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CD44 Regulates Survival and Memory Development in Th1 Cells

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SUMMARY

Optimal immunity to microorganisms depends upon the regulated death of clonally expanded effector cells and the survival of a cohort of cells that become memory cells. After activation of naive T cells, CD44, a widely expressed receptor for extracellular matrix components, is upregulated. High expression of CD44 remains on memory cells and despite its wide usage as a "memory marker," its function is unknown. Here we report that CD44 was essential for the generation of memory T helper 1 (Th1) cells by promoting effector cell survival. This dependency was not found in Th2, Th17, or CD8⁺ T cells despite similar expression of CD44 and the absence of splice variants in all subsets. CD44 limited Fas-mediated death in Th1 cells and its ligation engaged the phosphoinositide 3-kinase-Akt kinase signaling pathway that regulates cell survival. The difference in CD44-regulated apoptosis resistance in T cell subpopulations has important implications in a broad spectrum of diseases.

INTRODUCTION

In becoming memory cells, T cells undergo stages of dramatic expansion and contraction that depend upon regulated cell death and thereafter are maintained by survival signals from the environment. Survival of T cells during a response can be profoundly affected by the availability of costimulatory molecules and cytokines that modulate engagement of death pathways (Krammer et al., 2007). Once a response subsides, common gamma chain cytokines, such as interleukin-7 (IL-7) and IL-15, are essential to the homeostatic control of T cell memory (Boyman et al., 2007). However, as predominantly mobile populations, both effector and memory T cells have the potential to receive additional signals through adhesive interactions with the extracellular matrix (ECM) or other cells (Gilmore, 2005). CD44 is an adhesion molecule that is expressed by most cells and mediates binding to the ECM and other cells via its only known in vivo ligand, the glycosaminoglycan hyaluronic acid (HA) (Ponta et al., 2003). CD44 expression is upregulated on naive T cells after activation via the T cell receptor (TCR) and high expression is maintained indefinitely on memory cells (Puré and Cuff, 2001). As a consequence, elevated expression of CD44 is generally used to identify antigen-experienced T cells.

CD44 is associated with cell migration and together with HA has been implicated in numerous biological processes that are regulated by migrating cells (Ponta et al., 2003). The function of CD44 differs for different cell types and additional roles in the regulation of proliferation and apoptosis have been described (Hauptschein et al., 2005; Puré and Assoian, 2009). CD44 is the product of a single gene that gives rise to a family of HA-binding molecules by alternative exon RNA splicing (Lynch, 2004). In addition to the nonvariant or standard form of CD44, at least five isoforms are generated through translation of various combinations of 10 variable exons, which are inserted into a single site in the membrane proximal region of the extracellular domain. Additional cell type-specific posttranslational modifications of CD44 include differences in glycosylation (Camp et al., 1991). The variable forms of CD44 contribute to functional variations that allow for diverse interactions of cells with their environments through a variety of signaling events, which are not yet fully defined and can vary in different cell types (Puré and Assoian, 2009).

Whereas CD44 has the potential to participate in several processes associated with immune responses, the physiological functions of CD44 in T cells in vivo remain ill defined. It has been established that T cells bind HA, and that either HA binding or TCR signaling can augment the adhesive function and expression of CD44 (DeGrendele et al., 1997; Lesley et al., 1994). CD44 together with VLA-4 (α4 integrin) can regulate T cell migration into sites of inflammation (Nandi et al., 2004), and the association of these receptors correlates with enhanced T cell motility and survival after TCR stimulation in vitro (Marhaba et al., 2006). The binding of CD44 expressed on T cells to HA on the surface of dendritic cells (DCs) can promote cell clustering (Do et al., 2004) that can be blocked by HA inhibitors (Mummert et al., 2002). Although ligation of CD44 does not elicit proliferation of T cells, it can activate the TCR signaling-associated src family kinases Lck and Fyn. This suggests that induction of signaling events by CD44 impacts the T cell response (Rozsnyay, 1999), including that to TCR engagement (Föger et al., 2000). CD44 has been associated with both resistance (Marhaba et al., 2006; Naor et al., 2007; Wittig et al., 2000) and susceptibility (McKallip et al., 2002; Nakano et al., 2007) of activated T cells to apoptosis, suggesting that it participates in the control of expansion. However, although CD44 is broadly connected with the regulation of T cell responses, distinguishing direct roles in vivo has remained elusive, prompting us to study its function in CD4⁺ T cells.

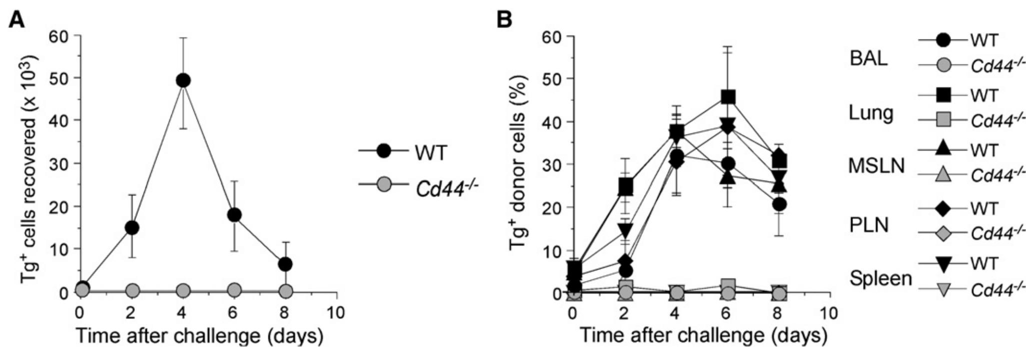

Figure 1. Requirement for CD44 in the Generation of Memory Responses in CD4+ T Cells

CFSE-labeled OT-II cells from WT (Ly5.1) and *Cd44* $^{-/-}$ (Thy1.1) mice were cotransferred (3×10^5 each) into C57BL/6 recipients (Ly5.2, Thy1.2) that were then infected with WSN-OVA_{II}. After 22 days, the recipients were challenged with HKx31-OVA_{II}.

(A) The recovery of Tg^+ WT and *Cd44* $^{-/-}$ cells in the MSLN from individual animals.

(B) The percentage Tg^+ cells in the $V\beta5^+$, $CD4^+$ population from BAL, lung, MSLN, PLN, and spleen (mean ± SEM, n = 3–4/group).

By using a murine model of influenza virus infection in which a Th1 cell response is induced, we found that memory in $CD4^+$ T cells failed to develop in the absence of CD44 engagement. Although the development of effectors appeared to proceed normally without CD44, $CD4^+$ T cells failed to survive because of apoptosis that engaged caspase-8, suggesting the involvement of extrinsic death-receptor signaling. Unexpectedly, Th1 cells, but not Th2, Th17, or activated $CD8^+$ T cells, showed a CD44 requirement for survival and resistance to apoptosis induced by Fas-engagement in vitro, which correlated with higher expression of Fas but not with differences in CD44. Further, ligation of CD44 in vivo enhanced Th1 cell accumulation and in vitro engaged the Akt kinase signaling pathway, which can promote survival of activated $CD4^+$ T cells (Jones et al., 2002; Varadhachary et al., 2001). Thus, CD44 maintained Th1 cells through active control, which could be mediated by physical contacts with HA in the ECM or on other cells. The results supported the concept that other subsets of T cells were less susceptible to death in part because of inherent differences in Fas expression (Varadhachary et al., 1999; Zhang et al., 1997). This differential regulation may permit strategies for immunotherapeutic targeting of Th1 cells in pathological responses to infections and in autoimmune diseases.

RESULTS

Loss of CD4+ T Cell Memory in the Absence of CD44

To investigate the role of CD44 in the development of immunity, we used an influenza model in which viral clearance from the lung epithelium depends upon a local T cell response. In an initial comparison of wild-type (WT) and CD44-deficient (*Cd44* $^{-/-}$) mice, we found that $CD4^+$ and $CD8^+$ lymphocyte subsets were normally represented in *Cd44* $^{-/-}$ mice, as previously reported (Schmits et al., 1997), because of additional compensatory HA binding receptors (Figure S1A available online; Naor et al., 2007). Moreover, we did not find differences in expression of several adhesion receptors, including CD62L, the integrins CD11a and CD49, CD45RB, and CD69 on $CD4^+$ T cells from 6-month-old animals (Figure S1B). Because CD44 is expressed by multiple cell types, the role of CD44 in $CD4^+$ T cells was directly assessed with WT and *Cd44* $^{-/-}$ mice crossed to OT-II TCR transgenic (Tg) mice whose $CD4^+$ T cells recognize a peptide of ovalbumin (OVA_{II} or $OVA_{323\text{-}339}$) (Barnden et al., 1998). Naive WT and *Cd44* $^{-/-}$ Tg $CD4^+$ T cells marked by expression of the $V\beta5$ chain of the TCR and by the allelic variants of Ly5.1 (CD45.1) or Thy1.1 (CD90.1), respectively, were isolated by negative selection from the lymphoid tissues of 6-week-old donors. The WT and *Cd44* $^{-/-}$ cells were coinjected in equal numbers into normal C57BL/6 mice (Thy1.2, Ly5.2) to provide an internal control for the response. The cells were titrated to the lowest number needed for a consistently detectable response (3×10^5), where all cells became engaged as measured by division that was detected by CFSE dilution. The recipients were then infected with the William Smith Neurotropic (WSN) influenza A virus (H1N1) expressing the OVA_{II} peptide (WSN-OVA_{II}) (Chapman et al., 2005). $CD4^+$ T cell memory development was assessed by challenging recipients 3 weeks later with the recombinant influenza virus Hong Kong Aichi.2.68 x31 (H3N2) that also expressed the OVA_{II} peptide (HKx31-OVA_{II}) (Thomas et al., 2006). The kinetics of cell expansion were evaluated as measured by the recovery of donor cells.

At the time of challenge, the frequencies of donor cells were very low. As anticipated, a memory response was observed from WT OT-II donor cells in the draining mediastinal lymph nodes (MSLN) as indicated by a >50-fold increase in the number of Tg^+ $CD4^+$ T cells on day 4, the peak of the response (Figure 1A). Dramatic increases in the frequencies of WT OT-II cells were also observed in the airways (retrieved by bronchoalveolar lavage, BAL) and lungs, as well as other lymphoid tissues (peripheral lymph nodes, PLN, and spleen) (Figure 1B). However, $CD4^+$ T cells from the *Cd44* $^{-/-}$ OT-II donors were undetectable in the MSLN (Figure 1A) and were present only in very low numbers systemically (Figure 1B). The data suggest that expression of CD44 on $CD4^+$ T cells might be necessary for either the appropriate development of memory cells or their capacity for expansion.

Unimpaired Induction of CD4+ T Cell Responses in the Absence of CD44

CD44 can regulate T cell migration via interactions with vascular endothelium through HA, which acts to initiate extravasation into

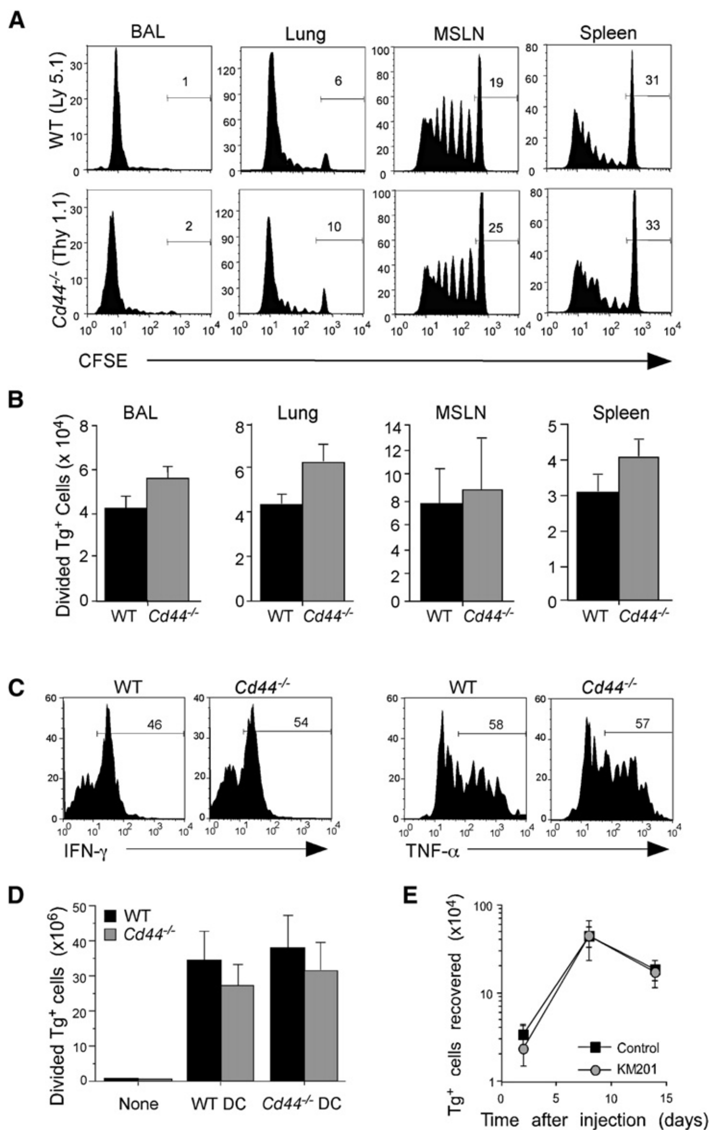

Figure 2. CD44 Independence of CD4$^+$ T Cell Priming

C57BL/6 mice were injected with CFSE-labeled WT and *Cd44*$^{-/-}$ OT-II cells (1.5 × 10^6 each) and infected with WSN-OVA$_{II}$.

(A) After 8 days, division of Tg$^+$ cells was analyzed by CFSE. The marker on each histogram shows the fraction of undivided cells.

(B) The average recoveries of Tg$^+$ donor cells that underwent one or more divisions on day 8 (mean ± SEM, n = 3–4/group).

(C) IFN-γ and TNF-α production by WT and *Cd44*$^{-/-}$ cells after overnight restimulation by OVA$_{II}$ peptide with splenic APC.

(D) CFSE-labeled WT and *Cd44*$^{-/-}$ OT-II cells were coinjected into C57BL/6 recipients as for (A) together with 2 × 10^5 CD11c$^+$, OVA$_{II}$ peptide-pulsed DCs from either WT or *Cd44*$^{-/-}$ C57BL/6 mice. Recovery of donor CD4$^+$ T cells that had undergone one or more divisions in the spleen 4 days later (mean ± SEM, n = 4/group).

(E) WT OT-II Th1 cells were generated with APC and OVA$_{II}$ peptide in the presence of the blocking anti-CD44 mAb, KM201, or control IgG. The cells were then injected into separate groups of C57BL/6 recipients (2 × 10^6/mouse). The donor Tg$^+$ cells recovered in the pooled lymph node (LN) and spleens of mice are shown at the indicated times after injection (mean ± SEM, n = 3–4/group).

tissue (DeGrendele et al., 1997; Nandi et al., 2004). Therefore, the absence of CD44 could generally affect the trafficking of *Cd44*$^{-/-}$ CD4$^+$ T cells. Thus, we evaluated the recovery of naive *Cd44*$^{-/-}$ and WT CD4$^+$ T cells with time after transfer to unimmunized hosts. The presence of comparable numbers of naive and WT cells (Figure S2A) in the lymphoid compartment and no differences in their distribution (data not shown) suggest normal homeostatic regulation and migration. Because inflammation could affect CD4$^+$ T cell trafficking and because cells from *Cd44*$^{-/-}$ mice display the normal responses to TCR activation in vitro (Schmits et al., 1997), we tested whether the failure of *Cd44*$^{-/-}$ CD4$^+$ T cells to generate a memory effector population could be attributed to defects in trafficking of naive or effector cells after influenza virus infection. Homing of naive or in vitro-activated OT-II effector CD4$^+$ T cells to either lymphoid or nonlymphoid tissues was unimpaired by the absence of CD44 irrespective of whether the recipients were naive or were infected several days previously (Figures S2B and S2C). Thus, although CD44 can regulate homing of CD4$^+$ T cells (DeGrendele et al., 1997), this function is not essential and/or is replaceable by other adhesion receptors.

Although differences in the responses of *Cd44*$^{-/-}$ and WT OT-II cells to TCR stimulation with peptide and antigen-presenting cells (APCs) were not observed in vitro, it is possible that CD44 contributes to the initial priming or responses of CD4$^+$ T cells in vivo. Therefore, the division of adoptively transferred WT and *Cd44*$^{-/-}$ Tg$^+$ cells was analyzed by CFSE dilution at the peak of the response to WSN-OVA$_{II}$. Here we used a higher dose of Tg$^+$ cells to observe potential differences in the kinetics of division. Similar kinetics of division in WT and *Cd44*$^{-/-}$ cells (Figure 2A) and distribution of cells in the lymphoid tissues and lungs as measured by recovery (Figure 2B) were observed. This suggests that CD44 is not essential for either the induction of a CD4$^+$ T cell response to influenza virus or for the migration of CD4$^+$ T cells. Furthermore, both populations exhibited a similar capacity to develop effector function as measured by secretion of IFN-γ and TNF-α (Figure 2C). Additionally, we directly confirmed that expression of CD44 was not required on either CD4$^+$ T cells or DCs to initiate a primary response in vivo, as shown by the induction of comparable numbers of dividing WT and *Cd44*$^{-/-}$ cells after immunization with OVA peptide-pulsed WT or *Cd44*$^{-/-}$ splenic DCs (Figure 2D). To further investigate whether blocking CD44 during Th1 cell priming could predispose the cells to death, OT-II cells were treated with the CD44 adhesion-blocking mAb, KM201 (Zheng et al., 1995), during in vitro culture with APCs and peptide under Th1 cell polarizing conditions. Cell recoveries at day 4 were similar to the isotype control-treated cultures, and exposure to KM201 did not negatively affect the ability of Th1 cells to persist (Figure 2E). The results show that initial activation, expansion, and effector development proceed normally in *Cd44*$^{-/-}$ CD4$^+$ T cells.

These findings suggest that the mechanisms which limit the development of memory in CD4$^+$ T cells could become manifest at the later stages of the primary response. Therefore, cell

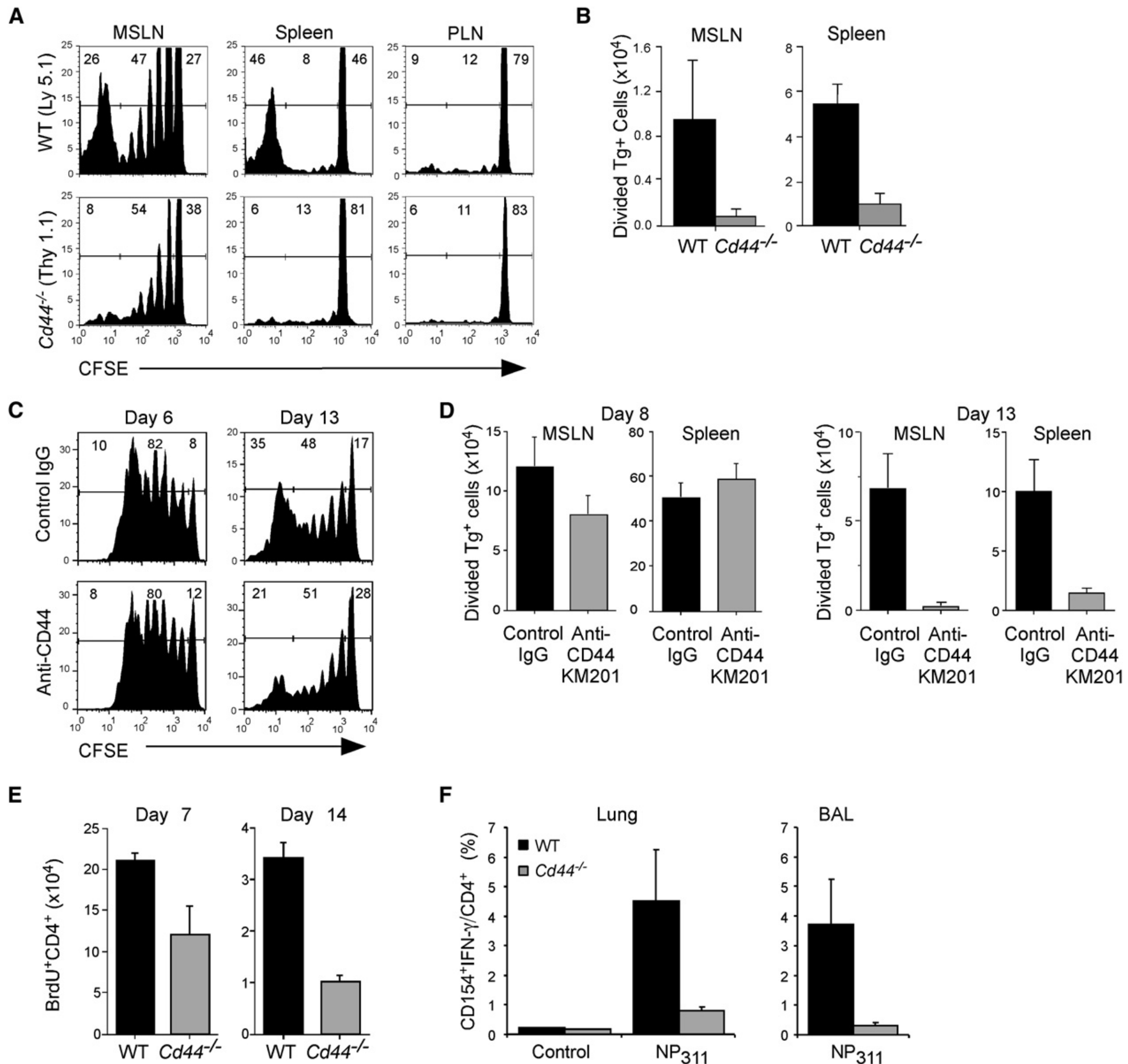

Figure 3. Loss of $CD4^+$ T Cell Effectors in the Absence of CD44 Engagement

C57BL/6 recipients were injected with CFSE-labeled WT and *Cd44*$^{-/-}$ OT-II cells (1.5×10^6 each) and infected with WSN-OVA_{II}.

(A and B) On day 13 after infection, division (A) and recovery (B) of Tg^+ cells was determined as for Figure 2 in the MSLN and spleen.

(C and D) Recipients of CFSE-labeled WT OT-II cells were injected with either KM201 anti-CD44 or control IgG at the time of cell transfer and infection with WSN-OVA_{II} influenza virus and three more times at 3 day intervals.

(C) The division of the donor cells on days 6 and 13.

(D) Recovery of donor $CD4^+$ T cells that had undergone one or more divisions in the MSLN and spleen 8 and 13 days after infection.

(E and F) WT and *Cd44*$^{-/-}$ mice were infected with PR8 influenza virus.

(E) The mice were treated with BrdU for 7 days before sampling. The recovery of $BrdU^+$ $CD4^+$ T cells is shown.

(F) On day 21 after infection, the virus-specific $CD4^+$ T cell response was assessed by intracellular staining of cells from the lungs and BALs after overnight culture with anti-CD28 in the presence or absence of NP_{311} peptide. Shown are the percentages of $CD154^+$IFN-γ^+ virus-specific $CD4^+$ T cells in the lung.

(B, D–F) Mean + SEM, n = 3–4/group.

division was analyzed at 13 days after infection. Divided *Cd44*$^{-/-}$ $CD4^+$ T cells failed to accumulate in the lymphoid tissues and were not detected in the lungs (Figures 3A and 3B). In order to prevent ligand binding by CD44, the KM201 mAb was administered, beginning at the time of WT cell transfer and infection. This did not affect the recovery of divided cells on day 6, but the accumulation of responding $CD4^+$ T cells on day 13 was inhibited when compared to mice that received control IgG (Figures 3C and 3D). This outcome is unlikely to be due to cytotoxic effects, because KM201 and several other anti-CD44 reagents did not elicit complement-mediated killing of activated OT-II cells (data not shown). The results suggest that CD44 participates in mechanisms that promote the survival of activated $CD4^+$ T cells.

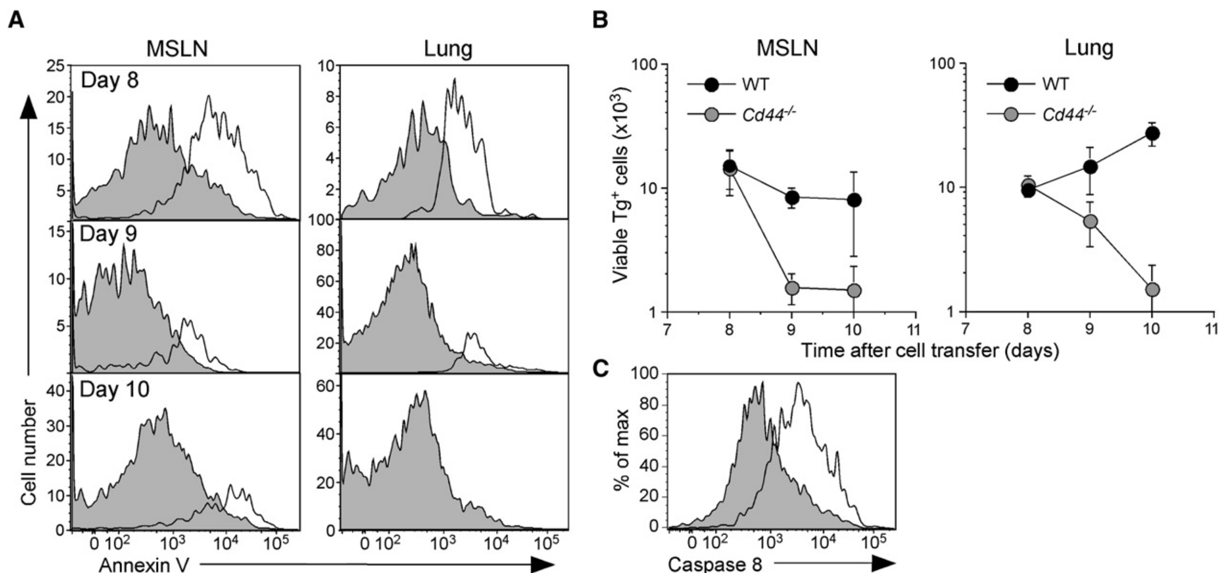

Figure 4. Induction of Apoptosis in Responding CD4⁺ T Cells Deficient in CD44

C57BL/6 recipients were given WT and *Cd44*$^{-/-}$ OT-II cells (1.5 × 10^6 each) and infected with WSN-OVA$_{II}$.

(A) Apoptosis was assessed by binding of Annexin V and exclusion of 7AAD by WT Tg$^+$ cells (shaded histograms) and *Cd44*$^{-/-}$ Tg$^+$ cells (open histograms) in the indicated tissues.

(B) Viable recoveries of WT and *Cd44*$^{-/-}$ donor Tg$^+$ cells in the MSLN and lungs (mean ± SEM, n = 5/group).

(C) Caspase 8 activation was assessed with a fluorophore-modified substrate in dispersed MSLN cells from recipients of WT and *Cd44*$^{-/-}$ CD4$^+$ T cells on day 7 after infection. The fluorescence induced by activated caspase 8 for WT Tg$^+$ cells (shaded histogram) and *Cd44*$^{-/-}$ Tg$^+$ cells (open histogram) is shown in 7AAD$^-$ Tg$^+$ population. The results are representative of those from six recipients.

Because the model used involves transfer of a relatively large number of Tg$^+$ CD4$^+$ T cells that might affect regulation, the responses of endogenous CD4$^+$ T cells from the lungs and MSLN of WT and *Cd44*$^{-/-}$ mice were assessed (Schmits et al., 1997) after infection with influenza virus with BrdU uptake (Figure 3E). The results showed that fewer divided CD4$^+$ T cells were present in the MSLN of *Cd44*$^{-/-}$ mice compared to WT mice on day 14. In addition, the lungs and BAL contained fewer virus-specific *Cd44*$^{-/-}$ CD4$^+$ T cells than WT CD4$^+$ T cells on day 21 (Figure 3F).

Responding *Cd44*$^{-/-}$ CD4$^+$ T Cells Die by Apoptosis

To assess whether apoptosis was evident in *Cd44*$^{-/-}$ CD4$^+$ T cells during expansion after infection, Annexin V binding was examined in cells from the MSLN and lungs (Figure 4A). Apoptotic cells were observed on day 8, followed by the disappearance of responding cells on day 9 (Figure 4B). Because CD44 has been associated with resistance to Fas-mediated cell death in various cell lines and tumor cells (Hauptschein et al., 2005; Mielgo et al., 2006), the activation of intracellular caspase 8 in *Cd44*$^{-/-}$ CD4$^+$ T cells was quantified. Activation characterizes the induction of extrinsic apoptotic death (Kim et al., 2006) and occurs as a consequence of the assembly of the death-inducing signaling complex (DISC). On day 7 after adoptive transfer, activated caspase 8 was markedly increased in *Cd44*$^{-/-}$ cells compared to WT cells from the MSLN (Figure 4C). Although changes in Bcl-2 family members correlate with the loss of CD44 and cell death in various cell types in vitro (Marhaba et al., 2003), no altered expression of the proapoptotic members Bim and BNIP-3 or the antiapoptotic members Bcl-2 and Bcl-xl was detected (Figure S3). The results support the hypothesis that death receptors participate in the mechanism of *Cd44*$^{-/-}$ CD4$^+$ T cell death in vivo and suggest a previously unidentified role of CD44 in maintaining CD4$^+$ effector T cells engaged in an immune response.

CD44 Can Regulate CD4$^+$ T Cell Survival in the Absence of Antigen

After influenza virus infection, antigen presentation to CD4$^+$ T cells is prolonged (Jelley-Gibbs et al., 2005), raising the possibility that TCR-mediated activation-induced cell death (AICD) occurs in the absence of CD44. We therefore asked whether death of *Cd44*$^{-/-}$ CD4$^+$ T cells occurred in the absence of overt signaling by antigen. To this end, activated effectors were generated from WT and *Cd44*$^{-/-}$ OT-II cells in culture. Both populations underwent comparable expansion and were similarly activated as measured by size (data not shown). Although WT and *Cd44*$^{-/-}$ effector cells showed similar distribution 1 day after coinjection into normal recipients, *Cd44*$^{-/-}$ CD4$^+$ T cells decayed more quickly than WT cells in the lungs and spleen (Figure 5A), as well as in the PLN, liver, and bone marrow (data not shown).

To confirm that impaired survival was not unique to Tg$^+$ cells or the viral response, we examined the decay of *Cd44*$^{-/-}$ effector CD4$^+$ T cells that were generated from polyclonal naive cells in vitro. Decay of *Cd44*$^{-/-}$ CD4$^+$ T cells compared to WT cells was pronounced (Figure 5B). The results support the concept that activated CD4$^+$ T cells that are unable to engage CD44 ligands fail to survive in the absence of CD44.

Because CD4$^+$ T cells develop into distinct subsets specified by different transcription factors and signature cytokines

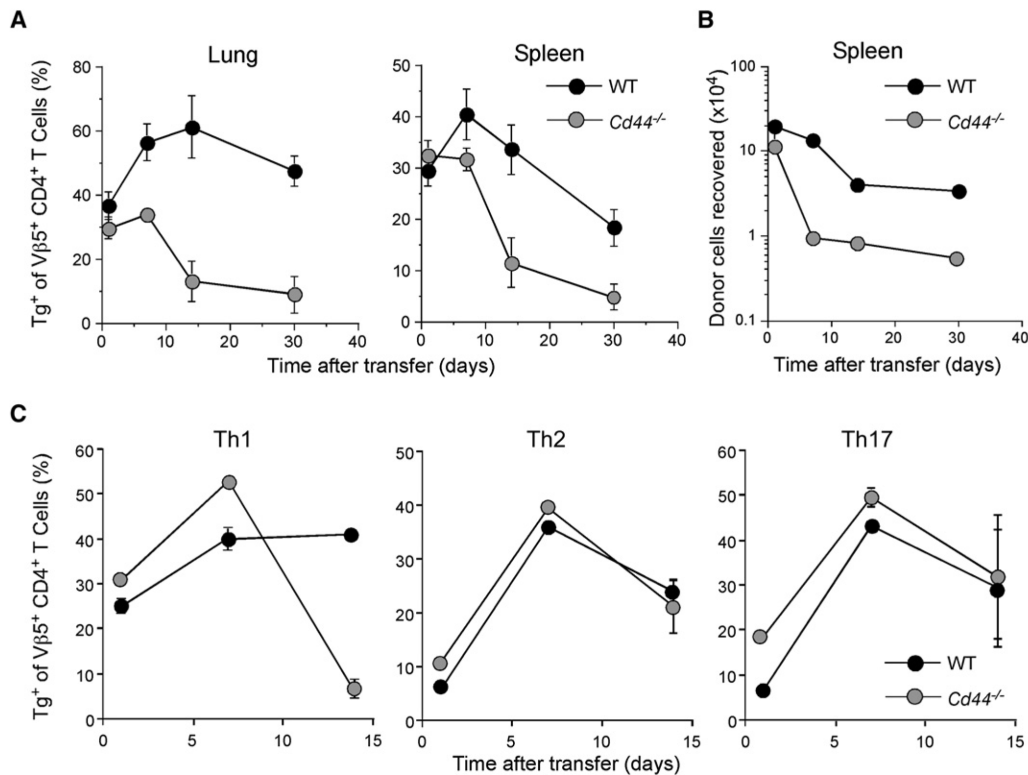

Figure 5. Impaired Survival of Activated CD4+ T Cells in Naive Recipients in the Absence of CD44

(A) WT and *Cd44*$^{-/-}$ OT-II cells were stimulated in vitro with APC and OVA peptide and then coinjected (1.5×10^6 each) into naive C57BL6 recipients. The frequencies of Tg+ cells, gated on the Vβ5+, CD4+ population in the lungs and spleen at the indicated times after cell transfer, are shown (mean ± SEM, n = 4/group).

(B) Polyclonal, non-Tg WT CD4+ T cells (Thy1.1, Ly5.2) and *Cd44*$^{-/-}$ CD4+ T cells (Thy1.2, Ly5.2) were stimulated with anti-CD3/anti-CD28 and coinjected into Ly5.1, Thy1.2 recipients in a dose of 1.5×10^6/recipient. The recovery of donor cells in the spleen is shown (mean ± SEM, n = 4/group).

(C) Th1, Th2, and Th17 cells were generated from OT-II cells with APC and OVA_{II} peptide. Allelically marked WT and *Cd44*$^{-/-}$ cells of each of the corresponding subsets were coinjected in a dose of 1.5×10^6 each into C57BL/6 recipients. Shown are the frequencies of donor cells recovered at the indicated times after cell transfer after gating on the Vβ5+, CD4+ population.

(Harrington et al., 2006), and the response to influenza virus is Th1 cell biased, we next asked whether the polarization of CD4+ effector T cells might affect CD44-dependent regulation of apoptosis. Th1, Th2, and Th17 WT and *Cd44*$^{-/-}$ cells were generated from naive CD4+ T cells with the appropriate cytokines. The effector phenotypes were confirmed by cytokine analysis with production of IFN-γ and TNF-α by Th1 cells, IL-17 by Th17 cells, and IL-4 and IL-10 by Th2 cells (Figure S4A). The CD4+ T cell subpopulations were similarly activated as indicated by the expression of CD11a and CD69 (Figure S4B). WT and *Cd44*$^{-/-}$ effectors of each subset were coinjected into C57BL/6 recipients. The donor cell recoveries at days 1, 7, and 14 revealed that only Th1 cells were affected by the absence of CD44 (Figure 5C). With Th1 and Th2 WT and *Cd44*$^{-/-}$ cells, no differences were observed in their ability to home to lymphoid tissues (Figure S4C) or to localize in the T cell areas of the spleens (Figure S4D). To determine whether CD44 might play a role in the survival of CD8+ T cells, TCR Tg+ OVA-specific OT-I or polyclonal CD8 effectors were generated from WT and *Cd44*$^{-/-}$ mice. In contrast to Th1 cells, the decay of CD8+ T cells in unimmunized recipients was not altered by the absence of CD44 (Figure S5). Thus, CD44 regulates CD4+ Th1 cells distinctly and plays a nonredundant role in the regulation of the Th1 cell response and ultimately their development into memory cells.

CD44 Limits Fas-Induced Death in Th1 Cells

The differential requirements of the CD4+ T cell subsets could not be attributed to CD44 expression, because this was equal between Th1 and Th2 cells (Figure 6A). Previous studies established that Th1 cells differ from Th2 cells with respect to regulation of apoptosis (Hur et al., 2007; Toscano et al., 2007; Zhang et al., 1997) and differential expression of CD44 isoforms has previously been associated with protection against death of various cell lines, including T cell lines (Mielgo et al., 2006). However, no splice variants were observed in Th1 and Th2 cells; only the standard form of CD44 was detected (Figure 6B; Figure S6A). In addition, no major differences in glycosylation that would lead to differences in the sizes of CD44 on Th1 versus Th2 cells were observed (Figure S6B).

CD44 has been associated with regulation of Fas (CD95)-mediated cell death (Hauptschein et al., 2005; Mielgo et al., 2006). We therefore compared expression of Fas and CD44 on Th1 and Th2 cells. Th1 cells expressed much higher amounts

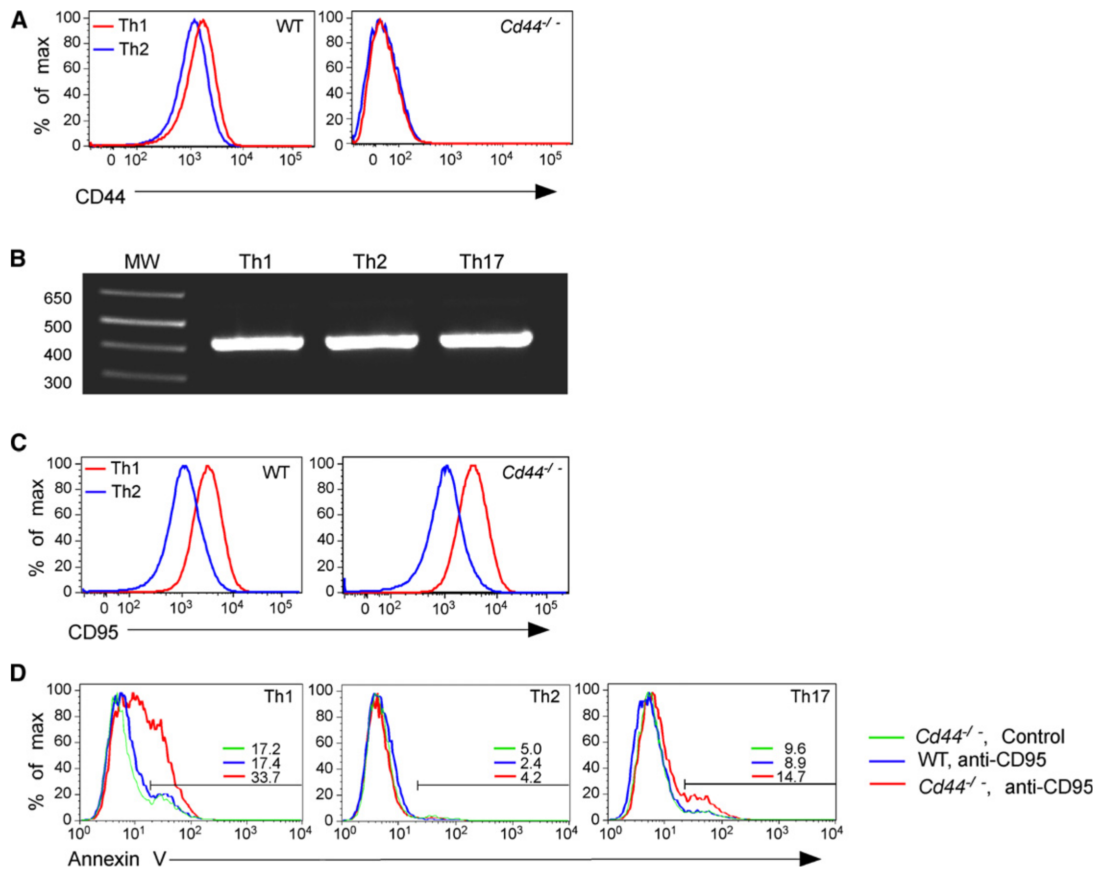

Figure 6. CD44s Regulates Fas-Mediated Death in Th1 Cells

(A) Th1 and Th2 cells were generated from WT and *Cd44*$^{-/-}$ OT-II Thy1.1 cells with OVA_{II} peptide and APC and tested for expression of CD44.

(B) Th1, Th2, and Th17 cells were generated from WT and *Cd44*$^{-/-}$ OT-II cells. RNA was isolated and tested for the presence of CD44 splice variants by RT-PCR with primers for the constant regions that flank the variant region. CD44 standard is 428 bp in size.

(C) Expression of Fas (CD95) on Th1 and Th2 cells.

(D) Th1, Th2, and Th17 cells were generated from WT and *Cd44*$^{-/-}$ $CD4^+$ T cells and recultured overnight in the presence or absence of plate-bound Fas mAb. The number after the colored bars indicate the percentages of cells undergoing apoptosis as indicated by binding of Annexin V and exclusion of 7AAD.

of Fas than Th2 cells, a difference that was also evident with *Cd44*$^{-/-}$ Th1 and Th2 cells (Figure 6C). Furthermore, incubation of in vitro-polarized $CD4^+$ T cells with anti-Fas elicited apoptosis in *Cd44*$^{-/-}$ Th1 cells, but not Th2 or Th17 cells (Figure 6D). This difference in Th1 cell susceptibility to apoptosis was detectable by 3 hr after Fas ligation (Figure S6C). Thus, CD44 and Fas could interact to limit death of Th1 cells by affecting extrinsic death receptor engagement or signaling. However, we were unable to detect a physical association of these molecules in the membranes of Th1, Th2, or Th17 cells by immunoprecipitation or fluorescence microscopy (data not shown).

CD44 Regulates Th1 Cell Survival by Engaging the PI3K-Akt Signaling Pathway

We next sought to determine whether signals that might contribute to $CD4^+$ T cell survival could be initiated by CD44. Therefore, we examined the recoveries of Th1 effector cells after transfer to unimmunized recipients that were treated with the agonist CD44 agonist antibody, IRAWB 14 (Lesley et al., 1993) or with the CD44 blocking antibody KM201. Compared to the IgG control, greater or reduced persistence of Th1 cells was observed after treatment with IRAWB 14 or KM201, respectively (Figure 7A). Thus, for Th1 cells, blocking CD44 binding to its ligand(s) inhibits survival, whereas signaling through CD44 enhances survival, implying a direct effect of CD44 engagement.

To test this prediction in an immune response, mice were treated with IRAWB 14 or control IgG at the time of transfer of WT OT-II cells and infection with the influenza virus. After IRAWB 14 treatment, there was a greater recovery of $CD4^+$ T cells engaged in the response by division as compared to the controls on day 13 after infection (Figure 7B). The results suggest that ligation of CD44 can promote the accumulation of $CD4^+$ T cells either by effects on survival, expansion, or both. However, IRAWB 14 did not promote proliferation of in vitro activated WT OT-II cells (data not shown), and other studies support the concept that ligation of CD44 without TCR signaling does not promote division of T cells (Marhaba et al., 2006). Thus, the data suggest that CD44 participates in maintaining survival of effector cells engaged in the response to influenza virus. To determine whether CD44 was required during the expansion versus contraction phase of the $CD4^+$ T cell response to influenza virus, IRAWB 14 or control Ig treatment was initiated on

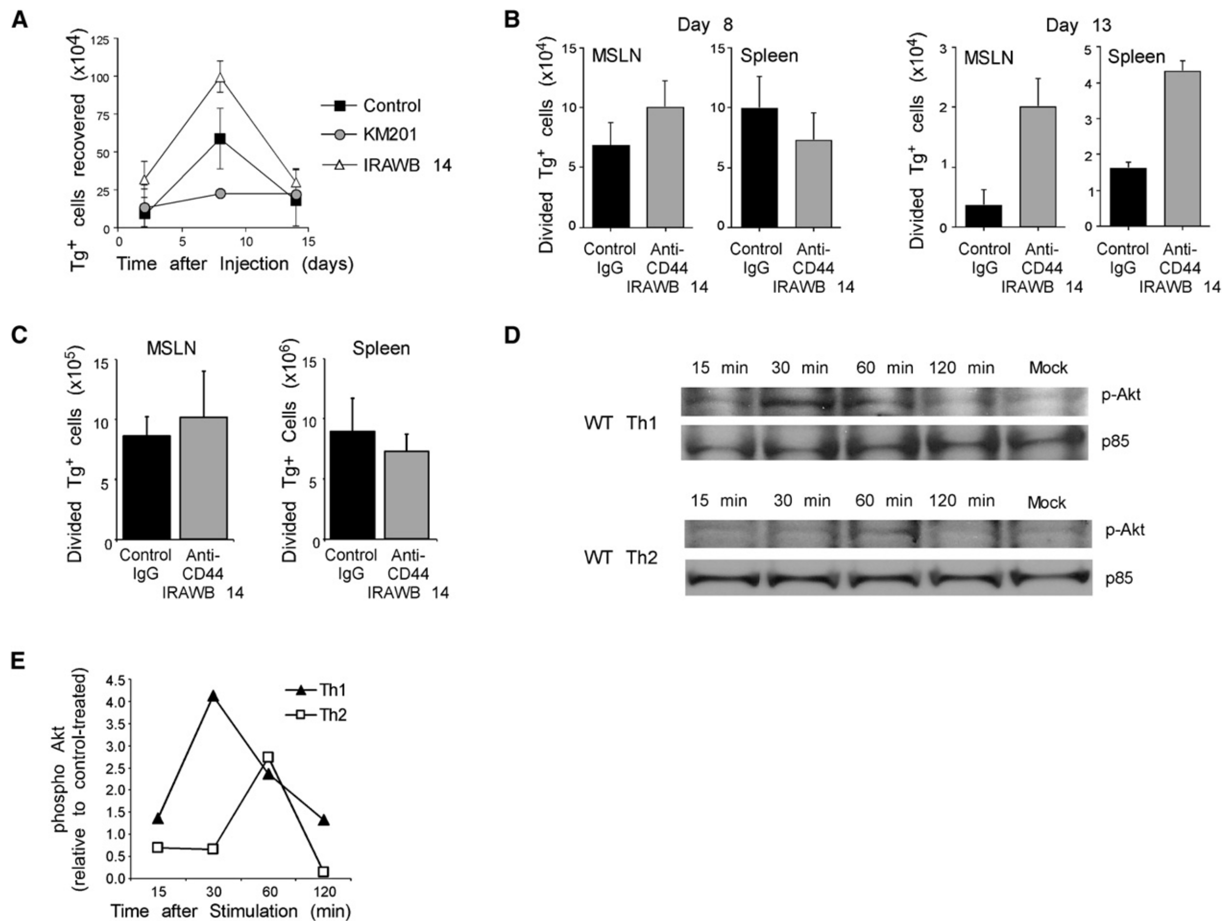

Figure 7. Requirement for CD44 Signaling in Th1 Cells

(A) WT OT-II Th1 cells were generated with APC and OVA_{II} peptide. The cells were then injected into C57BL/6 recipients (1.5×10^6/mouse) and treated with control IgG, KM201, or IRAWB 14 on the day of cell transfer, and three more times at 3 day intervals. The donor Tg^+ cells recovered in the pooled LN and spleens are shown at the indicated times after injection.

(B) C57BL/6 recipients of 1.5×10^6 CFSE-labeled WT OT-II cells were injected with either IRAWB 14 or control IgG at the time of cell transfer and infection with WSN-OVA_{II} influenza virus. The antibodies were administered three more times at 3 day intervals. The recoveries of donor $CD4^+$ T cells that had undergone one or more divisions in the MSLN and spleen were measured 8 and 13 days later.

(C) C57BL/6 recipients of 1.5×10^6 CFSE-labeled WT OT-II cells were injected with either IRAWB 14 or control IgG 8 days after infection with WSN-OVA_{II} influenza virus. The recoveries of donor $CD4^+$ T cells that had undergone one or more divisions in the MSLN and spleen were measured at 10 days after infection.

(A–C) Mean ± SEM, n = 3–4/group.

(D and E) Th1 and Th2 cells were generated from WT and $Cd44^{-/-}$ C57BL/6 $CD4^+$ T cells by stimulation with plate-bound anti-CD3 and anti-CD28. After resting for 1 day in rIL-7 and a further day without, the cells were cultured for the indicated times with plate-bound IRAWB 14 mAb.

(D) Phospho-Akt was detected by immunoblot and compared to the p85 subunit of PI3K as a loading control.

(E) Densitometry of phospho Akt on immunoblot data in (C). Results are represented as a ratio between band densities for IRAWB 14 and unstimulated control cells and are corrected for loading differences.

day 8 after WT OT-II cell transfer and infection of the recipient animals. No differences in the recoveries of OT-II cells were observed under these conditions on day 10 of the response (Figure 7C). These data imply that CD44-regulated survival signals are engaged during $CD4^+$ T cell expansion.

Ligation of CD44 can lead to activation of the PI3K-Akt pathway in some cell types (Klingbeil et al., 2009) and can inhibit Fas-mediated $CD4^+$ T cell death (Varadhachary et al., 2001) by interfering with DISC assembly (Jones et al., 2002). To determine whether ligation of CD44 might differentially signal in T cell subsets, Th1 and Th2 cells were generated from WT OT-II cells. After resting for 2 days, the cells were cultured with plate-bound IRAWB 14 or with the control IgG antibody. Phosphorylated Akt was measured as a downstream readout of PI3K activation. Phosphorylated Akt was induced in Th1 cells with peak expression of 30 min after ligation of CD44, whereas this response was lower in Th2 cells and was not observed until 60 min (Figures 7D and 7E). To confirm the CD44 dependence of PI3K induction, Akt phosphorylation in WT Th1 cells was compared to $Cd44^{-/-}$ Th1 cells in response to IRAWB 14. PI3K was engaged only in the WT Th1 cells and expression could be reduced with the PI3K inhibitor Ly294002 (Figure S7). The results support the concept that CD44 engagement elicits signaling that promotes survival in Th1 cells, which may be crucial in this subset because of the

high expression of Fas and the associated greater susceptibility to apoptosis by this pathway.

DISCUSSION

In this study, we identified a critical function of CD44 in the regulation of memory generation in Th1 CD4$^+$ T cells. Despite potential roles in migration and interactions with DCs, CD44 was not required for the initial induction of a primary immune responses in vivo or for the localization of naive or effector cells in either lymphoid or nonlymphoid tissues. This is probably because of redundancies in adhesion receptor usage that enable T cells to bypass its contribution and/or the ability of other HA binding receptors to perform these functions. However, CD44 plays a nonredundant role in regulating the survival of CD4$^+$ effector T cells in the influenza model, which is dominated by a Th1 cell response. Without engagement of CD44, effector cells that have progressed through several rounds of division died by apoptosis, whereas agonist signaling via CD44 during the expansion phase led to enhanced in vivo accumulation of effector cells. Thus, the generation of a memory population in Th1 cells most probably depends upon engagement of CD44 on responding effectors during the primary response. We showed that CD44 ligation activated the PI3K-Akt signaling pathway in Th1 cells. The mechanism by which CD44 activates PI3K remains to be explored, but could be due to constitutive association with the src family kinases Lck and Fyn (Rozsnyay, 1999) or to associations with β1 integrins that mediate the survival response (Lee et al., 2008; Marhaba et al., 2006; Nandi et al., 2004).

Th1 cells may uniquely require this survival signal through CD44 because of elevated Fas expression and an inherent ability to rapidly assemble the DISC in response to Fas trimerization (Varadhachary et al., 1999). Thus, we suggest that without engagement of CD44, the response to Fas ligation cannot be overcome. Such a mechanism may not be necessary in Th2 cells, and possibly other subsets of T cells, because of overall lower expression of Fas in addition to a greater capacity to engage PI3K-Akt in response to TCR signaling or costimulation (Varadhachary et al., 1999). It is of significance that activation of PI3K-Akt can block DISC formation by preventing the association of FADD and recruitment of pro-caspase 8 in CD4$^+$ T cells (Jones et al., 2002), and our studies lead us to favor this mechanism for regulation of Th1 cell survival.

Although CD44 can mediate resistance of tumor cells to apoptosis by death receptor ligation via FasL-Fas, DR5-TRAIL, and TNFR1-TNF-α by interfering with DISC assembly through the physical association of Fas and CD44 (Hauptschein et al., 2005), this interaction occurs through variant isoforms. Isoforms that include variants v6 and v9 are in close proximity with Fas in the membranes of transfected Jurkat cells and thereby prevent Fas trimerization (Mielgo et al., 2006). However, the lack of CD44 isoforms on CD4$^+$ T cells activated in vivo after influenza virus infection (data not shown) or on Th1, Th2, or Th17 cells generated in vitro, which differ in their susceptibility to Fas-mediated death in the absence of CD44, further argues against sequestration of Fas as the only mechanism that accounts for a selective function of CD44 in Th1 cells. The lack of CD44 isoforms on CD4$^+$ T cell subsets also argues against a mechanism whereby osteopontin binding to CD44 variants containing v7 leads to activation of NF-κB and prevents mitochondrial death controlled by the transcription factor Foxo3a, a regulator of Bim (Hur et al., 2007). Indeed, we did not detect changes of either pro- or antiapoptotic Bcl-2 family proteins in WT compared to *Cd44*$^{-/-}$ CD4$^+$ T cells. Although differences in glycosylation of Th2 cells have been reported to account for resistance to cell death compared to Th1 and Th17 cells (Toscano et al., 2007), the mechanism involves protection from binding of galectin-1, and we did not observe differences in the molecular weight of CD44 from Th1 and Th2 cells that would suggest significant differences in glycosylation. Because CD44 is upregulated on activated and memory CD8 cells, we did not anticipate differences in their regulation that would suggest independence from CD44-mediated survival signals. However, there are many differences in the regulation of CD4$^+$ and CD8$^+$ T cells, including in the programming to develop into memory cells after the initiation of a response (Kaech and Ahmed, 2001; van Stipdonk et al., 2003). Our data support the concept that internal signaling differences rather than external molecular variations account for the differences in regulation by CD44 on T cells.

A role for CD44 in regulating survival of CD4$^+$ T cells engaged in an immune response in vivo has not been previously examined directly. However, protection from TCR-mediated AICD by CD44 has been suggested by in vitro studies of in vivo primed cells (Marhaba et al., 2003). The results described herein, which show normal priming of CD4$^+$ T cells in vivo irrespective of the presence of CD44 on T cells or DCs, support the concept that engagement of CD44 in vivo is required for Th1 cells only after activation. Our results suggest that survival signals are transmitted in Th1 cells during the expansion phase of the effector response to influenza virus, which is profoundly compromised in the absence of CD44 or when adhesion binding of CD44 is blocked.

A CD44-dependent survival mechanism remained operative in activated Th1 cells that were withdrawn from overt Ag stimulation by transfer into naive recipients. This result suggests that TCR signaling in the context of an effector response and the production of proinflammatory cytokines, both of which can augment CD44 binding of HA (Marhaba et al., 2003), are not necessary for the function of CD44 in promoting apoptosis resistance. Indeed, in the absence of an immune response, agonist engagement of CD44 in vivo promoted enhanced accumulation of CD4$^+$ T cells. Our previous studies indicate that maintaining effector survival through costimulation can be key to the generation of robust memory in CD4$^+$ T cells (Linton et al., 2000, 2003). In this regard, we propose that CD44 can be viewed as an ECM-dependent, Th1 cell-specific "costimulatory" molecule that sustains effector cell responses through survival and thereby supports the development of memory.

The homeostasis of many cell types is regulated by contact dependence, and signals from the ECM can be crucial to prevent cells from undergoing anoikis or programmed cell death, which can be due to intrinsic death resulting in mitochondrial permeabilization, and extrinsic death that is initiated by death receptors (Gilmore, 2005). Although the molecular mechanisms that lead to PI3K activation and the downstream targets in Th1 cells remain to be defined, our results support the concept that physical contacts of Th1 cells with HA in the immediate environment

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regulate processes during which CD44-dependent survival signals are engaged. By promoting optimal survival of effector $CD4^+$ T cells engaged in an immune response (clonal burst), CD44 provides a previously unknown contribution to the development of T cell immunity in vivo.

EXPERIMENTAL PROCEDURES

Mice and Viruses

Cd44$^{-/-}$ mice (Schmits et al., 1997) were bred to B6PL-Thy mice and crossed to OT-I and OT-II TCR Tg mice in the vivarium at the Burnham Institute for Medical Research. These mice were also crossed to B6 Ly5.1 mice. C57BL/6 mice were purchased from Jackson Laboratories. All mice were males between 6 and 16 weeks of age. The mice were housed under specific pathogen-free conditions according to NIH guidelines. The use of the animals for the experiments described herein was approved by the IACUC review committee at the Burnham Institute.

All influenza viruses were grown in chicken eggs (10 days of embryonation) and titrated with MDCK cells for plaque-forming units (pfu) (Jelley-Gibbs et al., 2005). Infective doses elicited an optimal T cell response and were given i.n. in 30 μl. The WT influenza A viruses Puerto Rico/8/34 (PR8, H1N1) was given in a dose of 12.5 pfu. The engineered influenza A viruses William Smith Neurotropic/33 (WSN-OVA_{II}, H1N1) (Chapman et al., 2005) and Hong Kong Aichi/2/68 (HKx31-OVA_{II}, H3N2) (Thomas et al., 2006) that express the $OVA_{323-339}$ peptide recognized by OT-II $CD4^+$ T cells were given in doses of 1250 pfu and 112 pfu, respectively.

Antibodies for Cell Surface and Intracellular Proteins

Anti-Thy1.1 (CD90.1, OX-7) and -Ly5.1 (CD45.1, A20) were from BioLegend. The following antibodies were from eBioscience: Bcl2 (10C4), Bcl-xl (2H12), and Bnip3 (polyclonal rabbit), IFN-γ (XMG1.2) and TNF-α (MP6xt22). Anti-vβ5 (MR9) and anti-Fas (CD95, Jo2) were from BD Biosciences. Anti-BimS (14A8) was from Millipore. Cell lines producing the adhesion blocking anti-CD44 mAb, KM201, and the agonist/activating anti-CD44 mAb, IRAWB 14, were obtained from P. Kincade (Zheng et al., 1995). These mAb, anti-CD3 (145-2C11), and anti-CD28 (37.51) were produced and purified by BioLegend. Rat IgG was used as the control (Jackson ImmunoResearch).

Cell Preparation

$CD4^+$ and $CD8^+$ T cells were isolated from pooled spleen and lymph nodes by magnetic sorting (Imag, BD Biosciences) according to the manufacturer's protocol. To generate effector cells, naive T cells were cultured at 10^6/ml for 4 days with immobilized anti-CD3 (10 μg/ml) and anti-CD28 (5 μg/ml) and from TCR Tg mice with C57BL/6 splenic APCs (2×10^6/ml), and 5 μM OVA peptides (257-264, OT-I and 323-339, OT-II, Sigma-Genosys) (Harbertson et al., 2002). $CD4^+$ T subsets were polarized in the presence of rIL-2 (10 ng/ml) with anti-CD3 and anti-CD28, or with APCs and peptide for 4 days. Th1 cells were elicited with rIL-12 (5 ng/ml), and anti-IL-4 (11b11, 10 μg/ml) and Th2 cells with rIL-4 (10 ng/ml) and anti-IFN-γ (XMG1.2, 10 μg/ml). For Th17 cells, we used rIL-1b (10 ng/ml), rIL-6 (20 ng/ml), rTGF-β1 (1 ng/ml), TNF-α (10 ng/ml), and 10 μg/ml each of anti-IL-4 and anti-IFN-γ. Splenic DCs were enriched by centrifugation with 13.5% (w/v) histodenz (Sigma) (McLellan et al., 1995).

Adoptive Transfer and Detection of T Cells

T cells were injected i.v. into recipients in doses of $0.3–3.0 \times 10^6$ with donor and host combinations that differed by expression of Thy1 or Ly5. WT and *Cd44*$^{-/-}$ cells were coinjected in equal ratios. DCs pulsed for 2 hr with 10 μg/ml OVA peptide were injected in a dose of 1×10^5/recipient at the time of T cell transfer. After sacrifice, cells in the airways of recipient mice were collected by BAL. Cell suspensions of perfused lungs were obtained by digestion with collagenase D (Roche) at 10 mg/ml for 60 min at 37°C. Flow cytometry was used to detect Tg^+ $CD4^+$ T cells in these tissues, and in LN and spleen cells after fluorescent staining for CD4, Thy1.1, Ly5.1, and vβ5 (BD Biosciences). Viable lymphocyte recovery was determined by flow cytometry with propidium iodide uptake.

T Cell Responses

CFSE or BrdU labeling (Harbertson et al., 2002; Linton et al., 2003) were used to assess cell division. Intracellular staining was used to detect cytokine secretion by donor cells after overnight culture of lymphocyte suspensions from the MSLN with splenic APC and OVA_{II} peptide (Harbertson et al., 2002; Linton et al., 2003). After surface staining, the cells were permeabilized (BD Biosciences) and stained with anti-IFN-γ and -TNF-α. Annexin V and 7-Amino-actinomycin D (7AAD) staining were used to distinguish dead from dying cells by flow cytometry. To test caspase 8 activity, freshly isolated MSLN cells were incubated with CaspaLux 8-L_1D_2 (OncoImmunin, Inc) according to the manufacturer's protocol. Fas-mediated cell death was induced with plate-bound anti-Fas (10 μg/ml). In vitro treatment of $CD4^+$ T cells with anti-CD44 mAb or Rat IgG was done as soluble or plate bound (10 μg/ml) as indicated in the text.

Anti-CD44 Treatment In Vivo

KM201, IRAWB 14, or Rat IgG were administered in a dose of 200 μg/mouse on the day of cell transfer and three times thereafter spaced by 3 day intervals or given in a dose of 1 mg/mouse 8 days after cell transfer.

PCR for CD44 Isoforms

OTII $CD4^+$ T cells were enriched and polarized as described above and RNA isolated (RNeasy, QIAGEN). Primers spanning the CD44 isoform region (forward Exon 5 primer: CATCAGTCACAGACCTACCCAATTCC and reverse exon 16 primer CCAAGATGATGAGCCATTCTGGAATC) (Figure S6A) were used to distinguish isoforms by RT-PCR (GeneAmp, Applied Biosystems). Reverse transcriptase was for 60 min at 42°C via random hexamers and reverse primer, followed by PCR with the following conditions: 94°C 2 min, 64°C 30 s, 72°C 1 min 30 s (35 cycles).

Immunoblots for Akt

$CD4^+$ cells from WT or *Cd44*$^{-/-}$ mice were stimulated with plate-bound anti-CD3 plus anti-CD28 (5 μg/ml each) under Th1 or Th2 cell conditions for 4 days (see above), followed by 2 days of resting in RPMI culture medium with or without rIL-7 for the first 24 hr. To crosslink CD44, 10^6 cells were plated on IRAWB 14- or rat IgG-coated plates, incubated at 37°C for 15, 30, 60, or 120 min. After stimulation, cell lysate proteins were resolved through 4%–12% PAGE and transferred to nitrocellulose membranes. The membranes were probed and reprobed with Abs to phospho-Akt and p85 subunit of PI3K (Cell Signaling Technology), respectively. Densitometry on blots was performed with ImageJ (NIH).

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and can be found with this article online at doi:10.1016/j.immuni.2009.10.011.

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