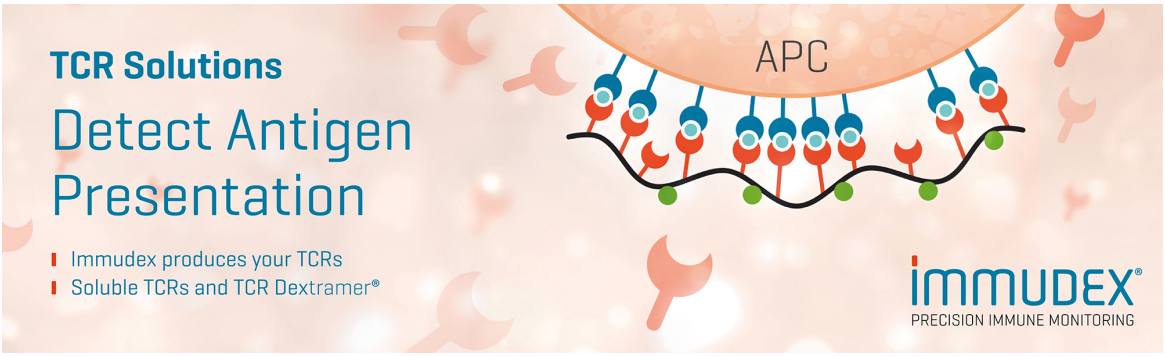

The Journal of Immunology

RESEARCH ARTICLE | NOVEMBER 15 2007

Memory CD8+ T Cells Require CD28 Costimulation[1] FREE

Annie B. Borowski; ... et. al

J Immunol (2007) 179 (10): 6494–6503.
https://doi.org/10.4049/jimmunol.179.10.6494

Related Content

Dendritic Cells and CD28 Costimulation Are Required To Sustain Virus-Specific CD8+ T Cell Responses during the Effector Phase In Vivo

J Immunol (April,2011)

CD28 costimulation promotes the production of Th2 cytokines.

J Immunol (January,1997)

CD28 Costimulation Is Essential for Human T Regulatory Expansion and Function

J Immunol (August,2008)

Downloaded from http://journals.aai.org/jimmunol/article-pdf/179/10/6494/1245361/zim02207006494.pdf by guest on 19 April 2024

Memory CD8$^+$ T Cells Require CD28 Costimulation[1]

Annie B. Borowski,* Alina C. Boesteanu,* Yvonne M. Mueller,* Caterina Carafides,* David J. Topham,† John D. Altman,‡ Stephen R. Jennings,* and Peter D. Katsikis[2]*

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 were 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-FLIPshort, 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

*Department of Microbiology and Immunology, Drexel University College of Medicine, Philadelphia, PA 19129; †Department of Microbiology and Immunology, University of Rochester School of Medicine and Dentistry, Rochester, NY 14642; and ‡Emory Vaccine Research Center, Atlanta, GA 30329

Received for publication May 31, 2007. Accepted for publication September 4, 2007.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This study was supported by Grants R01 AI66215, R01 AI46719, and R01 AI62437 from the National Institutes of Health (to P.D.K.).

[2] Address correspondence and reprint requests to Dr. Peter D. Katsikis, Department of Microbiology and Immunology and Institute for Molecular Medicine and Infectious Disease, Drexel University College of Medicine, 2900 Queen Lane, Philadelphia, PA 19129. E-mail address: Peter.Katsikis@DrexelMed.edu

[3] Abbreviations used in this paper: DC, dendritic cell; NP, nuclear protein; MLN, mediastinal lymph node.

Copyright © 2007 by The American Association of Immunologists, Inc. 0022-1767/07/$2.00

Downloaded from http://journals.aai.org/jimmunol/article-pdf/179/10/6494/1245361/zim02207006494.pdf by guest on 19 April 2024

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/6J (wild-type) and B6.129S4-Cd80^{tm1Shr}Cd86^{tm1Shr}/J (B7$^{-/-}$) mice were purchased from The Jackson Laboratory. B6.129-H2-*Ab1*^{tm1Gru}N12 (class II$^{-/-}$) mice were purchased from Taconic Farms. Mice were maintained at Drexel University College of Medicine in an 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/Puerto Rico/8/34 (PR8, H1N1) influenza type A strain, a recombinant viral strain of A/Aichi/2/68 and A/Puerto Rico/8/34 (HKx31, H3N2), and a recombinant strain of A/WSN/33 (WSN-OVA, H1N1) which expresses OVA were used. For HSV-1 experiments, HSV-1 Patton and an arbovirus expressing $gB_{498-505}$ were used. Influenza virus-specific MHC class I H-2D^b tetramer loaded with the immunodominant peptide $NP_{366-374}$ was prepared as previously described (32). The $NP_{366-374}$ peptide (ASNENMETM), the $OVA_{257-264}$ peptide (SIINFEKL) and the $gB_{498-505}$ peptide (SSIEFARL) were purchased from Anaspec. Anti-CD28 blocking Ab clone 37.51 and its isotype control were purchased from eBioscience. All Abs were functional grade (no azide/low endotoxin). Mice were treated with 100 μg i.p. on days 0, 2, and 4 of secondary infection.

Infection with influenza virus and HSV-1

Mice were primed i.p. with 12,000 hemagglutinin units (HAU) of influenza A virus strain PR8 and then rechallenged intranasally (i.n.) with 0.128 HAU of influenza A virus HKx31 strain in 20 μl of PBS for all blocking Ab experiments. All WSN-OVA i.n. infections were done with 6.4 HAU. For i.n. infections, mice were anesthetized by i.p. injection of avertin (2,2,2 tribromoethanol, 240 mg/kg; Acros).

For HSV-1 infections, mice were primed by rear footpad injection of 20 μl of 1.1×10^4 infectious units of arbovirus expressing HSV $gB_{498-505}$. Mice were rechallenged intradermal in both rear footpads with 20 μl of HSV-1 Patton strain (6×10^7 PFU/ml).

OT-I splenocyte adoptive transfer

For priming, splenocytes were isolated from the spleens of OT-I TCR-transgenic mice and total splenocytes (1×10^6 OT-I cells) were i.v. transferred into C57BL/6J (wild-type) mice in 100 μl of sterile injectable NaCl. For secondary studies, memory splenocytes were harvested and CD8$^+$ T cells were purified by negative selection (SpinSep CD8$^+$ T cell purification kit; Stem Cell Technologies). Fifty thousand memory $OVA_{257-264}$-specific CD8$^+$ T cells in 100 μl of sterile injectable saline were adoptively transferred by i.v. injection to wild-type and B7$^{-/-}$ mice. For trafficking and survival experiments, cells were labeled with 5 μM CFSE (Molecular Probes) for 5 min, washed, and then 4×10^5 memory $OVA_{257-264}$-specific CD8$^+$ T cells were adoptively transferred into wild-type and B7$^{-/-}$ mice. Animals were either infected with PR8 influenza virus as above or left uninfected. Animals were harvested 48 h later.

Lymphocyte isolation

Single-cell suspensions from lung, lymph node, and spleen were prepared as previously described (7). Cells were resuspended in RPMI 1640 complete medium (containing 10% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.2 mM L-glutamine.) Cells were then counted using acridine orange (3 μg/ml) and ethidium bromide (5 μg/ml; Sigma-Aldrich) to visualize live and dead cells under UV light.

Flow cytometry

Virus-specific CD8$^+$ T cells were quantitated and phenotyped by flow cytometry using MHC class I H-2D^b tetramers loaded with influenza virus nucleoprotein peptide ($NP_{366-374}$) and conjugated to APC as previously described (32). Abs used to phenotype virus-specific cells were anti-CD8-Alexa Fluor 405, anti-CD4-PE-Texas Red (Caltag Laboratories), anti-Bcl-2-PE, anti-Ki-67-FITC (BD Biosciences) anti-Bcl-x_L-PE (Southern Biotech Associates). OT-I cells were identified by staining for the TCR with anti-Va2-FITC and anti-Vb5.1-PE (BD Biosciences). For some experiments, Va2 TCR was stained with anti-Va2-biotin and streptavidin-PE-Texas Red (BD Biosciences). For intracellular IFN-γ staining, anti-CD8-PE-Cy5 and anti-IFN-γ-allophycocyanin (eBioscience) were used. Cells were stained as described previously (7). Cells were fixed with 1% paraformaldehyde and collected with a FACSCalibur using CellQuest or FACSAria using BD FACS Diva software (BD Biosciences) and then data were analyzed using FlowJo software (Tree Star).

Intracellular IFN-γ staining

Six-hour stimulations were performed in the presence or absence of 10 μg/ml influenza virus $NP_{366-374}$ peptide, $OVA_{257-264}$ peptide, or HSV-1 $gB_{498-505}$ peptide and 10 μg/ml brefeldin A. Cells were stained as described above for surface and intracellular staining. Samples were collected with a FACSCalibur using CellQuest (BD Biosciences). Data were analyzed using FlowJo software (Tree Star).

Cell cycle analysis

Lymphocytes were surface stained as described above, then permeabilized with 200 μl of Cytofix/Cytoperm (BD Biosciences) for 20 min. on ice. Cells were washed twice, then stained in 1 ml of propidium iodide at 50 μg/ml with RNase (100 μg; Molecular Probes) in Permwash (BD Biosciences) for 30 min at room temperature. Cells were washed with 2 ml of Permwash, spun down, and 2.5 ml was removed to leave 0.5 ml to run samples on the FACSCalibur (BD Biosciences). Data were analyzed using FlowJo software (Tree Star).

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 $NP_{366-374}$ peptide overnight. Target cells were then labeled with 100 μCi of ^{51}Cr for 75 min at 37°C, washed twice, and mixed (10^4 target cells/well) with varying 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 $\times$ *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 $\times$ (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′-TCTAACCGAGGTCGAAACGTA-3′) and random hexamers. Real-time assays were performed in triplicate with 5 μl of cDNA, 12.5 μl of 2$\times$ TaqMan Universal PCR Master Mix (Applied Biosystems), 900 nM influenza A virus sense primer (5′-AAGACCAATCCTGTCACCTCTGA-3′), 900 nM influenza A virus antisense primer (5′-CAAAGCGTCTACGCTGCAGTCC-3′), and 200 nM influenza A virus probe (FAM-5′-TTTGTGTTCACGCTCACCGT-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 ($TCID_{50}$) U/lung by comparison to an influenza viral stock standard curve run 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 preformed 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$^+$ T cell recall responses

We directly addressed the role for CD28 costimulation in the reactivation of memory CD8$^+$ T cells by using two different models

Downloaded from http://journals.aai.org/jimmunol/article-pdf/179/10/6494/1245361/zim02207006494.pdf by guest on 19 April 2024

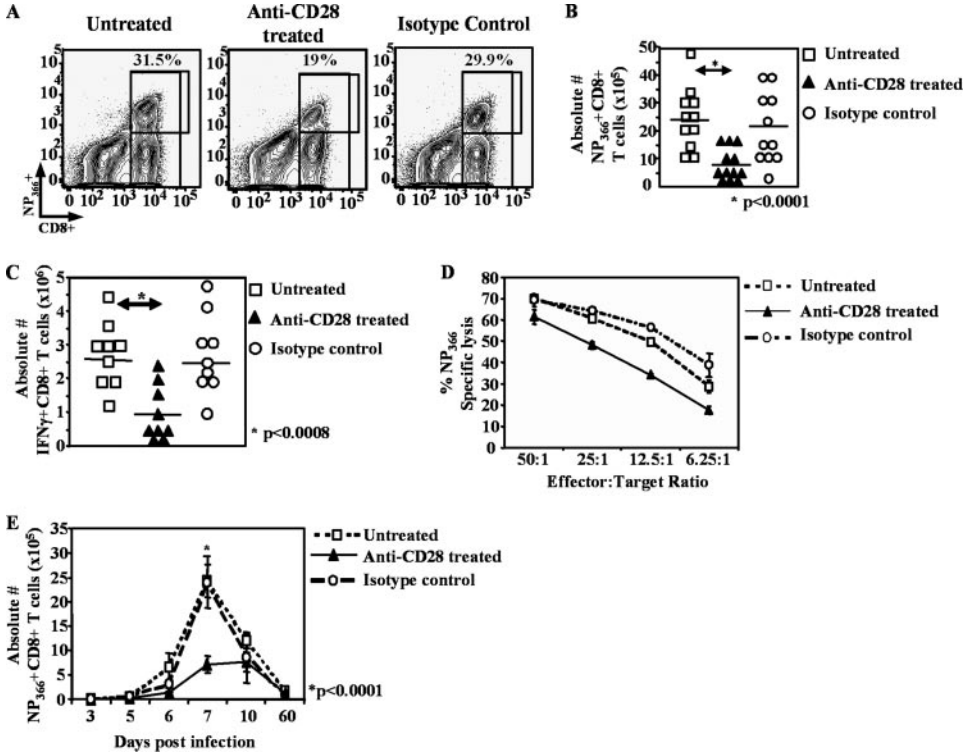

FIGURE 1. Blocking CD28 costimulation inhibits influenza virus-specific CD8$^+$ T cell recall responses. *A–E*, Blocking CD28 costimulation during secondary infection results in reduced virus-specific CD8$^+$ T cell responses. C57BL/6J (wild-type) mice were primed with PR8 influenza A virus and rechallenged 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-γ 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 nondepleting mAb that blocks CD28 in vivo (35). Indeed, anti-CD28 treatment did not deplete naive 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^b 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-γ 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. 1*C*). Blocking CD28 also reduced ex vivo cytotoxicity of lung lymphocytes against NP-peptide loaded targets in a ^{51}Cr release assay (Fig. 1*D*). 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 ($3.6 \pm 0.7 \times 10^6$ and $8.2 \pm 0.8 \times 10^6$ for anti-CD28 Ab-treated and untreated animals, respectively; $p < 0.001$;

Downloaded from http://journals.aai.org/jimmunol/article-pdf/179/10/6494/1245361/zim02207006494.pdf by guest on 19 April 2024

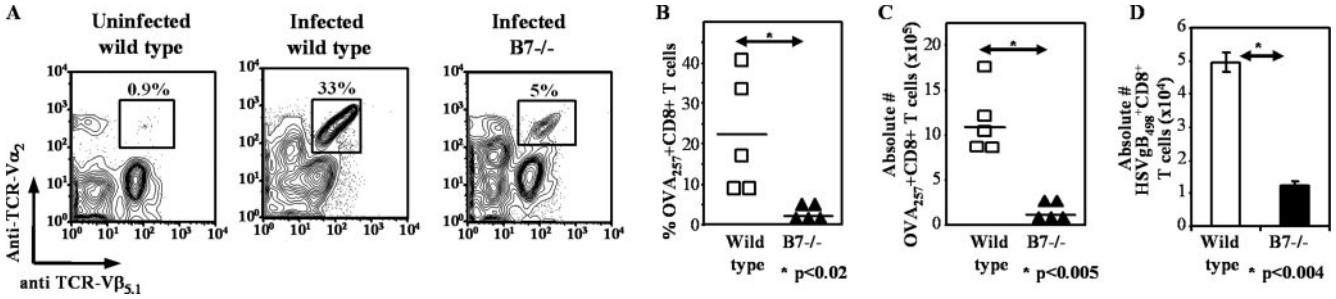

FIGURE 2. CD80 and CD86 are required for optimal virus-specific $CD8^+$ T cell recall responses. *A*, The secondary $CD8^+$ T cell response to influenza virus infection is reduced in B7-deficient mice. Following adoptive transfer of 5×10^4 day 60 memory $OVA_{257–264}$-specific $CD8^+$ T cells that were 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. $V\alpha2^+V\beta5.1^+CD8^+$ 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 5×10^4 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 Patton. Absolute numbers (mean ± SE) of day 7 splenic $gB_{498–505}$-specific $CD8^+$ T cells measured by intracellular staining for IFN-γ production following $gB_{498–505}$ peptide stimulation are shown ($n = 3$/group).

$n = 11$). Numbers of total pulmonary lymphocytes were not significantly different (data not shown).

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. 1*E*). 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 naive 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 5×10^4 day 60 memory $OVA_{257–264}$-specific $CD8^+$ T cells were transferred to both wild-type and $B7^{-/-}$ mice for secondary infection. In 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 ± 1.99% (mean ± SE) of $CD8^+$ T cells in $B7^{-/-}$ mice compared with 22.26 ± 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 ($p < 0.005$; $n = 5$; Fig. 2*C*). $OVA_{257–264}$-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 $0.4 \pm 0.6 \times 10^4$ compared with $1.14 \pm 0.9 \times 10^4$ 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 ± 3.87% vs 12.58 ± 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 describe 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 4×10^5 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

Downloaded from http://journals.aai.org/jimmunol/article-pdf/179/10/6494/1245361/zim02207006494.pdf by guest on 19 April 2024

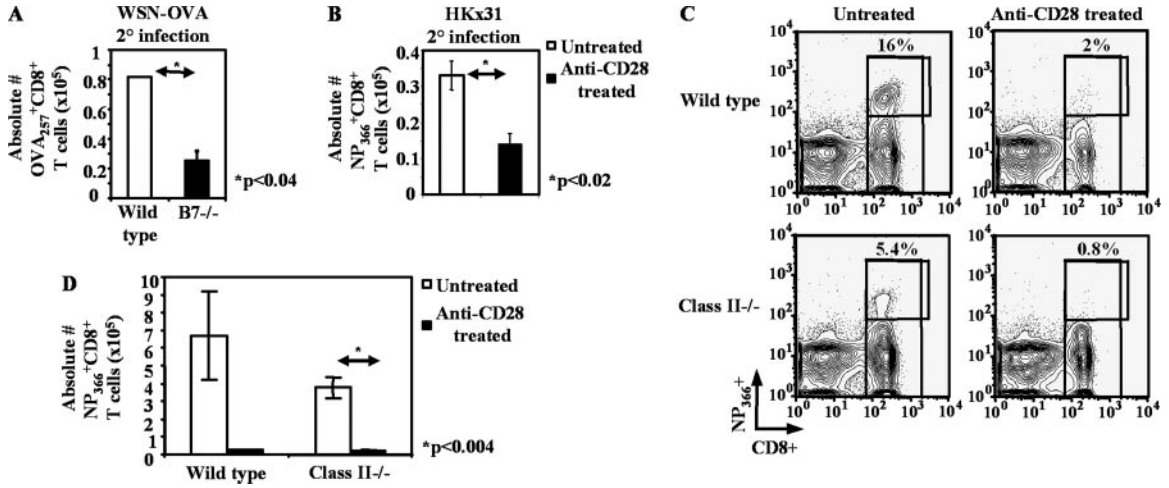

FIGURE 3. CD28 costimulation of CD8$^+$ T cells is required early following secondary influenza infection and is CD4$^+$ T cell independent. *A*, The $OVA_{257-264}$-specific CD8$^+$ T cell secondary response is reduced early during secondary infection following adoptive transfer of memory cells into B7$^{-/-}$ mice. Absolute numbers of day 3 MLN $OVA_{257-264}$-specific CD8$^+$ T cells shown (mean + SE shown, $n = 3$/group). *B*, Blocking CD28 costimulation during rechallenge results in a reduced secondary CD8$^+$ T cell response to influenza A virus early in the response. Absolute numbers of day 3 MLN $NP_{366-374}$-specific CD8$^+$ T cells shown (mean ± SE shown, $n = 3$/group). *C* and *D*, Blocking CD28 costimulation reduces the secondary CD8$^+$ T cell response in the absence of CD4$^+$ T cells. Wild-type and class II knockout mice were infected i.n. with HKx31 influenza virus following adoptive transfer of 5×10^4 day 60 memory CD8$^+$ T cells generated in wild-type mice primed with PR8 influenza. Mice were treated with blocking anti-CD28 Ab. *C*, Representative FACS plots of lung $NP_{366-374}$-specific CD8$^+$ T cells on day 7 after rechallenge ($n = 3$). Numbers in FACS plots indicate the percentage of pulmonary CD8$^+$ T cells that are $NP_{366-374}$-specific. *D*, Absolute numbers of day 7 lung influenza $NP_{366-374}$-specific CD8$^+$ T cells shown (mean ± SE shown, $n = 3$/group).

were harvested and the distribution of CFSE$^+$ $OVA_{257-264}$-specific CD8$^+$ T cells was determined. No differences between wild-type and B7$^{-/-}$ animals could be found in the numbers of CFSE$^+$ $OVA_{257-264}$-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 5×10^4 day 60 memory HSV $gB_{498-505}$-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 $gB_{498-505}$ epitope was reduced 4.5-fold in B7$^{-/-}$ mice (Fig. 2*D*). Wild-type animals had $4.96 \pm 0.3 \times 10^4$ HSV $gB_{498-505}$-specific CD8$^+$ T cells compared with $1.26 \pm 0.1 \times 10^4$ cells in B7$^{-/-}$ mice ($p < 0.004$; Fig. 2*D*). 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 $OVA_{257-264}$-specific CD8$^+$ T cell response on day 3 in the MLNs. We found that following adoptive transfer of 5×10^4 day 60 memory $OVA_{257-264}$-specific CD8$^+$ T cell and rechallenge, there was 4-fold fewer $OVA_{257-264}$-specific CD8$^+$ T cells as early as day 3 in the MLNs in B7$^{-/-}$ mice compared with wild-type mice ($0.25 \pm 0.06 \times 10^5$ and 0.82×10^5 OVA-specific CD8$^+$ T cells for B7$^{-/-}$ and wild-type mice, respectively; $n = 3$ in each group, $p < 0.04$; Fig. 3*A*). 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 $NP_{366-374}$-specific CD8$^+$ T cells in the MLNs on day 3 after rechallenge in anti-CD28 Ab-treated ($0.14 \pm 0.03 \times 10^5$ virus-specific CD8$^+$ T cells, $n = 3$) compared with untreated ($0.33 \pm 0.04 \times 10^5$ virus-specific CD8$^+$ T cells, $n = 3$) mice (Fig. 3*B*). 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 5×10^4 day 60 memory $NP_{366-374}$-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 $NP_{366-374}$-specific CD8$^+$ T cells response was only reduced 2-fold from $6.7 \pm 2.5 \times 10^5$ to $3.8 \pm 0.6 \times 10^5$ 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 $NP_{366-374}$-specific CD8$^+$ T cell response was reduced ~20-fold when CD28 was blocked in both class II$^{-/-}$ mice ($0.2 \pm 0.09 \times 10^5$, $n = 3$; $p < 0.004$ when compared with untreated class II$^{-/-}$ mice) and wild-type mice ($0.3 \pm 0.01 \times 10^5$, $n = 3$; $p < 0.004$ 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.

Downloaded from http://journals.aai.org/jimmunol/article-pdf/179/10/6494/1245361/zim02207006494.pdf by guest on 19 April 2024

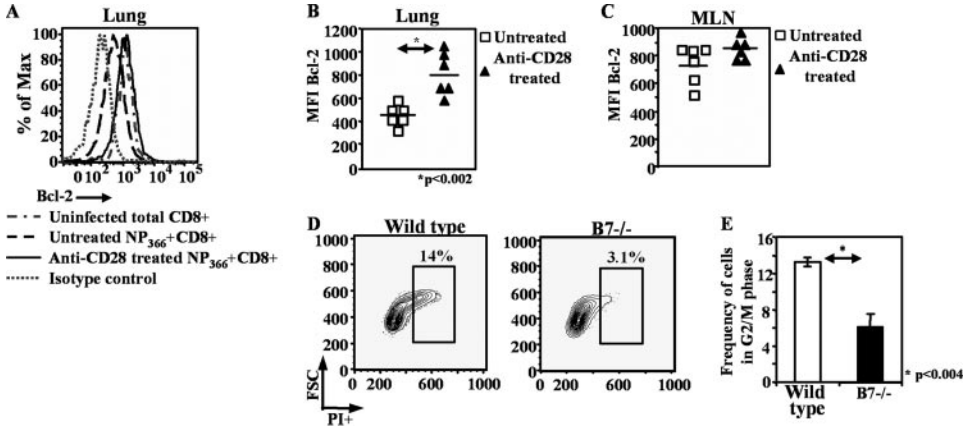

FIGURE 4. CD28 costimulation of memory $CD8^+$ T cells is required for the down-regulation of Bcl-2 and cell cycle progression. *A–C*, Bcl-2 fails to down-regulate in $NP_{366-374}$-specific $CD8^+$ T cells when CD28 costimulation is blocked during secondary infection. Day 7 Bcl-2 was measured by intracellular staining following HKx31 virus rechallenge of PR8 virus-primed mice that were treated with anti-CD28-blocking Ab during rechallenge. *A*, Histograms of Bcl-2 expression in lung $NP_{366-374}$-specific $CD8^+$ T cells shown are representative of two experiments and six animals. *B*, Bcl-2 expression in $NP_{366-374}$-specific $CD8^+$ T cells in the lungs. Each symbol represents an individual animal; bars, means. *C*, Bcl-2 expression in MLN $NP_{366-374}$-specific $CD8^+$ T cells of individual animals (each symbol) shown. Bars, Means. *D* and *E*, The absence of CD28 costimulation leads to cell cycle arrest at G_1-S. Day 60 $NP_{366-374}$-memory $CD8^+$ T cells from mice primed with PR8 influenza virus were transferred into wild-type and $B7^{-/-}$ mice. Mice were rechallenged i.n. with HKx31 influenza and pulmonary lymphocytes were examined on day 7 for cell cycle progression by staining with propidium iodide (PI). *D*, Representative FACS plots of lung $NP_{366-374}$-specific $CD8^+$ T cells showing reduced G_2-M phase cells; plots are representative of three animals per group. *E*, Frequency of lung $NP_{366-374}$-specific $CD8^+$ T cells in the G_2-M phase shown (mean ± SE shown, $n = 3$/group).

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-x_L (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-x_L 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. 4*B*) and MLNs (mean fluorescence intensity: anti-CD28 Ab-treated: 711 ± 96, untreated: 594 ± 98; Fig. 4*C*). 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 G_1-S stage of the cell cycle (Fig. 4, *D* and *E*) in $NP_{366-374}$-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 G_1-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: $35.3 \pm 8.4 \times 10^3$ and $3.3 \pm 0.3 \times 10^3$ $TCID_{50}$ per lung in anti-CD28 and isotype Ab-treated mice, respectively; Fig. 5*A*). 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 ($229 \pm 70 \times 10^4$ PFU/footpad; mean ± SE) compared with wild-type mice ($3.79 \pm 0.58 \times 10^4$ PFU/footpad; mean ± SE; Fig. 5*B*). These results indicate that CD28 costimulation is important for memory $CD8^+$ T cells to generate optimal secondary responses

Downloaded from http://journals.aai.org/jimmunol/article-pdf/179/10/6494/1245361/zim02207006494.pdf by guest on 19 April 2024

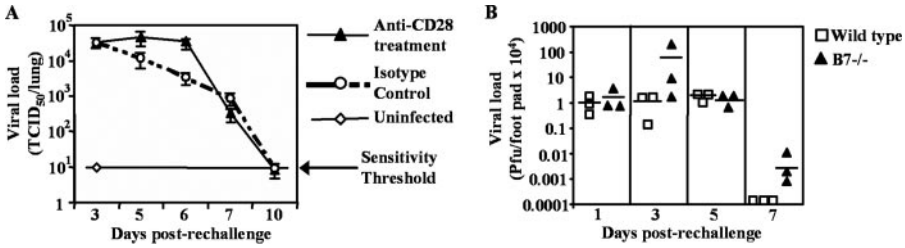

FIGURE 5. Impaired viral clearance by CD8$^+$ T cells lacking CD28 costimulation during pathogen rechallenge. *A*, Reduced clearance of influenza virus from lungs when CD28 costimulation is blocked. RNA was isolated from lungs of anti-CD28 or isotype control-treated C57BL/6J mice on the indicated days after secondary infection with HKx31 influenza A virus. Viral load was measured by TaqMan real-time PCR amplification of the influenza matrix protein using a FAM-labeled probe. Each time point is representative of triplicates from two or three animals per group. *B*, Lack of CD80 and CD86 result in impaired control of HSV-1 in footpads. Wild-type and B7$^{-/-}$ mice were infected with HSV-1 Patton following transfer of day 60 memory $gB_{498-505}$-specific CD8$^+$ T cells from HSV $gB_{498-505}$-expressing arbovirus infected mice. Footpads were examined for viral titers by plaque assay. Plaque assays were preformed in triplicate. Each symbol represents a single animal.

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 studies, cell size of secondary virus-specific CD8$^+$ T cells generated from memory cells did not differ between controls and anti-CD28 Ab-treated or B7$^{-/-}$ animals (data not shown). The Kim et al. (17) 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

Downloaded from http://journals.aai.org/jimmunol/article-pdf/179/10/6494/1245361/zim02207006494.pdf by guest on 19 April 2024

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 G_1-S stage of the cell cycle. Indeed, CD28 costimulation has been shown to enhance cell cycle progression by up-regulation of D 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 inhibitors INK and $p27^{Kip1}$, thus assisting with cell cycle progression (60–62). CD28 costimulation is able to promote survival at G_1 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 tolerize these memory cells (70). It is possible that the extent of Ag load in the transplantation studies overrides the ability to tolerize by CD28 costimulation, and NF-κB inhibition, which will mitigate TCR signaling (71), allows

Downloaded from http://journals.aai.org/jimmunol/article-pdf/179/10/6494/1245361/zim02207006494.pdf by guest on 19 April 2024

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 antigenic 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.

Acknowledgment

We thank Hao Shen (University of Pennsylvania) for providing us with OT-1-transgenic mice.

Disclosures

The authors declare no conflict of interest or financial interests.

References

1. Bretscher, P., and M. Cohn. 1970. A theory of self-nonself discrimination. *Science* 169: 1042–1049.
2. Lafferty, K. J., and A. J. Cunningham. 1975. A new analysis of allogeneic interactions. *Aust. J. Exp. Biol. Med. Sci.* 53: 27–42.
3. Harding, F. A., J. G. McArthur, J. A. Gross, D. H. Raulet, and J. P. Allison. 1992. CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones. *Nature* 356: 607–609.
4. Jenkins, M. K., P. S. Taylor, S. D. Norton, and K. B. Urdahl. 1991. CD28 delivers a costimulatory signal involved in antigen-specific IL-2 production by human T cells. *J. Immunol.* 147: 2461–2466.
5. June, C. H., J. A. Ledbetter, M. M. Gillespie, T. Lindsten, and C. B. Thompson. 1987. T-cell proliferation involving the CD28 pathway is associated with cyclosporine-resistant interleukin 2 gene expression. *Mol. Cell. Biol.* 7: 4472–4481.
6. Bertram, E. M., P. Lau, and T. H. Watts. 2002. Temporal segregation of 4-1BB versus CD28-mediated costimulation: 4-1BB ligand influences T cell numbers late in the primary response and regulates the size of the T cell memory response following influenza infection. *J. Immunol.* 168: 3777–3785.
7. Halstead, E. S., Y. M. Mueller, J. D. Altman, and P. D. Katsikis. 2002. In vivo stimulation of CD137 broadens primary antiviral CD8$^+$ T cell responses. *Nat. Immunol.* 3: 536–541.
8. Lumsden, J. M., J. M. Roberts, N. L. Harris, R. J. Peach, and F. Ronchese. 2000. Differential requirement for CD80 and CD80/CD86-dependent costimulation in the lung immune response to an influenza virus infection. *J. Immunol.* 164: 79–85.
9. Mittrucker, H.-W., M. Kursar, A. Kohler, R. Hurwitz, and S. H. E. Kaufmann. 2001. Role of CD28 for the generation and expansion of antigen-specific CD8$^+$ T lymphocytes during infection with *Listeria monocytogenes*. *J. Immunol.* 167: 5620–5627.
10. Shedlock, D. J., J. K. Whitmire, J. Tan, A. S. MacDonald, R. Ahmed, and H. Shen. 2003. Role of CD4 T cell help and costimulation in CD8 T cell responses during *Listeria monocytogenes* infection. *J. Immunol.* 170: 2053–2063.
11. Suresh, M., J. K. Whitmire, L. E. Harrington, C. P. Larsen, T. C. Pearson, J. D. Altman, and R. Ahmed. 2001. Role of CD28–B7 interactions in generation and maintenance of CD8 T cell memory. *J. Immunol.* 167: 5565–5573.
12. Tuosto, L., and O. Acuto. 1998. CD28 affects the earliest signaling events generated by TCR engagement. *Eur. J. Immunol.* 28: 2131–2142.
13. Viola, A., and A. Lanzavecchia. 1996. T cell activation determined by T cell receptor number and tunable thresholds. *Science* 273: 104–106.
14. Kirchhoff, S., W. W. Muller, M. Li-Weber, and P. H. Krammer. 2000. Up-regulation of c-FLIPshort and reduction of activation-induced cell death in CD28-costimulated human T cells. *Eur. J. Immunol.* 30: 2765–2774.
15. Sperling, A. I., J. A. Auger, B. D. Ehst, I. C. Rulifson, C. B. Thompson, and J. A. Bluestone. 1996. CD28/B7 interactions deliver a unique signal to naive T cells that regulates cell survival but not early proliferation. *J. Immunol.* 157: 3909–3917.
16. Bachmann, M. F., A. Gallimore, S. Linkert, V. Cerundolo, A. Lanzavecchia, M. Kopf, and A. Viola. 1999. Developmental regulation of Lck targeting to the CD8 coreceptor controls signaling in naive and memory T cells. *J. Exp. Med.* 189: 1521–1530.
17. Kim, S. K., K. S. Schluns, and L. Lefrancois. 1999. Induction and visualization of mucosal memory CD8 T cells following systemic virus infection. *J. Immunol.* 163: 4125–4132.
18. Sprent, J., and C. D. Surh. 2002. T cell memory. *Annu. Rev. Immunol.* 20: 551–579.
19. Tough, D. F., P. Borrow, and J. Sprent. 1996. Induction of bystander T cell proliferation by viruses and type I interferon in vivo. *Science* 272: 1947–1950.
20. Zhang, X., S. Sun, I. Hwang, D. F. Tough, and J. Sprent. 1998. Potent and selective stimulation of memory-phenotype CD8$^+$ T cells in vivo by IL-15. *Immunity* 8: 591–599.
21. Croft, M., L. M. Bradley, and S. L. Swain. 1994. Naive versus memory CD4 T cell response to antigen: memory cells are less dependent on accessory cell costimulation and can respond to many antigen-presenting cell types including resting B cells. *J. Immunol.* 152: 2675–2685.
22. London, C. A., M. P. Lodge, and A. K. Abbas. 2000. Functional responses and costimulator dependence of memory CD4$^+$ T cells. *J. Immunol.* 164: 265–272.
23. Shahinian, A., K. Pfeffer, K. P. Lee, T. M. Kundig, K. Kishihara, A. Wakeham, K. Kawai, P. S. Ohashi, C. B. Thompson, and T. W. Mak. 1993. Differential T cell costimulatory requirements in CD28-deficient mice. *Science* 261: 609–612.
24. Bevan, M. J., and P. J. Fink. 2001. The CD8 response on autopilot. *Nat. Immunol.* 2: 381–382.
25. Kaech, S. M., and R. Ahmed. 2001. Memory CD8$^+$ T cell differentiation: initial antigen encounter triggers a developmental program in naive cells. *Nat. Immunol.* 2: 415–422.
26. Murali-Krishna, K., J. D. Altman, M. Suresh, D. J. Sourdive, A. J. Zajac, J. D. Miller, J. Slansky, and R. Ahmed. 1998. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. *Immunity* 8: 177–187.
27. Turka, L. A., J. A. Ledbetter, K. Lee, C. H. June, and C. B. Thompson. 1990. CD28 is an inducible T cell surface antigen that transduces a proliferative signal in CD3$^+$ mature thymocytes. *J. Immunol.* 144: 1646–1653.
28. Vacchio, M. S., J. A. Williams, and R. J. Hodes. 2005. A novel role for CD28 in thymic selection: elimination of CD28/B7 interactions increases positive selection. *Eur. J. Immunol.* 35: 418–427.
29. Zheng, X., J.-X. Gao, X. Chang, Y. Wang, Y. Liu, J. Wen, H. Zhang, J. Zhang, Y. Liu, and P. Zheng. 2004. B7-CD28 interaction promotes proliferation and survival but suppresses differentiation of CD4$^-$CD8$^-$ T cells in the thymus. *J. Immunol.* 173: 2253–2261.
30. Belz, G. T., N. S. Wilson, C. M. Smith, A. M. Mount, F. R. Carbone, and W. R. Heath. 2006. Bone marrow-derived cells expand memory CD8$^+$ T cells in response to viral infections of the lung and skin. *Eur. J. Immunol.* 36: 327–335.
31. Zammit, D. J., L. S. Cauley, Q. M. Pham, and L. Lefrancois. 2005. Dendritic cells maximize the memory CD8 T cell response to infection. *Immunity* 22: 561–570.
32. Altman, J. D., P. A. H. Moss, P. J. R. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael, and M. M. Davis. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274: 94–96.
33. Ward, C. L., M. H. Dempsey, C. J. Ring, R. E. Kempson, L. Zhang, D. Gor, B. W. Snowden, and M. Tisdale. 2004. Design and performance testing of quantitative real time PCR assays for influenza A and B viral load measurement. *J. Clin. Virol.* 29: 179–188.
34. Smith, P. M., R. M. Wolcott, R. Chervenak, and S. R. Jennings. 1994. Control of acute cutaneous herpes simplex virus infection: T cell-mediated viral clearance is dependent upon interferon-γ (IFN-γ). *Virology* 202: 76–88.
35. Prilliman, K. R., E. E. Lemmens, G. Palioungas, T. G. Wolfe, J. P. Allison, A. H. Sharpe, and S. P. Schoenberger. 2002. Cutting edge: a crucial role for B7-CD28 in Transmitting T help from APC to CTL. *J. Immunol.* 169: 4094–4097.
36. Flynn, K. J., J. M. Riberdy, J. P. Christensen, J. D. Altman, and P. C. Doherty. 1999. In vivo proliferation of naive and memory influenza-specific CD8$^+$ T cells. *Proc. Natl. Acad. Sci. USA* 96: 8597–8602.
37. Krummel, M. F., and J. P. Allison. 1995. CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. *J. Exp. Med.* 182: 459–465.
38. Walunas, T. L., D. J. Lenschow, C. Y. Bakker, P. S. Linsley, G. J. Freeman, J. M. Green, C. B. Thompson, and J. A. Bluestone. 1994. CTLA-4 can function as a negative regulator of T cell activation. *Immunity* 1: 405–413.
39. Badovinac, V. P., K. A. Messingham, A. Jabbari, J. S. Haring, and J. T. Harty. 2005. Accelerated CD8$^+$ T-cell memory and prime-boost response after dendritic-cell vaccination. *Nat. Med.* 11: 748–756.
40. Arriola, E. L., A. M. Rodriguez-Lopez, J. A. Hickman, and C. M. Chresta. 1999. Bcl-2 overexpression results in reciprocal downregulation of Bcl-x_L and sensitizes human testicular germ cell tumours to chemotherapy-induced apoptosis. *Oncogene* 18: 1457–1464.
41. Behrens, T. W., and D. L. Mueller. 1997. Bcl-x and the regulation of survival in the immune system. *Immunol. Res.* 16: 149–160.
42. Chao, D. T., and S. J. Korsmeyer. 1998. BCL-2 family: regulators of cell death. *Annu. Rev. Immunol.* 16: 395–419.
43. Chao, D. T., G. P. Linette, L. H. Boise, L. S. White, C. B. Thompson, and S. J. Korsmeyer. 1995. Bcl-x_L and Bcl-2 repress a common pathway of cell death. *J. Exp. Med.* 182: 821–828.
44. Grayson, J. M., A. J. Zajac, J. D. Altman, and R. Ahmed. 2000. Cutting edge: increased expression of Bcl-2 in antigen-specific memory CD8$^+$ T cells. *J. Immunol.* 164: 3950–3954.
45. Chen, Q. M., J. Liu, and J. B. Merrett. 2000. Apoptosis or senescence-like growth arrest: influence of cell-cycle position, p53, p21 and bax in H_2O_2 response of normal human fibroblasts. *Biochem. J.* 347: 543–551.
46. Kim, R. 2005. Unknotting the roles of Bcl-2 and Bcl-x_L in cell death. *Biochem. Biophys. Res. Commun.* 333: 336–343.
47. Sasaki, M., T. Kumazaki, H. Takano, M. Nishiyama, and Y. Mitsui. 2001. Senescent cells are resistant to death despite low Bcl-2 level. *Mech. Ageing Dev.* 122: 1695–1706.
48. Schmitt, C. A. 2003. Senescence, apoptosis and therapy: cutting the lifelines of cancer. *Nat. Rev. Cancer.* 3: 286–295.

Downloaded from http://journals.aai.org/jimmunol/article-pdf/179/10/6494/1245361/zim02207006494.pdf by guest on 19 April 2024

49. Scholzen, T., and J. Gerdes. 2000. The Ki-67 protein: from the known and the unknown. *J. Cell Physiol.* 182: 311–322.
50. Graham, M. B., and T. J. Braciale. 1997. Resistance to and recovery from lethal influenza virus infection in B lymphocyte-deficient mice. *J. Exp. Med.* 186: 2063–2068.
51. Liang, S., K. Mozdzanowska, G. Palladino, and W. Gerhard. 1994. Heterosubtypic immunity to influenza type A virus in mice: effector mechanisms and their longevity. *J. Immunol.* 152: 1653–1661.
52. Ndejembi, M. P., J. R. Teijaro, D. S. Patke, A. W. Bingaman, M. R. Chandok, A. Azimzadeh, S. G. Nadler, and D. L. Farber. 2006. Control of memory CD4 T cell recall by the CD28/B7 costimulatory pathway. *J. Immunol.* 177: 7698–7706.
53. Bertram, E. M., W. Dawicki, B. Sedgmen, J. L. Bramson, D. H. Lynch, and T. H. Watts. 2004. A switch in costimulation from CD28 to 4-1BB during primary versus secondary CD8 T cell response to influenza in vivo. *J. Immunol.* 172: 981–988.
54. Mandelbrot, D. A., M. A. Oosterwegel, K. Shimizu, A. Yamada, G. J. Freeman, R. N. Mitchell, M. H. Sayegh, and A. H. Sharpe. 2001. B7-dependent T-cell costimulation in mice lacking CD28 and CTLA4. *J. Clin. Invest.* 107: 881–887.
55. Masopust, D., V. Vezys, A. L. Marzo, and L. Lefrancois. 2001. Preferential localization of effector memory cells in nonlymphoid tissue. *Science* 291: 2413–2417.
56. Moyron-Quiroz, J. E., J. Rangel-Moreno, L. Hartson, K. Kusser, M. P. Tighe, K. D. Klonowski, L. Lefrancois, L. S. Cauley, A. G. Harmsen, F. E. Lund, and T. D. Randall. 2006. Persistence and responsiveness of immunologic memory in the absence of secondary lymphoid organs. *Immunity* 25: 643–654.
57. Moyron-Quiroz, J. E., J. Rangel-Moreno, K. Kusser, L. Hartson, F. Sprague, S. Goodrich, D. L. Woodland, F. E. Lund, and T. D. Randall. 2004. Role of inducible bronchus associated lymphoid tissue (iBALT) in respiratory immunity. *Nat. Med.* 10: 927–934.
58. van Panhuys, N., R. Perret, M. Prout, F. Ronchese, and G. Le Gros. 2005. Effector lymphoid tissue and its crucial role in protective immunity. *Trends Immunol.* 26: 242–247.
59. Boonen, G. J., A. M. van Dijk, L. F. Verdonck, R. A. van Lier, G. Rijksen, and R. H. Medema. 1999. CD28 induces cell cycle progression by IL-2-independent down-regulation of $p27^{kip1}$ expression in human peripheral T lymphocytes. *Eur. J. Immunol.* 29: 789–798.
60. Appleman, L. J., A. Berezovskaya, I. Grass, and V. A. Boussiotis. 2000. CD28 costimulation mediates T cell expansion via IL-2-independent and IL-2-dependent regulation of cell cycle progression. *J. Immunol.* 164: 144–151.
61. Kovalev, G. I., D. S. Franklin, V. M. Coffield, Y. Xiong, and L. Su. 2001. An important role of CDK inhibitor $p18^{INK4c}$ in modulating antigen receptor-mediated T cell proliferation. *J. Immunol.* 167: 3285–3292.
62. Wolfraim, L. A., and J. J. Letterio. 2005. Cutting edge: $p27^{Kip1}$ deficiency reduces the requirement for CD28-mediated costimulation in naive $CD8^+$ but not $CD4^+$ T lymphocytes. *J. Immunol.* 174: 2481–2484.
63. Boise, L. H., A. J. Minn, P. J. Noel, C. H. June, M. A. Accavitti, T. Lindsten, and C. B. Thompson. 1995. CD28 costimulation can promote T cell survival by enhancing the expression of Bcl-x_L. *Immunity* 3: 87–98.
64. Alam, S. M., P. J. Travers, J. L. Wung, W. Nasholds, S. Redpath, S. C. Jameson, and N. R. Gascoigne. 1996. T-cell-receptor affinity and thymocyte positive selection. *Nature* 381: 616–620.
65. Zinkernagel, R. M., M. F. Bachmann, T. M. Kundig, S. Oehen, H. Pirchet, and H. Hengartner. 1996. On immunological memory. *Annu. Rev. Immunol.* 14: 333–367.
66. Servet-Delprat, C., P. O. Vidalain, H. Bausinger, S. Manie, F. Le Deist, O. Azocar, D. Hanau, A. Fischer, and C. Rabourdin-Combe. 2000. Measles virus induces abnormal differentiation of CD40 ligand-activated human dendritic cells. *J. Immunol.* 164: 1753–1760.
67. Morrow, G., B. Slobedman, A. L. Cunningham, and A. Abendroth. 2003. Varicella-zoster virus productively infects mature dendritic cells and alters their immune function. *J. Virol.* 77: 4950–4959.
68. Chaudhry, A., S. R. Das, A. Hussain, S. Mayor, A. George, V. Bal, S. Jameel, and S. Rath. 2005. The Nef protein of HIV-1 induces loss of cell surface costimulatory molecules CD80 and CD86 in APCs. *J. Immunol.* 175: 4566–4574.
69. Majumder, B., M. L. Janket, E. A. Schafer, K. Schaubert, X. L. Huang, J. Kan-Mitchell, C. R. Rinaldo, Jr., and V. Ayyavoo. 2005. Human immunodeficiency virus type 1 Vpr impairs dendritic cell maturation and T-cell activation: implications for viral immune escape. *J. Virol.* 79: 7990–8003.
70. Adams, A. B., M. A. Williams, T. R. Jones, N. Shirasugi, M. M. Durham, S. M. Kaech, E. J. Wherry, T. Onami, J. G. Lanier, K. E. Kokko, et al. 2003. Heterologous immunity provides a potent barrier to transplantation tolerance. *J. Clin. Invest.* 111: 1887–1895.
71. Schulze-Luehrmann, J., and S. Ghosh. 2006. Antigen-receptor signaling to nuclear factor κB. *Immunity* 25: 701–715.

Downloaded from http://journals.aai.org/jimmunol/article-pdf/179/10/6494/1245361/zim02207006494.pdf by guest on 19 April 2024