{+/+} mice. **C**, Gating on naive phenotype (CD44^{low}) CD8 T cells from uninfected mice or D^bNP₃₉₆-specific CD8 T cells from day 8 (effector) or day 65 (memory) postinfection reveals reduced binding of PNA on CD43^{-/-} effector and memory CD8 T cells in comparison to CD43^{+/+}.

Table I. Summary of mAb binding of S7 and IB11 on naive and activated CD4 and CD8 T cells

mAb	CD4		CD8		
	CD44 ^{low}	CD44 ^{high}	CD44 ^{low}	Effector	Memory
S7	-/+	++	++	++	++
IB11	-/+	+++	-/+	+++	+

Reduced binding of PNA on effector and memory T cells in CD43^{-/-} mice

We have previously demonstrated that virus-specific T cells become PNA^{high}, and this increased PNA reactivity may result from increased neuraminidase activity in activated T cells (2). Alterations in expression or activity of one or more sialyltransferases could also affect PNA binding (19, 20). We examined PNA binding on T cells derived from CD43^{-/-} mice after challenge with LCMV. Effector and memory cells were gated for expression of CD8 and D^bNP₃₉₆₋₄₀₄, while naive cells were gated on CD44^{low} CD8 T cells from uninfected mice. While PNA binding to effector T cells was markedly up-regulated compared with naive cells from both +/+ and -/- mice, the level of binding to effector and memory -/- CD8 T cells was significantly reduced compared with similar populations of +/+ cells (~30–40%) (Fig. 1C). While prior work has demonstrated that CD43 is a PNA-binding glycoprotein on thymocytes (21), the data presented in this work demonstrate that CD43 expression contributes to PNA binding by activated peripheral T cells. This suggests that the glycosylation status of CD43 on peripheral T cells is dynamically regulated during a viral infection in vivo. Because PNA binding to peripheral T cells is reduced but not abolished in the CD43^{-/-} mice, expression of other O-glycosylated proteins such as CD8 most likely contribute to PNA binding (21–23).

Modest role for CD43 in costimulation in vivo

Cross-linking CD43 has been shown to play a role in the costimulation of T cells in vitro (12); however, CD43^{-/-} T cells have been shown to be hyperproliferative in vitro (8, 13). We examined the CD8 T cell response on day 8 in CD43^{-/-} mice after acute infection with LCMV. Wild-type mice show the peak of expansion approximately day 8 postinfection. While CD43^{-/-} mice show similar percentages of virus-specific CD8 T cells (Fig. 1), the total cell number is slightly reduced, accounting for an ~1.5- to 2-fold reduction in the numbers of virus-specific T cells in CD43^{-/-} mice (Fig. 2, A and B). Both groups of mice show undetectable virus titers in day-8 serum, liver, kidney, and spleen (data not shown). CTL-specific lysis was measured, as well as intracellular cytokine production, and no differences were seen in CD43^{-/-} mice, even when data were normalized for CD8 T cell numbers (Fig. 2C and data not shown). CD8 T cells specific to subdominant epitopes D^bgp276–306 and K^bNP₂₀₅₋₂₁₂ were also reduced in magnitude in CD43^{-/-} compared with CD43^{+/+} mice (Fig. 2D). These results demonstrate a modest effect in the expansion of naive CD8 T cells, supporting a role in costimulation. However, this slight decrease in expansion has little overall effect on the acute immune response, as viral clearance is unaffected.

Role for CD43 in trafficking of CD8 T cells to tissues

CD43 has been shown to play a role in the trafficking of T cells and monocytes to sites of inflammation (10, 11). However, it is also purported to play an antiadhesive role in lymphocyte trafficking, suggesting increased trafficking of lymphocytes to some tissues in

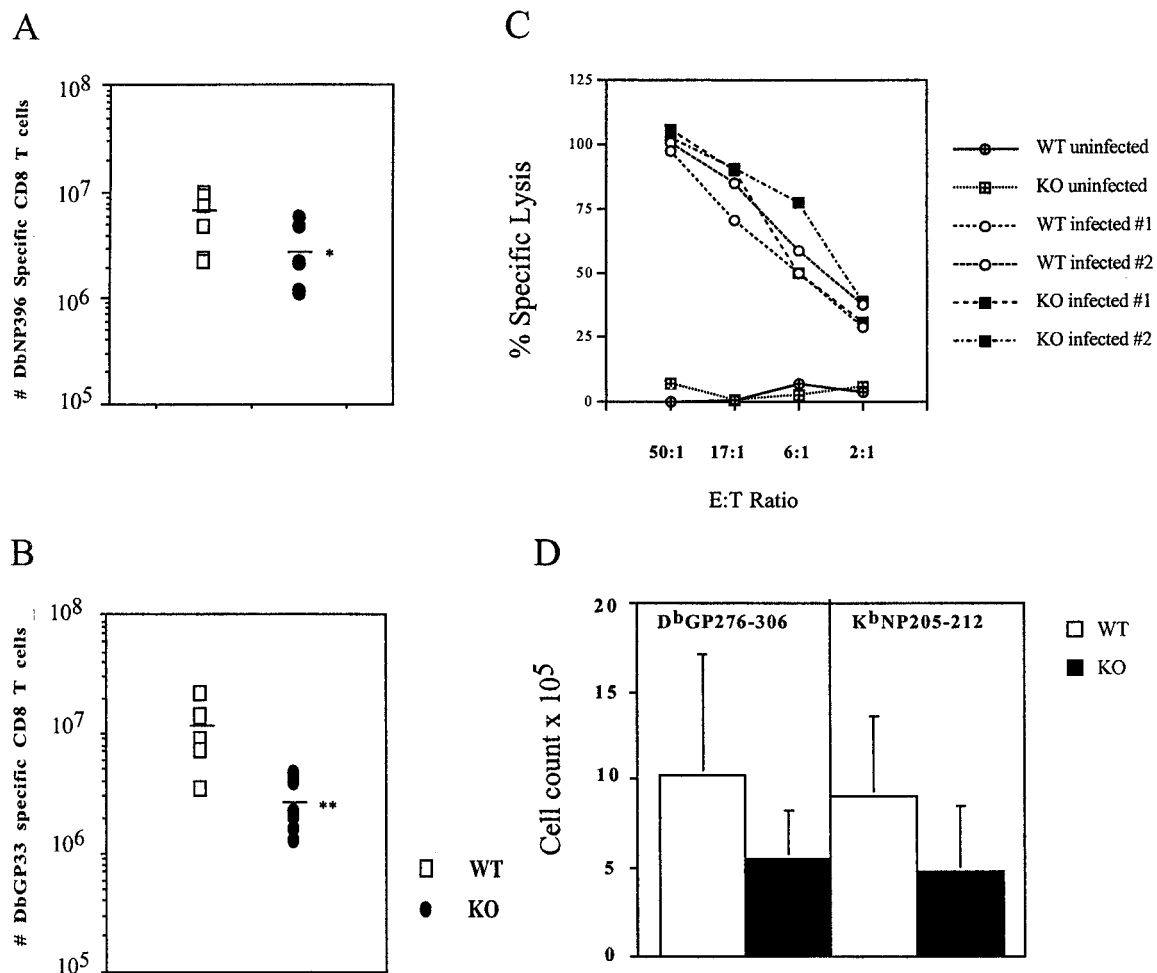


FIGURE 2. Minimal role for CD43 in generation of a primary LCMV-specific CD8 T cell response. *A* and *B*, Splenocytes from CD43^{+/+} and CD43^{-/-} mice challenged with 2×10^5 PFU LCMV-Armstrong were stained with D^bNP₃₉₆₋₄₀₄-specific and D^bgp33-41-specific MHC class I tetramers and the total number of specific CD8 T cells was determined on day 8 postinfection. CD43^{-/-} showed reduced numbers, ~1.5- to 2-fold compared to CD43^{+/+} ($n = 7$). *, $p < 0.5$; **, $p < 0.01$ (Student's *t* test). *C*, Splenocytes were cultured with virally infected target cells for 5 h at the indicated E:T ratio and ⁵¹Cr release was measured. Both ^{+/+} and ^{-/-} mice showed similar cytolytic killing of target cells. Similar results were obtained when data were normalized for CD8-specific responses. *D*, CD8 T cell-specific responses to LCMV subdominant epitopes D^bgp276-306 and K^bNP₂₀₅₋₂₁₂ were analyzed by intracellular cytokine staining on day 8 postinfection.

the absence of CD43 (9). We examined the role of CD43 in trafficking of CD8 T cells to the brain of i.c. infected mice. In this model, infiltration of LCMV-specific CD8 T cells to the meninges of i.c. infected mice results in mortality, and the onset of symptoms leading to mortality is rapid and acute (reviewed in Ref. 24). CD43^{+/+} or CD43^{-/-} mice were infected with 1×10^2 or 1×10^5 PFU LCMV-Armstrong i.c. and survival was examined. Although both ^{+/+} and ^{-/-} mice demonstrated 100% mortality regardless of the dose used, CD43^{-/-} mice showed a delay in the onset of mortality at both high and low viral doses (Fig. 3A and data not shown). These results suggested that, while functional, the CD43^{-/-} CD8 T cells may be defective in their ability to traffic to the brain.

We examined this in more detail by analyzing T cell responses in isolated mononuclear cells from brain vs spleen at day 6 post-challenge i.c. At this time point, while CD43^{-/-} and CD43^{+/+} mice showed similar numbers of Ag-specific CD8 T cells in the spleen, CD43^{-/-} mice showed fewer virus-specific CD8 T cells in isolated mononuclear cells from brain (Fig. 3B). By comparing CD44^{high} CD8 T cells in the spleen and the brain as a ratio, we saw that this difference in activated CD8 T cells in the brain exceeds the difference seen in the spleen and suggests this difference is

more than that seen from costimulation only (data not shown). Immunohistochemistry of brain sections confirmed that CD43^{-/-} mice had reduced CD8 T cell infiltrates in the leptomeninges (Fig. 3, *C* and *D*), as well as choroid plexus and ventricular ependyma. These results suggest that CD43 plays a significant role in the trafficking of CD8 T cells to the CNS. Additionally, it supports previously published work using Ab blockade of CD43 to prevent T cell infiltration to inflamed pancreatic islets and salivary and lacrimal glands of nonobese diabetic mice (10, 25). However, this defect in trafficking appears to be tissue specific, as we saw no defect in trafficking of T cells to the liver of infected CD43^{-/-} mice (as shown later). Furthermore, CD43^{-/-} mice rejected skin allografts at days 10-13, similar to CD43^{+/+} mice, suggesting no defect in trafficking of CD43^{-/-} T cells to skin. Thus, different tissues may have different CD43-dependent mechanisms of T cell migration during immune responses.

CD43 plays a negative regulatory role in the down-modulation of the immune response

When we examined the kinetics of the immune response in CD43^{-/-} mice we saw a modest role in the expansion phase of the

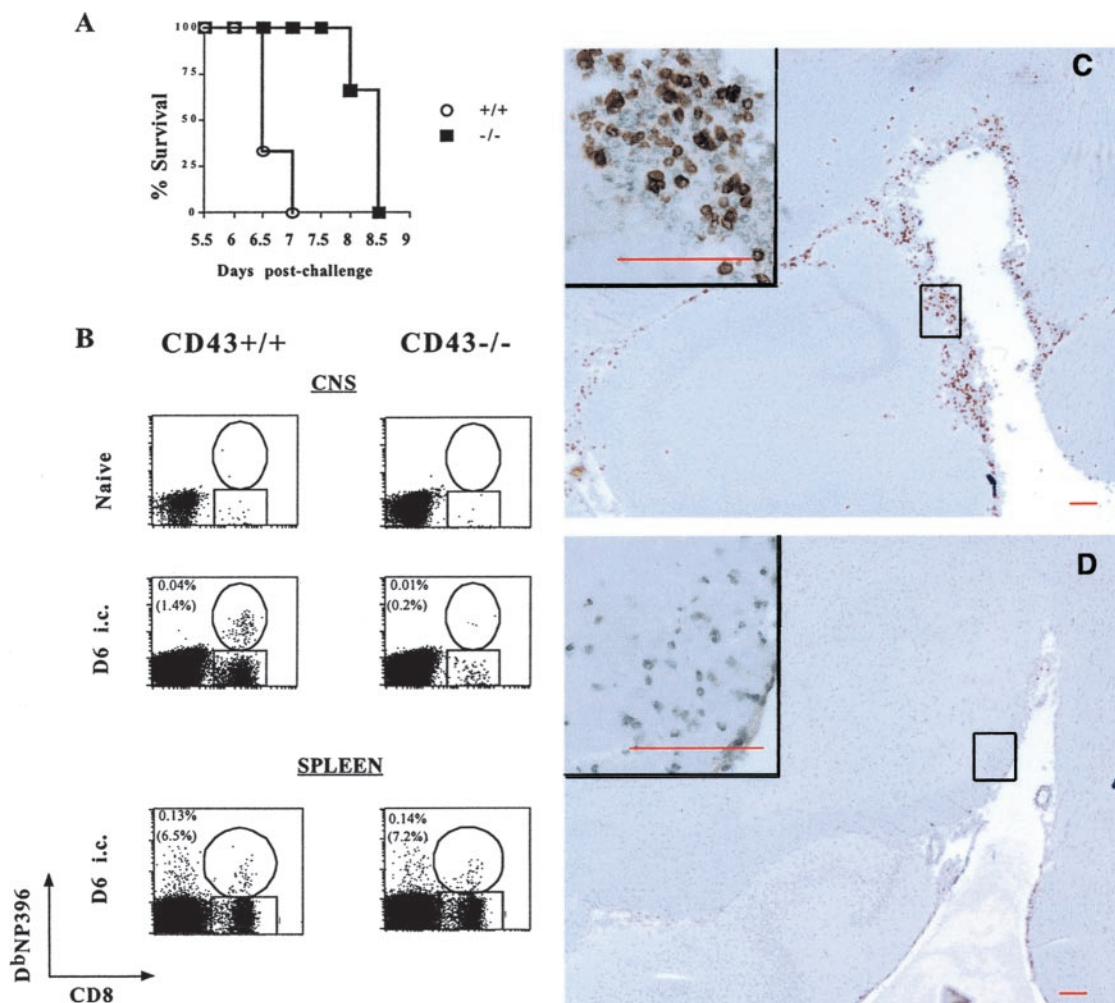


FIGURE 3. Role for CD43 in trafficking of CD8 T cells to CNS. *A*, Mice were infected with 100 PFU Armstrong i.c. and the percentage of survival was measured. CD43^{-/-} mice showed increased survival time relative to CD43^{+/+} mice of ~24–36 h, even at higher infection doses (data not shown). While death is delayed, all CD43^{-/-} mice eventually succumb to disease. *B*, On day 6 postinfection i.c., CD8 T cell responses were analyzed by flow cytometry in splenocytes and CNS mononuclear cells. While similar responses were seen in the spleen at this time, CD43^{-/-} mice showed reduced Ag-specific CD8 T cells in CNS mononuclear cells by flow cytometry. The upper number indicates the percentage of total Ag-specific cells; the lower number in parentheses indicates the percentage of CD8 T cells. *C* and *D*, Photomicrographs of sagittal brain sections from mice at 6 days postinfection showing CD8⁺ T cell infiltrates in the meninges of CD43^{+/+} mice (*C*) vs CD43^{-/-} mice (*D*). Scale bar = 100 μ m. Uninfected mice showed few CD8 T cells in both CD43^{+/+} and CD43^{-/-} mice (data not shown).

response but, surprisingly, a significant delay in the down-modulation or waning of the response (Fig. 4). Days 8–15 following LCMV challenge are characterized by a precipitous loss in the numbers of Ag-specific T cells, usually resulting in an ~10-fold reduction of Ag-specific T cells (26, 27). On day 15 postinfection, CD43^{-/-} mice showed consistently higher numbers of activated CD8 T cells, demonstrating only an ~2-fold reduction in Ag-specific T cells, suggesting a defect in the death or increased proliferation of these cells (Fig. 4).

This delay in the down-modulation of the response is not specific to viral responses, because we observe this in allospecific responses as well. In skin allograft responses, the peak of the allospecific T cell response is around day 12 and wanes by day 16 (28). When CD43^{-/-} mice were given an allogeneic skin graft (H-2^d), they rejected the graft with similar kinetics to CD43^{+/+}, approximately days 10–13 postgraft (Fig. 5). However, they had slightly reduced numbers of allospecific T cell responses (1.7-fold) at the peak of the response (day 12), showing that, similar to the viral infection data, there is a modest effect on the generation of the T cell response (Fig. 5). During the contraction phase, by day 16,

CD43^{-/-} mice showed increased allospecific T cell numbers relative to wild-type mice (Fig. 5). Taken together with the acute LCMV response, the absence of CD43 resulted in a delay in the waning or contraction of the T cell response, resulting in longer persistence of Ag-specific T cells in vivo.

Increased numbers of activated T cells not due to increased proliferation

To determine whether this increase in numbers of activated T cells during the contraction phase was due to increased proliferation of ^{-/-} T cells, CD43^{+/+} or CD43^{-/-} mice were infected with LCMV-Armstrong, and on days 8–15 postinfection they were given BrdU in their drinking water. On day 15 postinfection, mice were sacrificed and BrdU incorporation was examined in CD8 T cells from the spleen, liver, and PBMC. CD43^{-/-} virus-specific CD8 T cells showed no increased BrdU incorporation in any of the tissue compartments examined, suggesting no increased proliferation during the contraction phase, even though increased numbers of Ag-specific CD8 T cells were observed (Fig. 6). These data

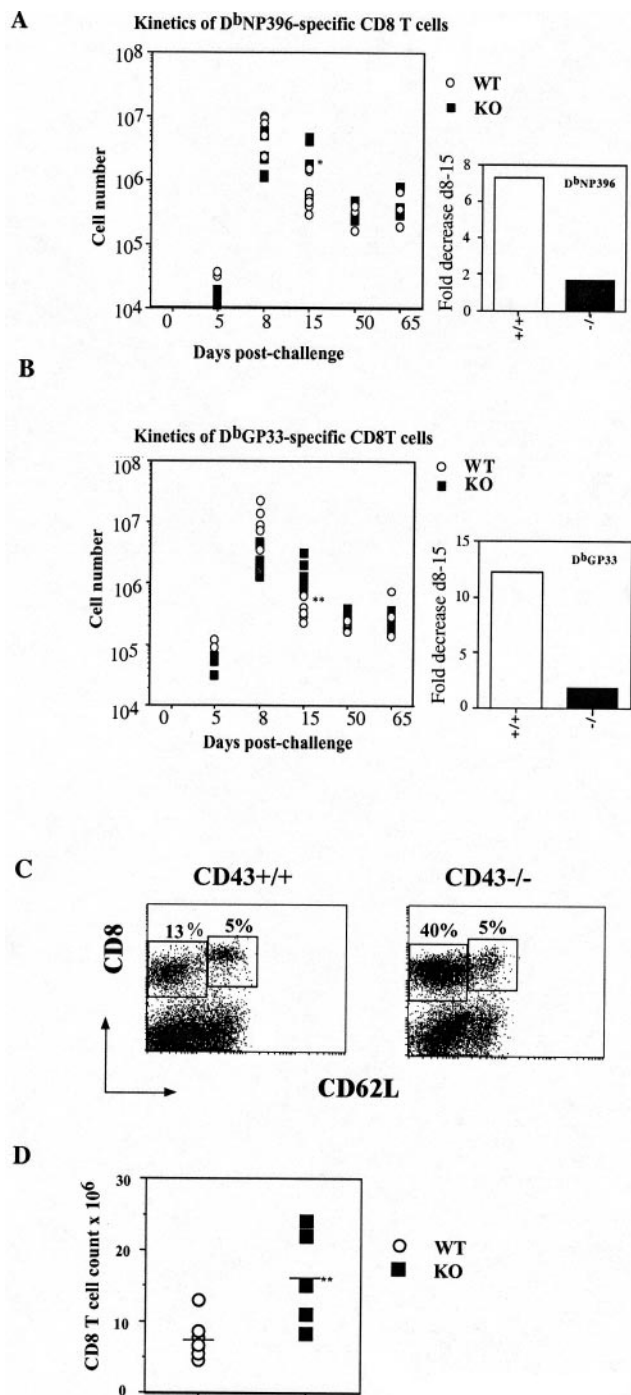


FIGURE 4. Kinetics of D^bgp33-specific and D^bNP₃₉₆-specific CD8 T cell responses in CD43^{+/+} vs CD43^{-/-} mice suggest a role for CD43 in down-modulation of immune response. Mice were infected with 2 × 10⁵ LCMV-Armstrong i.p. and at days 5, 8, 15, 50, and 65 postinfection, and virus-specific splenic CD8 T cells were quantitated by MHC class I tetramers D^bNP₃₉₆ (A) or D^bgp33 (B). The expansion of virus-specific CD8 T cells was slightly reduced in CD43^{-/-} mice. However, during the contraction phase (days 8–15), the decline or loss of virus-specific CD8 T cell numbers is delayed in CD43^{-/-} relative to CD43^{+/+} mice (*, p < 0.05; **, p < 0.01). While the fold decrease in CD43^{+/+} mice during days 8–15 is ~10-fold, the fold decrease in CD43^{-/-} mice is ~2-fold. Both CD43^{+/+} and CD43^{-/-} mice showed similar virus-specific CD8 T cells in the memory pool on days 50 and 65, suggesting that this is a transient defect. On day 15 postinfection CD43^{-/-} mice show increased percentages (C) and numbers (D) of activated phenotype CD8 T cells (CD62L^{low}) compared to CD43^{+/+} mice (p < 0.01). Each symbol represents one mouse with a minimum of n = 3 for each genotype at each time point.

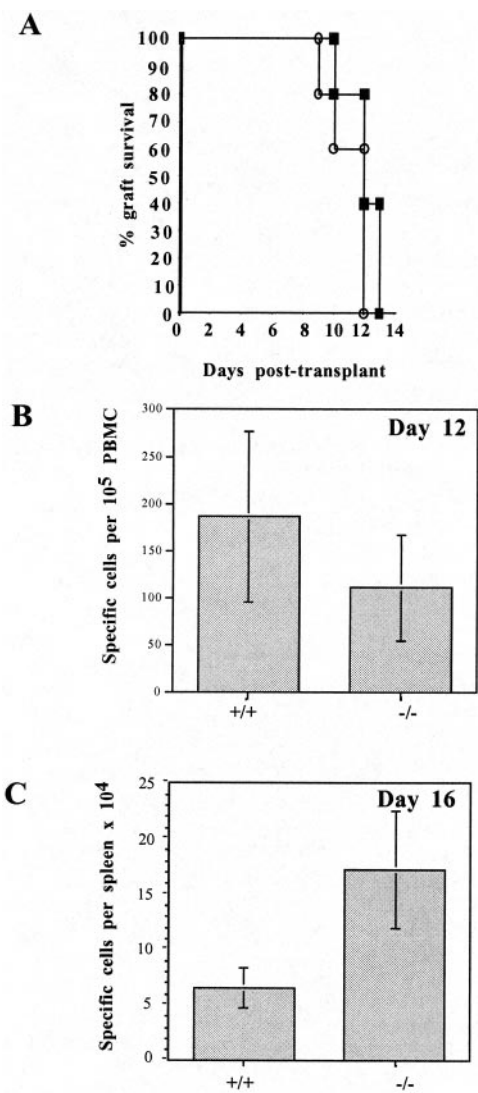


FIGURE 5. Defective down-modulation in allospecific responses. A, CD43^{+/+} mice (○) and CD43^{-/-} mice (■) received H-2^d skin allografts and skin graft survival was measured. Both CD43^{+/+} and CD43^{-/-} mice rejected grafts with similar kinetics between days 10 and 13. On days 12 (B) and 16 (C) after receiving grafts, allospecific responses were measured by IFN-γ ELISPOT assay. CD43^{-/-} mice show slightly decreased numbers of allospecific responses in the PBMC on day 12 after graft, the peak of the response in wild-type mice (1.5- to 2-fold). B, During the waning of the response on day 16, CD43^{-/-} mice show increased numbers of allospecific splenic T cells compared to CD43^{+/+}. Data from one of two similar experiments using +/+ (n = 5) and -/- (n = 5) are shown.

argue against increased proliferation of CD43^{-/-} CD8 T cells during the contraction phase. However, the defect in the contraction phase is not permanent, as immune mice at days 50 and 65 show similar numbers of Ag-specific CD8 T cells (Fig. 4). Rather, it is a transient defect or delay.

Increased Bcl-2 expression and decreased apoptosis of virus-specific -/- CD8 T cells

We examined whether this delay in the contraction phase was due to decreased death, because we saw no evidence of increased proliferation. We examined bcl-2 levels in Ag-specific effector CD8 T cells from CD43^{-/-} mice. Bcl-2 has been characterized as an antiapoptotic protein and its overexpression has been found to increase resistance to apoptosis (29). Following viral infection, bcl-2

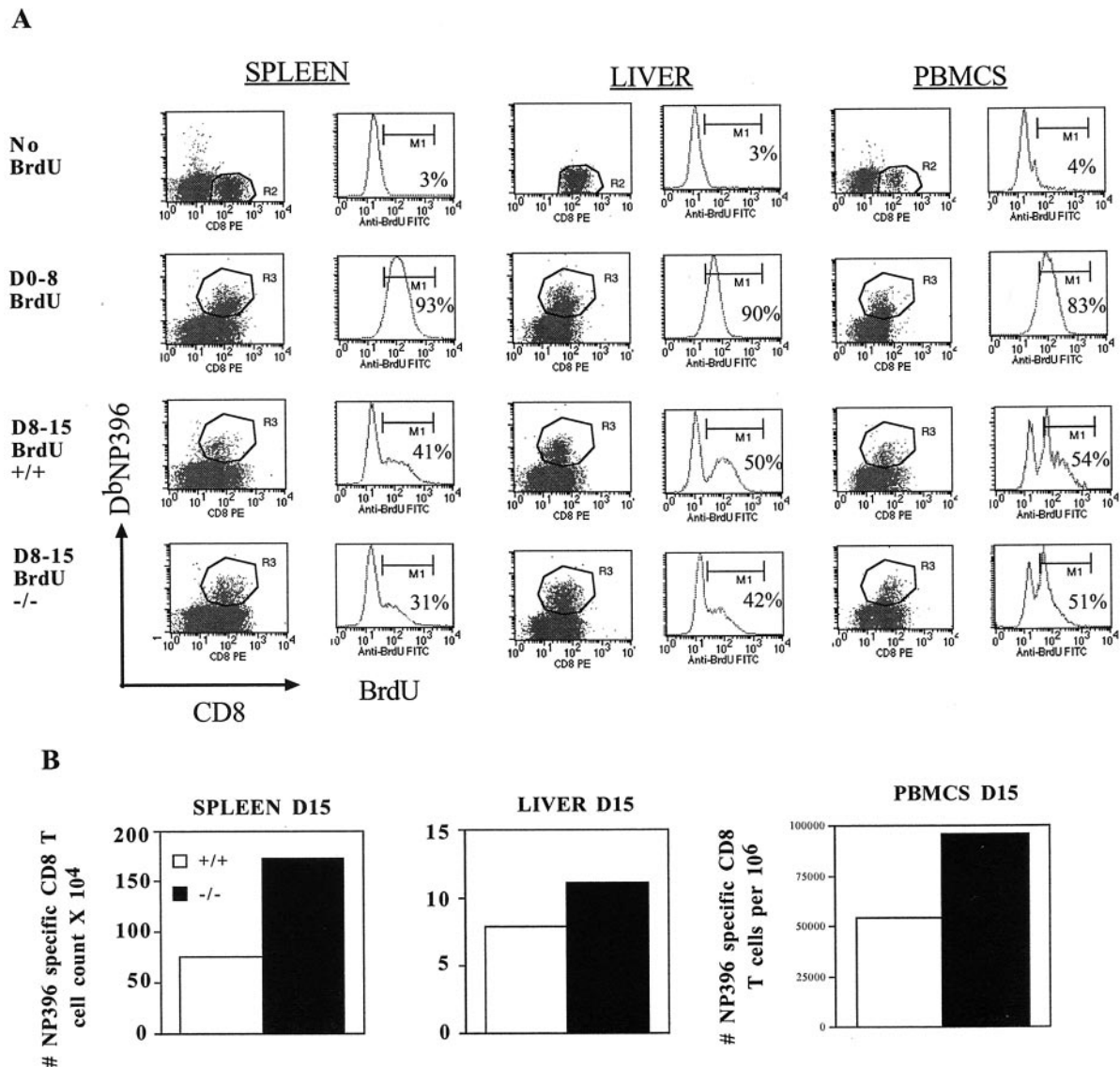


FIGURE 6. Increased numbers of activated CD8 T cells in CD43^{-/-} mice during the contraction phase is not due to increased proliferation. **A**, Mice were infected with 2×10^5 PFU LCMV-Armstrong and given 1 mg/ml BrdU in drinking water for days 0–8 (control) or days 8–15 (CD43^{+/+} or CD43^{-/-}). Ag-specific responses were analyzed in lymphocytes from spleen, liver, and PBMC, and BrdU incorporation was measured. Analysis of a representative animal is shown. Analysis of CD43^{-/-} animals revealed no increased BrdU incorporation relative to CD43^{+/+}, suggesting no increased proliferation of Ag-specific CD8 T cell on days 8–15. **B**, D^bNP₃₉₆-specific CD8 T cell responses in the spleen, liver, and PBMCs of CD43^{+/+} and CD43^{-/-} mice show increased numbers on day 15 in each compartment analyzed in CD43^{-/-} mice ($n = 3$).

expression has been found to decrease on effector cells compared with naive T cells and then increase again on memory T cells to higher levels than on naive (27, 30). CD43^{-/-} effector virus-specific T cells showed increased levels of bcl-2 compared with CD43^{+/+} (Fig. 7). However, directly ex vivo, we saw similar percentages (3–6%) of apoptotic Ag-specific CD8 T cells by Annexin V and 7-AAD at day 8, 10, or 12 (data not shown). Because apoptotic cell uptake is very efficient in vivo, we examined death of effector Ag-specific CD8 T cells from +/+ or -/- mice after in vitro culture. Culturing of an equal number of spleen cells overnight revealed 2- to 3-fold increased percentages of Annexin V⁺7-AAD⁺ cells in +/+ vs -/- effector CD8 T cells (Fig. 7). Taken together with our results showing no increased BrdU incorporation of -/- CD8 T cells, these results suggest that the increased numbers of Ag-specific CD8 T cells during the contraction phase of an immune response in CD43^{-/-} mice is most likely the result of a defect in apoptosis of effector CD8 T cells. These

data would support studies suggesting a role for CD43 in apoptosis of leukocytes (31, 32).

Chronic infection results in increased mortality in CD43^{-/-} mice

We examined chronic infection in CD43^{+/+} vs CD43^{-/-} mice using a persistent strain of LCMV, clone 13. Because the virus is not cytopathic, LCMV is a classic model of immunopathology. Tissue damage that occurs following infection is entirely dependent on the immune response (reviewed in Ref. 24). Previous reports have suggested that functional inactivation or down-modulation of immune responses during chronic infection are critical for host survival (33–35). When previously uninfected adult mice are infected with LCMV clone 13 i.v., the virus replicates and disseminates rapidly (36). It can persist in the serum for up to 100 days, and remains indefinitely in some tissues. When CD43^{-/-} mice were infected with LCMV clone 13 they showed similar

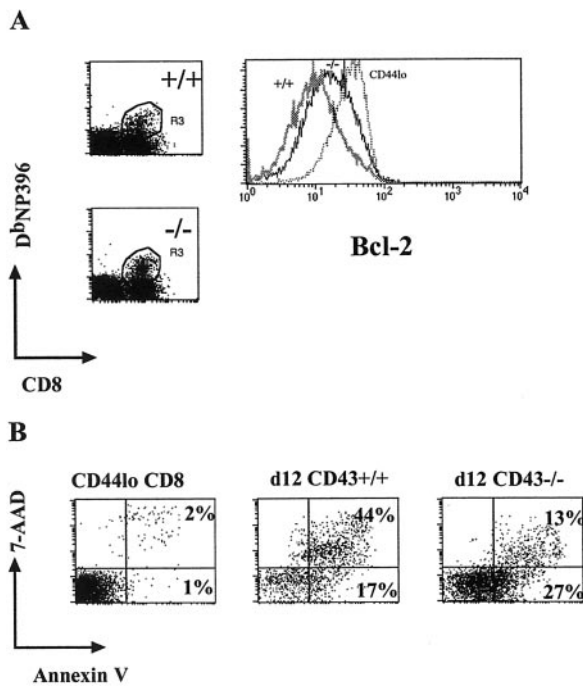


FIGURE 7. Increased Bcl-2 levels and decreased death of Ag-specific CD8 T cells from CD43^{-/-} mice. *A*, Virus-specific CD8 T cells from CD43^{+/+} mice (thick line) and CD43^{-/-} mice (thin line) were assayed for Bcl-2 levels ex vivo by FACS on day 10. D^bNP_{396–404}-specific effector CD8 T cells from CD43^{+/+} mice show reduced Bcl-2 levels relative to naive CD44^{low} CD8 T cells (dotted line), as previously shown. CD43^{-/-} Ag-specific CD8 T cells show higher levels of Bcl-2 compared to CD43^{+/+}. *B*, Splenocytes from LCMV-infected (day 12) CD43^{+/+} and CD43^{-/-} were cultured overnight and apoptotic cells were assayed by 7-AAD and Annexin V staining. Analysis of virus-specific CD8 T cells revealed decreased percentages of 7-AAD⁺Annexin V⁺ cells in CD43^{-/-} mice compared to CD43^{+/+}. Similar results were seen from cultures on days 8 and 10.

Ag-specific responses in spleen, liver, and PBMC compartments at day 8 (Fig. 8A). Furthermore, day 8 postinfection viral titers in the serum of +/+ and -/- mice are comparable (Fig. 8B). However, CD43^{-/-} mice showed dramatically increased disease following day 7 postinfection and increased mortality relative to wild-type mice (Fig. 8C). Together with the data from acute infection showing persistence of activated CD8 T cells during the contraction phase in vivo, these data support a negative regulatory role of CD43 on activated CD8 T cells. During chronic viral infection, CD43 appears to play a critical negative regulatory role during down-modulation, and in its absence immunopathology may result, causing increased mortality. These results argue for a very dynamic role of the CD43 glycoprotein during an immune response reflective of the dynamic modification through the expression and glycosylation it undergoes during an immune response. On naive T cells it may play a more positive regulatory role in costimulation and trafficking, while on effector T cells it may play a more negative regulatory role in mediating the death of effector T cells.

Discussion

Putative mechanisms underlying positive and negative regulatory roles of CD43

The exact role of CD43 has not been well defined, due to its seemingly contradictory roles in several processes such as cell adhesion and costimulation. We propose that, depending on the state of activation of the T cell, CD43 may have positive or negative regulatory

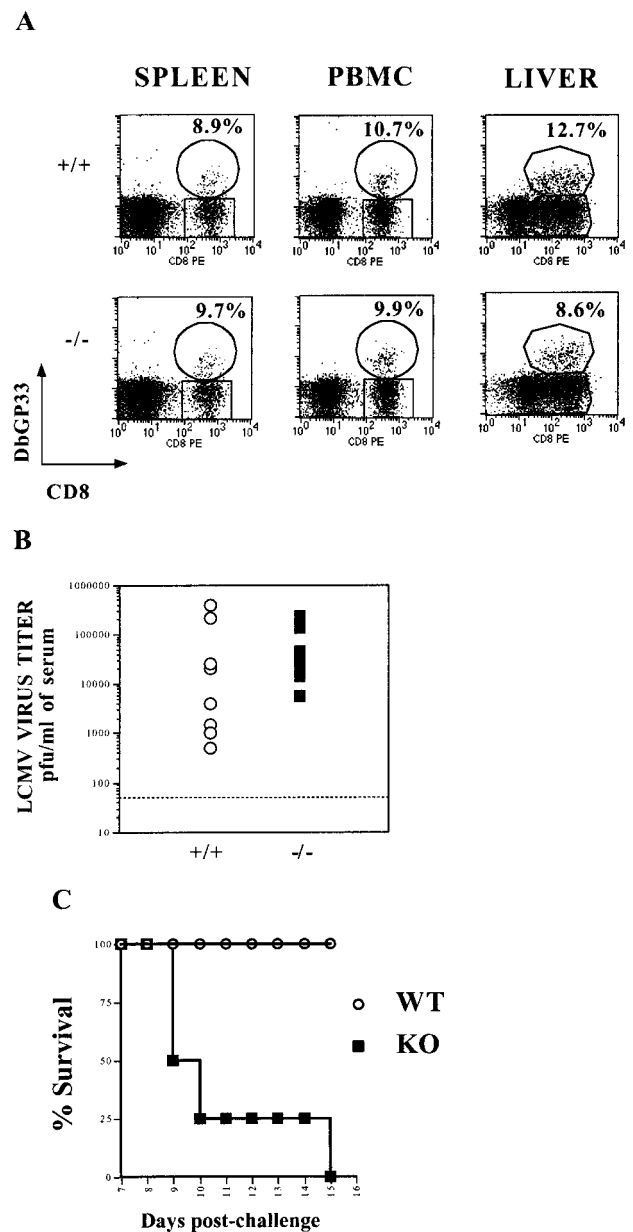


FIGURE 8. Increased mortality following chronic LCMV infection. *A*, CD43^{+/+} and CD43^{-/-} mice were infected with LCMV clone 13, which causes chronic infection. On day 8, spleen, liver, and PBMCs were harvested, and similar Ag-specific responses were seen. *B*, LCMV virus titer on day 8 postchallenge in the serum was measured by plaque assay and CD43^{+/+} and CD43^{-/-} mice showed similar viral loads ($n = 10$). The dashed line represents the limit of detection for this assay. *C*, After infection with LCMV clone 13, CD43^{-/-} mice show more disease by day 7 and become moribund beginning on day 8 postinfection. In three experiments, survival was 10 of 10 for +/+ and 0 of 10 for -/-.

roles during an immune response. On a naive T cell, the 115-kDa glycoform is abundantly sialylated, and this large negatively charged glycoprotein seems to play a negative regulatory role in T cell homing (9) and activation in vitro (8). However, our work suggests that, on activated T cells, CD43 and other cell surface glycoproteins become hypsialylated. This reduction in sialic acid content could result in reduced binding to endogenous lectins such as the recently described CD43 counterreceptor Siglec-1 (37), a sialic acid-binding lectin, and enhanced binding to lectins such as MMGL (38) and galectin-1 (39) that recognize terminal galactose residues on cell surface glycoproteins. Thus, depending on the type of ligand/receptor interactions

being studied, differential glycosylation of CD43 could control the type or magnitude of specific T cell interactions.

Interestingly, CD43 expression as assayed by S7 binding is differentially regulated on CD4 vs CD8 T cells. CD8 T cells show constitutive S7 binding on both CD44^{high} and CD44^{low} CD8 T cells, while CD4 T cells show S7 binding on CD44^{high} but not CD44^{low} T cells (40). However, CD4 T cells and CD8 T cells show increased binding of 1B11 on CD44^{high} cells. This differential expression of CD43 may play a role in differential activation and clearance rates of CD8 T cells and CD4 T cells (41).

Our finding showing no increase in 1B11 binding of CD43^{-/-} effector CD8 T cells *in vivo* extends previous work noting low-level binding to thymocytes and Con A blasts of CD43^{-/-} mice (7). This group saw 1B11 reactivity in CD43^{-/-} mice on a subset of lymph node cells and noted low-level binding to a small population of CD8 splenocytes in CD43^{-/-} mice as data not shown. They suggested this 1B11 reactivity was binding an epitope on CD45RB that is independent of core 2 *O*-glycans. Importantly, they clearly state this binding on blasts and thymocytes is much less than 1B11 binding on wild-type cells, which agrees with our work on Ag-specific CD8 T cells. Our data show clearly that on virus-specific effector and memory CD8 T cells generated after an *in vivo* challenge the up-regulated binding of 1B11 is dependent on CD43 expression.

Mortality in mice following LCMV infection *i.c.* has been shown to be critically dependent on functional CD8 T cell infiltration of the brain (reviewed in Ref. 24). We show that CD43^{-/-} mice infected with LCMV-Armstrong *i.c.* show a delay in mortality, and this delay is consistent with our findings showing reduced numbers of CD8 T cell infiltrates in the CNS of these mice. These results are suggestive of a defect in CD8 T cell trafficking to the CNS, but they do not rule out alternative mechanisms. One report examining CD43 expression in brain tissue of normal and Alzheimer's disease cases demonstrated the expression of CD43 on human microglia (42). It is possible that the reduction in CD8 T cell numbers in the CNS of CD43^{-/-} mice could be secondary to a defect in microglial cells. Thus, the contribution of other cell types to the defect seen in these mice remains to be determined.

Recent cross-linking studies have suggested that T cell activation through CD43 leads to *vav* tyrosine phosphorylation and induces Cbl-serine phosphorylation (4, 43). Cbl and cbl-b have been shown to negatively regulate T cell activation (44–46). Interestingly, Wiskott-Aldrich syndrome protein (WASp) is purported to be a downstream signaling target of the cbl-b signaling cascade (44). CD43 was earlier characterized because of its disrupted expression/glycosylation on leukocytes from patients suffering from the X-linked immunodeficiency Wiskott-Aldrich syndrome (47, 48). It has been demonstrated that mutation(s) in WASp is the causative mutation for this disease (49). T cells from WASp^{-/-} mice fail to form effective TCR caps, resulting in defects in signaling through the TCR (50). Recent work has shown that CD43 is excluded from the TCR-APC synapse and that this movement of CD43 away from the synapse is mediated by ezrin-radixin-moesin-dependent mechanisms (14–17). Thus, the dysregulation of CD43 in patients with Wiskott-Aldrich syndrome may serve as some sort of compensatory mechanism in T cell activation in these patients due to cytoskeleton considerations. Future studies will aim at examining TCR signaling in Ag-specific effector and memory CD43^{+/+} and CD43^{-/-} CD8 T cells.

Finally, it is interesting to note that the high-molecular mass glycoform of CD43 remains high on T cells during chronic infection in both mice (T. Onami and R. Ahmed, unpublished data) and humans (51). During the course of an acute infection, CD43 on activated T cells seems to serve to regulate the numbers of acti-

vated T cells that remain during the waning of the response. Perhaps during a chronic infection CD43 may serve to regulate numbers and/or responsiveness of the T cell to persisting Ag. Future studies will examine these questions in more detail.

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