Memory CD8+ T Cells Require CD28 Costimulation

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Memory CD8\(^+\) T Cells Require CD28 Costimulation\(^1\)

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CD8\(^+\) T cells are a critical component of the adaptive immune response against infections and tumors. A current paradigm in immunology is that naive CD8\(^+\) T cells require CD28 costimulation, whereas memory CD8\(^+\) T cells do not. We show here, however, that during viral infections of mice, costimulation is required in vivo for the reactivation of memory CD8\(^+\) T cells. In the absence of CD28 costimulation, secondary CD8\(^+\) T cell responses are greatly reduced and this impairs viral clearance. The failure of CD8\(^+\) T cells to expand in the absence of CD28 costimulation is CD4\(^+\) T cell help independent and is accompanied by a failure to down-regulate Bcl-2 and by cell cycle arrest. This requirement for CD28 costimulation was shown in both influenza A and HSV infections. Thus, contrary to current dogma, memory CD8\(^+\) T cells require CD28 costimulation to generate maximal secondary responses against pathogens. Importantly, this CD28 requirement was shown in the context of real infections where multiple other cytokines and costimulators may be up-regulated. Our findings have important implications for pathogens, such as HIV and measles virus, and tumors that evade the immune response by failing to provide CD28 costimulation. These findings also raise questions about the efficacy of CD8\(^+\) T cell-based vaccines against such pathogens and tumors. The Journal of Immunology, 2007, 179: 6494–6503.

The activation of naive T cells requires receiving two signals from professional APC. The first signal is provided via the TCR following engagement of Ag presented in the context of MHC. The second, costimulatory signal, is provided by CD28 following binding to its ligands B7-1 (CD80) or B7-2 (CD86) on the APC. These two signals form the basis of the two-signal theory of lymphocyte activation (1, 2). CD28 is the most well-characterized costimulatory molecule. Signaling through CD28 enhances naive T cell activation, proliferation, and survival (3–5). The costimulatory signal through CD28 has been shown, through the use of CD28-deficient mice and treatment with receptor antagonists, to be a necessary signal for the development of primary CD8\(^+\) T cell responses to most pathogens (6–11). During primary CD8\(^+\) T cell responses, CD28 acts by amplifying the signal received through the TCR to lower the threshold of activation (12, 13) and by enhancing the survival of T cells via up-regulation of antiapoptotic proteins, such as Bcl-x\(_L\) and c-FLIP\(_{short}\), to protect them from activation-induced cell death (14, 15).

Although the role of CD28 costimulation during primary response has been clearly defined, the role such costimulation plays during a recall responses to pathogens is unknown. A current paradigm of immunology postulates that, in contrast to naive T cells, the reactivation of memory CD8\(^+\) T cells does not depend on CD28 costimulation (16–18). This is based on in vitro studies (16–18) and the lower thresholds of activation that memory CD8\(^+\) T cells exhibit (19, 20). A similar reduced CD28 costimulation requirement has also been suggested for memory CD4\(^+\) T cells (21, 22). The in vivo requirement of memory CD8\(^+\) T cells for CD28 costimulation, however, has never been directly addressed. Although CD28-deficient animals have been useful in determining the function of CD28 during primary CD8\(^+\) T cell responses, this model is not appropriate for looking at secondary responses since the primary response in these animals is greatly reduced (7, 10, 23) and this leads to deficiencies in memory generation (24–26). This has made studying the effect of CD28 costimulation on memory CD8\(^+\) T cell response upon rechallenge using CD28-deficient mice impossible. Additionally, although CD28-deficient mice have normal numbers of B and T cell populations (23), one cannot exclude that given the importance of CD28 costimulation in thymic T cell development (27–29) lack of CD28 during T cell development could lead to defective mature T cells, which complicates studying memory responses in these mice.

Recent studies have indicated that dendritic cells (DC)\(^3\) are required for optimal secondary CD8\(^+\) T cell responses (30, 31). DC are required for the generation of optimal CD8\(^+\) T cells responses to secondary infection with influenza virus, vesicular stomatitis virus, and Listeria monocytogenes (30, 31). This DC requirement suggests that memory CD8\(^+\) T cell reactivation requires either costimulation or cytokines derived from DC, the precise nature of which is currently unknown.

In the present study, using two different approaches and two different viruses, we show that following infection in vivo memory CD8\(^+\) T cells require CD28 costimulation during reactivation to reach maximal expansion and effective pathogen clearance. Lack of CD28 costimulation results in high Bcl-2 levels and cell cycle arrest. The above findings challenge the current paradigm on the requirement of CD28 costimulation by memory CD8\(^+\) T cells and raise important questions for vaccines and immune responses in the context of real infections.
against viruses and tumors that evade the immune system by failing to provide CD28 costimulation.

Materials and Methods

Animals and reagents
Female 6- to 8-wk-old specific-pathogen-free C57BL/6 (wild-type) and B6.129S4-Cd8d<sup>−/−</sup>/Cd8e<sup>−/−</sup>/Cd8f<sup>−/−</sup> (B7<sup>−/−</sup>) mice were purchased from The Jackson Laboratory. B6.129-H2-Ab<sup>−/−</sup> (G9N212; class II<sup>−/−</sup>) mice were purchased from Taconic Farms. Mice were maintained at Drexel University College of Medicine and The American Association for the Accreditation of Laboratory Animal Care-certified barrier facility and acclimated for 1 wk before use. All animal work was performed following approval from the Institutional Animal Care and Use Committee. For influenza virus experiments, the A/Porto Rico/8/34 (PR8, H1N1) influenza type A strain, a recombinant viral strain of A/Hong Kong/31/1968 (H3N2), and a recombinant strain of A/Wisconsin/35 (WSN, H1N1) which expresses OVA were used. For HSV-1 experiments, HSV-1 Patton and an arbovirus expressing gB<sub>498–505</sub> were used. Influenza virus-specific MHC class I H-2<sup>D</sup>-tetramer loaded with the immunodominant peptide NP<sub>366–374</sub> was prepared as previously described (32). The NP<sub>366–374</sub> peptide (AS NENMETY), the OVA<sub>257–264</sub> peptide (SIINFEKL) and the gB<sub>498–505</sub> peptide (SSIEFARL) were purchased from Anaspec. Anti-CD28 blocking Ab (CD28-biotin, Stem Cell Technologies). Fifty thousand memory OVA257–264-specific CD8<sup>+</sup>/H9262<sup>+</sup> were labeled with 5 μM of CFPSE (Molecular Probes) for 5 min, washed, and then 4 μl of 10<sup>5</sup> infectious units of arbovirus expressing HSV gB<sub>498–505</sub>. Mice were rechallenged intradermal in both rear footpads with 20 μl of HSV-1 Patton strain (6 × 10<sup>7</sup> PFU/ml).

OT-I splenocyte adoptive transfer

For priming, splenocytes were isolated from the spleens of OT-I TCR-transgenic mice and total splenocytes (1 × 10<sup>7</sup> OT-I cells) were i.v. transferred into C57BL/6 (wild-type) mice in 100 μl of sterile injectable NaCl. For secondary experiments, memory splenocytes were harvested and killed T cells were purified by negative selection (SpinSep CD8<sup>−</sup> cell purification kit; Stem Cell Technologies). Fifty thousand memory OVA<sub>257–264</sub>-specific CD8<sup>+</sup> T cells in 100 μl of sterile injectable saline were adoptively transferred by i.v. injection to wild-type and B7<sup>−/−</sup> mice. For trafficking and survival experiments, cells were labeled with 5 μM CFSE (Molecular Probes) for 5 min, washed, and then 4 μl of 10<sup>5</sup> infectious units of arbovirus expressing HSV gB<sub>498–505</sub>. Mice were rechallenged intradermal in both rear footpads with 20 μl of HSV-1 Patton strain (6 × 10<sup>7</sup> PFU/ml).

Cytotoxicity assay

Ex vivo cytotoxicity of lung cells was measured against target EL-4 cells that were loaded with 1 μg/ml MHC class I NP<sub>366–374</sub> peptide overnight. Target cells were then labeled with 100 μCi of 31<sup>Cr</sup> for 75 min at 37°C, washed twice, and mixed (10<sup>4</sup> target cells/well) with various dilutions of effector cells in 96-well V-bottom Costar culture plates (Corning Glass). Plates were incubated for 6 h at 37°C, then centrifuged for 5 min at 500 × g. Maximum lysis was obtained by the addition of 5% Triton X-100 solution. Thirty microliters of the supernatant was transferred to a 96-well Lumaplate (Packard Instrument). Lumaplates were dried overnight and counted with a Top Count-NXT luminescence scintillation counter (Packard Instrument). Percent lysis was calculated from the formula: 100 × (cpm experimental – cpm spontaneous)/(cpm maximum – cpm spontaneous).

Determination of influenza virus viral loads

Lung tissue was frozen in 1 ml of TRIzol (TRI-Reagent; Molecular Research Center, Cincinnati, OH) at −20°C. Tissue was homogenized in TRIzol reagent, on ice, using a polytron blade homogenizer. RNA was extracted using the Molecular Research Center protocol followed by cleanup of the RNA with a Qiagen RNeasy Kit. cDNA synthesis was performed using both a specific primer (5′-TCTAACCAGGTTCGAAGAAGTGA-3′) and random hexamers. Real-time assays were performed in triplicate with 5 μl of cDNA, 12.5 μl of 2× Taqman Universal PCR Master Mix (Applied Biosystems), 900 nM influenza A virus sense primer (5′- AAGACCAATCTTGACATGTCGTA-3′), 900 nM influenza A virus anti-sense primer (5′- CAAAGCTTACCGAGTCGTA-3′), and 200 nM influenza A virus probe (5′- TTCAGTCTGCACCTCGTCC-3′, TAMRA) (33). All primers were specific for the influenza A virus matrix protein. Amplification and detection were performed using an Applied Biosystems Prism 7900HT sequence detection system with SDS 2.2.1 software (Applied Biosystems) at the following conditions: 2 min at 50°C and 10 min at 95°C, then 45 cycles of 15 s at 95°C and 1 min at 60°C. Viral loads were calculated as 50% tissue culture infectious dose (TCID<sub>50</sub>)/μg by comparison to an influenza viral stock curve by linear regression in every assay.

HSV-1 viral titer plaque assay

The rear footpad was frozen in 1 ml of complete RPMI 1640 (10% FBS) at −80°C. Plaque assay on tissue homogenate was performed as previously described (34).

Statistical analysis

Mann-Whitney U test, Student’s t test, nonparametric Wilcoxon signed-rank test for paired samples, and Shapiro-Wilk W test for normality were used for statistical analysis with the JMP statistical analysis program (SAS). Values of p < 0.05 were to be considered significant.

Results

CD28 costimulation is required for optimal influenza virus-specific CD8<sup>+</sup> T cell recall responses

We directly addressed the role for CD28 costimulation in the reactivation of memory CD8<sup>+</sup> T cells by using two different models...
FIGURE 1. Blocking CD28 costimulation inhibits influenza virus-specific CD8\(^+\) T cell recall responses. A–E, Blocking CD28 costimulation during secondary infection results in reduced virus-specific CD8\(^+\) T cell responses. C57BL/6d (wild-type) mice were primed with PR8 influenza A virus and rechallenged 60 days later with HKx31 influenza A virus i.n. CD28 costimulation was blocked with anti-mouse CD28 clone 37.51 and day 7 lung NP\(_{366-374}\)-specific CD8\(^+\) T cells were measured. A, Representative FACS plots of lung NP\(_{366-374}\)-specific CD8\(^+\) T cells, representative of four independent experiments shown. The percentage of pulmonary CD8\(^+\) T cells that is NP\(_{366-374}\)-specific is shown in the FACS plots. B, Absolute number of lung NP\(_{366-374}\)-specific CD8\(^+\) T cells shown. Each symbol represents a single animal. Bars, Mean. C, Absolute number of pulmonary virus-specific CD8\(^+\) T cells measured by intracellular staining for IFN-\(\gamma\) following NP\(_{366-374}\) peptide stimulation. Each symbol represents an individual animal and bars represent the mean. D, Cytotoxicity of lung lymphocytes is reduced when CD28 costimulation is blocked during influenza virus rechallenge. Ex vivo cytotoxicity against NP\(_{366-374}\)-peptide-loaded target cells shown. Data are representative of three experiments and nine animals per group. E, Blocking CD28 costimulation during secondary infection results in a reduced peak CD8\(^+\) T cell response, not a delay in the kinetics. Pulmonary NP\(_{366-374}\)-specific CD8\(^+\) T cells were measured on days 3, 5, 6, 7, 10, and 60 following rechallenge by flow cytometry using NP\(_{366-374}\)-loaded MHC class I tetramer (\(n = 3\)/group).

where CD28 costimulation was lacking: 1) blocking costimulation with a mAb against CD28 during rechallenge of primed animals and 2) rechallenging wild-type and B7\(^-/-\) mice which had received memory CD8\(^+\) T cells by adoptive transfer.

In our first model, we blocked CD28 costimulation with a non-depleting mAb that blocks CD28 in vivo (35). Indeed, anti-CD28 treatment did not deplete naïve CD62L\(^+\)CD44\(^+\) CD8\(^+\) T cells or total CD8\(^+\) or CD4\(^+\) T cells from spleens of uninfected animals nor splenic memory NP\(_{366-374}\)-specific CD8\(^+\) T cells from unchallenged animals (data not shown). The immunodominant peptide NP\(_{366-374}\)-specific CD8\(^+\) T cell response was examined by staining with NP\(_{366-374}\)-loaded MHC class I H2-D\(d\) tetramers. In mice primed i.p. with H1N1 PR8 influenza virus and rechallenged 60 days later i.n. with H3N2 HKx31 strain, when CD28 was blocked during rechallenge, there was a 3-fold reduction in the number of lung NP\(_{366-374}\)-specific CD8\(^+\) T cells (7.12 \(\pm\) 1.7 \(\times\) 10\(^5\) cells, mean\(\pm\)SE, \(n = 11\)) on day 7, the peak of the secondary response (36), compared with untreated animals (22.23 \(\pm\) 3.4 \(\times\) 10\(^5\), \(n = 12\), \(p < 0.0001\)) or isotype control-treated animals (23.94 \(\pm\) 5.2 \(\times\) 10\(^5\), \(n = 11\), \(p < 0.0001\)) (Fig. 1, A and B). Percentages of lung NP\(_{366-374}\)-specific CD8\(^+\) T cells were also decreased in anti-CD28 Ab-treated mice (18.4 \(\pm\) 2.0% vs 30.7 \(\pm\) 3.6% vs 32.3 \(\pm\) 3.2% of CD8\(^+\) T cells for anti-CD28-treated, untreated, and isotype control-treated respectively, \(n = 11–12\) for each group; \(p < 0.001\)). A similar reduction in absolute numbers was also found when the lung NP\(_{366-374}\)-specific CD8\(^+\) T cells response was measured by NP\(_{366-374}\) peptide stimulation and intracellular IFN-\(\gamma\) staining, with 26.3 \(\pm\) 3.2 \(\times\) 10\(^5\) NP\(_{366-374}\)-specific CD8\(^+\) T cells for untreated mice and 8.9 \(\pm\) 2.7 \(\times\) 10\(^5\) virus-specific CD8\(^+\) T cells for anti-CD28-treated mice, respectively (\(p < 0.0008; n = 9\); Fig. 1C). Blocking CD28 also reduced ex vivo cytotoxicity of lung lymphocytes against NP-peptide loaded targets in a \(^{51}\)Cr release assay (Fig. 1D). The mediastinal lymph nodes (MLNs) of mice treated with the anti-CD28 blocking Ab also had a reduced number of NP\(_{366-374}\)-specific CD8\(^+\) T cells compared with untreated animals (0.28 \(\pm\) 0.09 \(\times\) 10\(^5\) and 0.76 \(\pm\) 0.18 \(\times\) 10\(^5\) NP\(_{366-374}\)-specific CD8\(^+\) T cells anti-CD28 Ab-treated and untreated animals, respectively; \(p < 0.02; n = 11\)). Anti-CD28 Ab also reduced NP\(_{366-374}\)-specific CD8\(^+\) T cells to a similar degree in spleen (3.8 \(\pm\) 3.2 \(\times\) 10\(^5\) and 14.6 \(\pm\) 12.5 \(\times\) 10\(^5\) NP\(_{366-374}\)-specific CD8\(^+\) T cells anti-CD28 Ab treated and untreated animals, respectively; \(p < 0.02; n = 11\)). Isotype control Ab had no effect on the numbers in MLNs and spleen (data not shown). The frequency of CD62L\(^+\)CD44\(^+\) and CD62L\(^-\)CD44\(^-\) within NP\(_{366-374}\)-specific CD8\(^+\) T cells did not change on day 7 with anti-CD28 Ab treatment in any of the tissues examined (lung, MLNs, and spleen), indicating that we were not selectively affecting effector cells or central memory cells (data not shown). Total CD8\(^+\) T cells were also significantly reduced in the lungs of anti-CD28-treated animals (3.6 \(\pm\) 0.7 \(\times\) 10\(^5\) and 8.2 \(\pm\) 0.8 \(\times\) 10\(^5\) for anti-CD28 Ab-treated and untreated animals, respectively; \(p < 0.001\).
FIGURE 2. CD80 and CD86 are required for optimal virus-specific CD8\(^+\) T cell recall responses. A, The secondary CD8\(^+\) T cell response to influenza virus infection is reduced in B7-deficient mice. Following adoptive transfer of \(5 \times 10^5\) day 60 memory OVA\(_{257-264}\)-specific CD8\(^+\) T cells that were generated by i.n. infection of wild-type mice with WSN-OVA flu virus, wild-type, and B7/− mice were rechallenged i.n. with WSN-OVA influenza A virus and harvested on day 7. Representative FACS plots of CD8\(^+\) CD3\(^+\) gated lymphocytes shown. Vxav257–264 CD8\(^+\) T cells are OVA\(_{257-264}\)-specific CD8\(^+\) T cells. Numbers in plots indicate the percentage of pulmonary CD8\(^+\) T cells that is OVA\(_{257-264}\)-specific. B, The percentage of pulmonary CD8\(^+\) T cells that are OVA\(_{257-264}\)-specific CD8\(^+\) T cells are shown. Each symbol depicts one animal. The horizontal line depicts the mean. C, Absolute numbers of day 7 pulmonary OVA\(_{257-264}\)-specific CD8\(^+\) T cells shown. Individual animals depicted by each symbol. Horizontal lines show the mean. All data above from two independent experiments. D, Memory HSV-1-specific CD8\(^+\) T cells require CD28 costimulation. Mice were primed with arbovirus-expressing HSV-1 gB\(_{498-505}\) peptide and 5 \(\times 10^5\) day 60 memory gB\(_{498-505}\)-specific CD8\(^+\) T cells were transferred to C57BL/6J and B7/− mice, which were then rechallenged by footpad injection of HSV-1 Patten. Absolute numbers (mean \pm SE) of day 7 spleen gB\(_{498-505}\)-specific CD8\(^+\) T cells measured by intracellular staining for IFN-γ production following gB\(_{498-505}\) peptide stimulation are shown (\(n = 3\) group).

To exclude that the above described reduced secondary CD8\(^+\) T cell response to influenza on peak day 7 was not due to altered kinetics of the response, we performed a kinetic study of the secondary virus-specific CD8\(^+\) T cell response by examining days 3, 5, 6, 7, 10, and 60 after rechallenge. The kinetics for expansion and contraction in anti-CD28-treated mice matched those seen in the untreated and isotype control-treated mice (Fig. 1E). Thus, the reduced day 7 pulmonary secondary CD8\(^+\) T cell response was not due to a delay in the kinetics of the response but truly reflected a decrease in the magnitude of the response. Finally, there was no difference in the number of memory cells seen on day 60 in the lungs, spleen, or MLNs (data not shown), indicating that CD28 costimulation is not required for the generation of secondary memory cells. The question however of the quality of this secondary memory generated in the absence of CD28 costimulation remains open.

Since the T cell inhibitory CTLA-4 signal (37, 38) is unaffected when blocking CD28, the reduced CD8\(^+\) T cell response described above may have been the result of augmented CTLA-4 signaling due to increased availability of CD80 and CD86 ligands for CTLA-4 binding. To address this issue, we used B7/− mice that are deficient in the expression of both CD80 and CD86 and cannot provide CD28 or CTLA-4 signaling. To overcome the fact that these mice are unable to optimally prime naïve T cells and generate normal primary responses and equal memory, we performed adoptive transfer of equal numbers of memory CD8\(^+\) T cells into wild-type and B7-deficient mice. Importantly, in this model, memory was generated following lung infection of animals with virus.

Determining the requirement of CD28 costimulation for memory CD8\(^+\) T cells generated following an infection of animals was important because the presence of inflammation can affect the quantity and potentially quality of memory generated (39) and may alter costimulation requirements. For these studies, we used OT-I TCR-transgenic T cells that are specific for an OVA\(_{257-264}\) peptide (SIINFEKL). One million naive OT-I TCR-transgenic T cells were adoptively transferred i.v. into wild-type mice, the recipients were infected i.n. with H1N1 WSN-OVA influenza virus that expresses the OVA\(_{257-264}\) peptide, and then 5 \(\times 10^5\) day 60 memory OVA\(_{257-264}\)-specific CD8\(^+\) T cells were transferred to both wild-type and B7/− mice for secondary infection. This model, lack of CD28 costimulation during rechallenge of memory CD8\(^+\) T cells resulted also in decreased frequency and absolute numbers of OVA\(_{257-264}\)-specific CD8\(^+\) T cells on day 7, the peak of the response (36). At day 7 after challenge, the frequency of lung OVA\(_{257-264}\)-specific CD8\(^+\) T cells was only 2.68 \pm 1.99% (mean \pm SE) of CD8\(^+\) T cells in B7/− mice compared with 22.26 \pm 14.48% for wild-type mice (\(p < 0.02; n = 5\); Fig. 2, A and B). The absolute numbers of OVA-specific CD8\(^+\) T cells in the lung of B7/− mice were 1.31 \pm 1.12 \times 10^5 compared with 11.29 \pm 3.77 \times 10^5 in wild-type animals (\(p < 0.05\); n = 5; Fig. 2C). OVA\(_{257-264}\)-specific CD8\(^+\) T cells were also reduced in MLNs and spleen. The absolute numbers of MLN OVA-specific CD8\(^+\) T cells in B7/− mice was 0.4 \pm 0.6 \times 10^5 compared with 1.14 \pm 0.9 \times 10^5 in wild-type animals (\(p < 0.02; n = 3\)–4). In spleen, B7/− mice had 5.30 \pm 0.51 \times 10^5 OVA-specific CD8\(^+\) T cells compared with 9.18 \pm 0.49 \times 10^5 in wild-type mice (\(p < 0.005\); n = 5). Frequencies of OVA-specific CD8\(^+\) T cells were also reduced in MLNs and spleen of B7/− mice compared with wild-type animals (data not shown). The frequency and absolute numbers of total CD8\(^+\) T cells in lung were also significantly lower in B7/− animals compared with wild-type animals (28.18 \pm 3.87% vs 12.58 \pm 0.53% for wild-type and B7/−, respectively, \(n = 5\), \(p < 0.001\); 2.75 \pm 0.65 \times 10^6 and 1.51 \pm 0.96 \times 10^6 for wild-type and B7/−, respectively, \(n = 5\), \(p < 0.05\)), and this most likely reflects the greatly reduced numbers of Ag-specific CD8\(^+\) T cells in the lung. CD8\(^+\) T cells in MLNs and spleen however did not differ between wild-type and B7/− mice (data not shown). Lung, MLN, and spleen lymphocyte numbers were not significantly different between wild-type and B7/− animals (data not shown).

In the experiments described above, we had to exclude that the lack of CD28 costimulation could be affecting either survival or trafficking of memory CD8\(^+\) T cells into effector sites and secondary lymphoid organs. Rapid death of memory CD8\(^+\) T cells or their inability to traffic to either effector sites or lymphoid tissue in B7/− mice after adoptive transfer would confound our findings. To test this, we transferred 4 \times 10^6 CFSE-labeled day 60 memory OVA\(_{257-264}\)-specific CD8\(^+\) T cells into either uninfected or infected wild-type or B7/− mice. Forty-eight hours later, animals
were harvested and the distribution of CFSE+ OVA257–264-specific CD8+ T cells was determined. No differences between wild-type and B7−/− animals could be found in the numbers of CFSE+ OVA257–264-specific CD8+ T cells in the lungs or spleens of these animals (data not shown). These findings indicate that under steady state and inflammation due to infection, neither survival nor trafficking of memory CD8+ T cells is affected by the absence of B7-1 and B7-2.

**HSV-specific CD8+ T cell recall responses require CD28 costimulation**

Having clearly established the requirement for CD28 costimulation in influenza infection, we then examined the role of CD28 costimulation in the reactivation of memory CD8+ T cells to a different virus, namely, the DNA virus HSV-1. Similar to our findings using influenza virus, we demonstrate that memory HSV-1-specific CD8+ T cells also require CD28 costimulation. When \(5 \times 10^7\) day 60 memory HSV gB498–505-specific CD8+ T cells were transferred into wild-type and B7−/− animals and these animals were then footpad rechallenged with HSV-1, the secondary CD8+ T cell response in the draining popliteal lymph nodes against the HSV gB498–505 epitope was reduced 4.5-fold in B7−/− mice (Fig. 2D). Wild-type animals had 4.96 ± 0.3 × 10^4 HSV gB498–505-specific CD8+ T cells compared with 1.26 ± 0.1 × 10^4 cells in B7−/− mice (\(p < 0.004\); Fig. 2D). Therefore, the requirement for CD28 in the reactivation of memory CD8+ T cells is not restricted to the influenza virus response but rather exists for different classes of viruses. Whether this costimulation is also required for other pathogens such as bacteria remains however unknown.

**CD28 costimulation of CD8+ T cells is required early following secondary influenza infection and is CD4+ T cell independent**

To determine how early costimulation is required, we examined the OVA257–264-specific CD8+ T cell response on day 3 in the MLNs. We found that following adoptive transfer of \(5 \times 10^8\) day 60 memory OVA257–264-specific CD8+ T cell and rechallenging, there was a 4-fold fewer OVA257–264-specific CD8+ T cells as early as day 3 in the MLNs in B7−/− mice compared with wild-type mice (0.25 ± 0.06 × 10^5 and 0.82 ± 0.10^5 OVA-specific CD8+ T cells for B7−/− and wild-type mice, respectively; \(n = 3\) in each group, \(p < 0.04\); Fig. 3A). To confirm this finding in wild-type animals, we blocked costimulation during rechallenge with anti-CD28 mAb in wild-type mice that had been primed with H1N1 PR8 influenza virus and rechallenged 60 days later with H3N2 HKx31 influenza virus and found a 2-fold reduction in the number of NP366–374-specific CD8+ T cells in the MLNs on day 3 after rechallenge in anti-CD28 Ab-treated (0.14 ± 0.03 × 10^5 virus-specific CD8+ T cells, \(n = 3\)) compared with untreated (0.33 ± 0.04 × 10^5 virus-specific CD8+ T cells, \(n = 3\)) mice (Fig. 3B). These results show that CD28 costimulation is crucial early after the reactivation of memory CD8+ T cells for their expansion.

In the studies described above, the lack of CD28 costimulation could be directly affecting CD8+ T cells or indirectly by affecting the CD4+ T cell response and reducing the help these cells provide to CD8+ T cells. To determine this, we adoptively transferred \(5 \times 10^6\) day 60 memory NP366–374-specific CD8+ T cells into MHC class II-deficient mice (these mice lack CD4+ T cells) and then blocked CD28 during rechallenge. In untreated animals, the peak of the secondary lung NP366–374-specific CD8+ T cell response was only reduced 2-fold from 6.7 ± 2.5 × 10^5 to 3.8 ± 0.6 × 10^5 in the absence of CD4+ T cell help (Fig. 3, C and D), indicating that memory CD8+ T cells require some help. However, the lung NP366–374-specific CD8+ T cell response was reduced ~20-fold when CD28 was blocked in both class II−/− mice (0.2 ± 0.09 × 10^5, \(n = 3\); \(p < 0.004\) when compared with untreated class II−/− mice) and wild-type mice (0.3 ± 0.01 × 10^5, \(n = 3\); \(p < 0.004\) when compared with untreated wild-type mice) (Fig. 3, C and D). These findings clearly show that CD28 is not acting indirectly through CD4+ T cells and most likely CD28 is having a direct effect on memory CD8+ T cells.
CD28 costimulation of CD8⁺ T cells is required for the down-regulation of Bcl-2 and cell cycle progression

CD28 costimulation is known to enhance the survival of T cells during a primary response by increasing the expression of Bcl-xL (15). We therefore examined the expression of this antiapoptotic molecule along with its reciprocally regulated antiapoptotic counterpart, Bcl-2 (40–43). Expression of these molecules was detected by intracellular staining and flow cytometry. We found that, as expected, both anti-CD28-treated and B7-deficient mice had decreased Bcl-xL expression in reactivated memory virus-specific CD8⁺ T cells in the lung and MLNs on day 7 after rechallenge (data not shown). Surprisingly, Bcl-2 expression in reactivated memory virus-specific CD8⁺ T cells was increased in both anti-CD28-treated (Fig. 4, A–C) and B7-deficient mice (data not shown). Normally, Bcl-2 is down-regulated upon naive CD8⁺ T cell activation following viral infection (44). The increase in Bcl-2 was seen in both the lung (mean fluorescence intensity: anti-CD28 Ab-treated: 803 ± 76 and untreated: 457 ± 31) (Fig. 4B) and MLNs (mean fluorescence intensity: anti-CD28 Ab-treated: 711 ± 96, untreated: 594 ± 98; Fig. 4C). Similar increases were seen in the B7⁻/⁻ mice (data not shown). The increased Bcl-2 expression may be the result of the CD8⁺ T cells not being fully activated; however, this is unlikely since the increased Bcl-2 is found in lung virus-specific CD8⁺ T cells that obviously have undergone a number of divisions. These results therefore indicate that CD28 signaling may be directly involved in the down-regulation of Bcl-2.

The reduced secondary CD8⁺ T cell response seen in the absence of CD28 costimulation could either be due to an increase in apoptotic death or reduced proliferation of reactivated memory CD8⁺ T cells. We therefore examined lung and MLN virus-specific CD8⁺ T cells at days 3–7 after infection for the presence of activated caspase 3, an indicator of apoptosis, and expression of Ki-67, a cell cycle protein that indicates ongoing proliferation. Expression of these two markers was examined throughout a kinetic study using the CD28-blocking Ab model. We found no difference in caspase 3 or Ki-67 in the virus-specific CD8⁺ T cells of untreated or anti-CD28-treated animals throughout the kinetics examined (data not shown). However, the increased levels of Bcl-2 in the absence of CD28 costimulation that we found, may be affecting cell cycle and this may account for the decrease in virus-specific CD8⁺ T cells, since Bcl-2 overexpression has been shown to result in cell cycle arrest (45–48). Indeed, cell cycle analysis showed an arrest at the G1-S stage of the cell cycle (Fig. 4, D and E) in NP₆₆₆₋₇₄-specific CD8⁺ T cells in B7-deficient mice compared with wild-type mice. Since Ki-67 is expressed at all active stages of the cell cycle, including the G1-S phase (49), this would account for why Ki-67 was not decreased when we blocked CD28 costimulation.

Impaired viral clearance by CD8⁺ T cells lacking costimulation during secondary pathogen rechallenge

Memory CD8⁺ T cells play a critical role in viral clearance during secondary infection (50, 51). To determine the physiological relevance of a lack of CD28 costimulation during memory CD8⁺ T cell reactivation, influenza viral loads in the lungs of infected mice were measured by real-time PCR. We found that in the lungs of mice that had been treated with the CD28-blocking Ab, the virus persisted at maximum levels 2 days longer than in isotype control-treated animals. On day 6, anti-CD28-treated animals had a 10-fold higher lung viral load over that of isotype control-treated animals (mean ± SE: 35.3 ± 8.4 × 10⁶ and 3.3 ± 0.3 × 10⁴ TCID₅₀ per lung in anti-CD28 and isotype Ab-treated mice, respectively; Fig. 5A). The importance of CD28 signaling for memory CD8⁺ T cells to control viral rechallenge was also shown by measuring viral titers in the footpad following rechallenge with HSV-1. In the absence of CD28 costimulation, increased viral titers were found on day 3 of the secondary HSV-1 infection in B7⁻/⁻ mice (229 ± 70 × 10⁴ PFU/footpad; mean ± SE) compared with wild-type mice (3.79 ± 0.58 × 10⁴ PFU/footpad; mean ± SE; Fig. 5B). These results indicate that CD28 costimulation is important for memory CD8⁺ T cells to generate optimal secondary responses during a secondary pathogen rechallenge.
and control viral load at early time points after secondary infections. Therefore, the lack of CD28 costimulation may have important pathophysiological consequences since viral titers remain higher longer.

**Discussion**

CD28 costimulation has a clearly defined role in the activation of naive CD8$^+$ T cells during a first encounter with a foreign pathogen by providing signals to the T cell which enhance activation, survival, and proliferation (3–5). The current paradigm in immunology is that this costimulatory signal is not required for the reactivation of memory CD4$^+$ and CD8$^+$ T cells since these cells have a lower threshold for activation and proliferate and respond more quickly to secondary challenge (16, 17, 21, 22). A major caveat of these studies is that most were performed in vitro and this may not necessarily reflect the in vivo complexity of the immune response. Kim et al. (17) did examine the in vivo requirement of CD28 costimulation by CD8$^+$ T cells and concluded no role in the secondary CD8$^+$ T cell response for CD28 signaling. This was based on in vivo stimulation of memory transgenic T cells by feeding Ag to mice and simultaneously treating with CTLA-4-Ig. The effect of blocking costimulation, however, was assessed by measuring blastogenesis and not quantifying the secondary response. Since we detect cell cycle arrest when memory CD8$^+$ T cells are stimulated in the absence of CD28 costimulation, it is very possible that increased cell size and proliferation are disassociated when CD28 costimulation is lacking. Indeed, in our study however did show that CD11c expression was partially blocked by CTLA-4-Ig, indicating that CD28 costimulation was playing some role. A recent study examining in vivo memory CD4$^+$ T cells rechallenged with peptide suggested that CD28 is required in vivo for the maximal secondary response (52). A question with all of the above is whether CD28 is really required for memory T cells responses under conditions of an infection where presumably inflammation will up-regulate many other potential costimulatory factors and cytokines. A study by Bertram et al. (53) suggested that memory CD8$^+$ T cells do not require CD28 costimulation but require 4-1BB costimulation during influenza virus rechallenge. This study examined CD28$^{-/-}$ animals treated with an agonist anti-4-1BB Ab during the primary response and then determined the secondary response which was restored. A problem with this study was that rechallenge was performed at day 21 after infection when memory is not fully formed and contraction is still ongoing (36). Another problem is that anti-4-1BB Ab was most likely still present during such an early rechallenge, and therefore it is not that memory CD8$^+$ T cells from CD28$^{-/-}$ mice do not require CD28 costimulation, but rather that during a secondary response 4-1BB stimulation can substitute CD28 costimulation. Indeed, we have previously shown that primary CD8$^+$ T cell responses are greatly reduced in CD28$^{-/-}$ mice and that these can be restored by anti-4-1BB agonist Ab (7). Thus, exogenous 4-1BB stimulation may also substitute for CD28 when the latter is lacking in memory cells. Although our current study clearly shows that CD28 costimulation is required by memory cells in the setting of viral infections, this does not however exclude that potential requirement for additional costimulatory signals such as 4-1BB costimulation, something suggested by Bertram et al. (53). Finally, our current study shows that memory CD8$^+$ T cells when rechallenged by viral infection require CD28 costimulation. In its absence, memory CD8$^+$ T cells suboptimally expand and fail to rapidly control virus, suggesting an important physiological relevance for such a costimulation requirement.

In this study, we used two different methods to directly examine the in vivo role of CD28 costimulation in the generation of secondary CD8$^+$ T cell responses to influenza A and HSV-1 viruses. By blocking CD28 with a specific mAb and examining secondary responses in mice deficient in the costimulatory ligands CD80 and CD86, we found that CD28 costimulation is crucial in the generation of optimal secondary CD8$^+$ T cell responses. Thus, our findings challenge a long-standing paradigm of immunology that dictates that memory CD8$^+$ T cells have little or no requirement for CD28 costimulation. We blocked CD28 in addition to using B7$^{-/-}$ animals, since there may be alternative receptors for B7-1 and B7-1 other than CD28 and CTLA-4 (54) and therefore just using the B7$^{-/-}$ mice would not have identified CD28 as the signaling receptor. The finding that memory T cells require CD28 costimulation is of particular importance for memory CD8$^+$ T cells because it suggests that maximal responses to pathogens will require APC even in the case of pathogens that do not infect APC. For CD4$^+$ T cells, this may not be much of a problem since Ag presentation occurs by the exogenous pathway, but for memory CD8$^+$ T cells that depend primarily on the endogenous pathway this suggests that cross-presentation is critical for such non-APC-infecting pathogens. Our findings suggest a DC requirement for memory CD8$^+$ T cells and this is supported by recent studies which indicated that DC are indeed required for optimal secondary CD8$^+$ T cell responses against viruses (30, 31). Our data suggest that one of the crucial signals provided by these DC during a secondary response is CD28 costimulation. Where the DC-memory CD8$^+$ T cell encounter and CD28 costimulation is occurring is currently unclear, because memory CD8$^+$ T cells can be found in...
both lymphoid and nonlymphoid tissues (55). A recent study has shown that memory influenza virus-specific CD8\(^+\) T cells can be reactivated even in the absence of secondary lymphoid organs (56), suggesting that inducible lymphoid structures such as inducible BALT (57, 58) are the sites where DC provide CD28 costimulation to memory CD8\(^+\) T cells. Such an in situ reactivation of memory CD8\(^+\) T cells at the site of infection may maximize their protective effect as these cells expand locally and do not have to recirculate to get to the site of infection which would be the case if their reactivation was occurring at the draining lymph nodes.

Our studies also examined the mechanism by which CD28 contributes to the reactivation of memory CD8\(^+\) T cells. We found that absence of CD28 costimulation during secondary infection results in a reduction in the number of virus-specific CD8\(^+\) T cells responding rather than a delay in the kinetic of the response. Additionally, kinetic studies showed that CD28 costimulation plays a role early during the reactivation of memory CD8\(^+\) T cells, as lack of CD28 signaling results in a reduced expansion of virus-specific CD8\(^+\) T cells in the draining lymph nodes as early as 3 days after infection. Furthermore, we found that when costimulation is absent, virus-specific CD8\(^+\) T cells were arrested at the G1-S stage of the cell cycle. Indeed, CD28 costimulation has been shown to enhance cell cycle progression by up-regulation of cyclins (59), the activation of CDK4-CDK6 complexes (60), and the phosphorylation of retinoblastoma proteins (60). CD28 also inhibits negative regulators of the cell cycle, such as the CDK inhibitor INK and p27\(^kip1\), thus assisting with cell cycle progression (60–62). CD28 costimulation is able to promote survival at G1 by up-regulating the antiapoptotic molecule Bcl-x\(_L\) (63). Thus, CD28 costimulation plays an essential role in proliferation and cell cycle progression and lack of this signal may therefore lead to cell cycle arrest.

Our results also show that Bcl-2 expression is increased in virus-specific CD8\(^+\) T cells when CD28 costimulation is blocked. This could either be the result of a failure to down-regulate Bcl-2 or a selection of high Bcl-2 cells when CD28 signaling is absent. This latter situation could arise if CD28 is providing some non-Bcl-2-related survival signals and in the absence of CD28 signaling Bcl-2\(_{high}\) cells have a survival advantage and accumulate. We believe this latter possibility is less likely because we did not detect an increase of ex vivo apoptosis when we blocked CD28 and as we discussed above we found evidence of cell cycle arrest. Given, however, the difficulty of detecting in vivo apoptosis, this possibility cannot be fully ruled out. Bcl-2 is traditionally thought of as being important for cell survival. The overexpression of Bcl-2 however has also been shown to induce cellular senescence and G\(_1\) cell cycle arrest (45, 48) by inhibiting the activity of CDK2 complexes (47). Therefore, it is possible that in the absence of costimulation, Bcl-2 is not down-regulated and that this overexpression of Bcl-2 in the virus-specific CD8\(^+\) T cells leads to an arrest in the cell cycle. Thus, it seems that CD28 costimulation plays an important role in the down-regulation of Bcl-2 and cell cycle progression when memory CD8\(^+\) T cells are rechallenged with Ag. Our findings also put forth the notion that not only is CD28 costimulation required for the up-regulation of Bcl-x\(_L\) to aid in survival, but additionally for the down-regulation of Bcl-2 to enhance cell cycle progression. In fact, the idea of reciprocal regulation of Bcl-2 and Bcl-x\(_L\) expression has previously been proposed (40–43). This indicates an important balance that seems to be controlled by CD28 signaling and is crucial for optimal cell cycle progression and proliferation to ensure that optimal CD8\(^+\) T cell recall responses are generated.

One could argue that CD28 costimulation is required by low-affinity TCR-bearing memory CD8\(^+\) T cells and that high-affinity TCR-expressing memory CD8\(^+\) T cells do not require such costimulation. Our studies using the OT-I OVA\(_{257–264}\)-specific TCR-transgenic memory T cells, which are known to be high-affinity binders to OVA\(_{257–264}\) peptide MHC class I complexes (64), clearly show that CD28 costimulation is required for high-affinity TCR-expressing memory CD8\(^+\) T cells. Thus, the CD28 requirement for memory CD8\(^+\) T cells we demonstrate here is most likely independent of TCR affinity.

Lacking CD28 costimulation during rechallenge resulted in an increase of influenza virus and HSV-1 viral loads. In the absence of CD28 costimulation, the secondary CD8\(^+\) T cell response seems to resemble more a primary response in both magnitude and viral clearance than a secondary response. Although the virus was cleared successfully even in the absence of CD28 signaling, the ability of the virus to persist longer at maximal viral loads could have critical consequences. Normally, influenza A virus infection is controlled and limited to the upper respiratory tract. However, if the virus spreads to the lower respiratory tract, it can result in severe desquamation of the bronchial and alveolar epithelium which can result in the death of the host (65). Thus, the persistence of virus at high levels when the secondary CD8\(^+\) T cell response is impaired could result in increased damage to host tissues. Our studies therefore suggest that CD28 costimulation is critical for the generation of protective secondary CTL responses.

Our findings also have important implications in view of a number of pathogens that have evolved to evade host immune responses by down-modulating CD80 and CD86 on the surface of APC. For example, the measles virus is able to infect DC and inhibit CD40 ligand-dependent maturation, thereby inhibiting the up-regulation of CD80 and CD86 costimulatory ligands (66). Varicella zoster virus establishes productive infection of immature DC and is able to selectively down-regulate expression of CD80 and CD86 (67). HIV-1 Nef and Vpr proteins have also been shown to impair CD80 and CD86 expression to evade host immune responses (68, 69). Thus, a requirement for CD28 costimulation in memory CD8\(^+\) T cell reactivation could have important implications in terms of vaccines and therapeutic applications for these types of pathogens. Vaccines that efficiently generate memory CD8\(^+\) T cells may nevertheless prove inefficient against viruses that inhibit CD28 costimulation of memory cells. In contrast, providing CD28 costimulation against such viruses may allow memory cells to be fully activated. The requirement for CD28 costimulation by memory CD8\(^+\) T cells may have important implications for tumor-specific CD8\(^+\) T cells since tumors are known to fail to provide CD28 costimulation. Memory established after therapy and tumor load reduction may not be fully activated when recurrence occurs in patients. Our findings also raise the question whether tolerance-inducing therapies such as CTLA-4-Ig may also interfere with protection provided by infection- or vaccine-induced memory responses to pathogens by impairing the reactivation of these memory cells. Another question in the transplantation setting is whether allospecific memory CD8\(^+\) T cells require CD28 costimulation. Adams et al. (70) found that allospecific memory CD8\(^+\) T cells generated by repeated stimulation with viruses contributed to allograft rejection and were not rendered tolerant by treatment with CTLA-4-Ig and anti-CD40 ligand. This suggests that allospecific and virus-specific memory CD8\(^+\) T cells may have different requirements for CD28 costimulation. It should be noted, however, that if costimulation blockade is combined with inhibition of NF-κB, then tolerance can be induced in allospecific memory CD8\(^+\) T cells (70). Neither costimulation blockade or NF-κB inhibition alone is sufficient to tolerate these memory cells (70). It is possible that the extent of Ag load in the transplantation studies overrides the ability to tolerate by CD28 costimulation, and NF-κB inhibition, which will mitigate TCR signaling (71), allows...
tolerization by dampening the level of TCR stimulation. Thus, the difference between allospecific- and virus-specific CD8+ T cells requirement for CD28 costimulation may be due to different antigen load between the transplant and infection settings.

In summary, our data show a critical role for CD28 costimulation in the expansion of memory CD8+ T cells, the generation of maximal secondary response, and effective clearing of virus. In the absence of CD28 costimulation, memory cells fail to down-regulate Bcl-2 and are cell cycle arrested. The CD28 costimulation requirement of memory CD8+ T cells could affect secondary responses generated from memory CD8+ T cells induced by infection but also from vaccines and tumors. Furthermore, our findings indicate that vaccines may be ineffective against pathogens that evade the host immune response by manipulating CD28 costimulation but also that strategies to stimulate CD28 during reinfection may enhance responses against such pathogens.

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Disclosures
The authors declare no conflict of interest or financial interests.

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