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Costimulation Requirements for Antiviral CD8+ T Cells Differ for Acute and Persistent Phases of Polyoma Virus Infection

Christopher C. Kemball,2,* Eun D. Han Lee,2† Eva Szomolanyi-Tsuda, ‡ Thomas C. Pearson, † Christian P. Larsen, ‡ and Aron E. Lukacher3*

The requirement for costimulation in antiviral CD8+ T cell responses has been actively investigated for acutely resolved viral infections, but it is less defined for CD8+ T cell responses to persistent virus infection. Using mouse polyoma virus (PyV) as a model of low-level persistent virus infection, we asked whether blockade of the CD40 ligand (CD40L) and CD28 costimulatory pathways impacts the magnitude and function of the PyV-specific CD8+ T response, as well as the humoral response and viral control during acute and persistent phases of infection. Costimulation blockade or gene knockout of either CD28 or CD40L substantially dampened the magnitude of the acute CD8+ T cell response; simultaneous CD28 and CD40L blockade severely depressed the acute T cell response, altered the cell surface phenotype of PyV-specific CD8+ T cells, decreased PyV VP1-specific serum IgG titers, and resulted in an increase in viral DNA levels in multiple organs. CD28 and CD40L costimulation blockade during acute infection also diminished the memory PyV-specific CD8+ T cell response and serum IgG titer, but control of viral persistence varied between mouse strains and among organs. Interestingly, we found that CD28 and CD40L costimulation is dispensable for generating and/or maintaining PyV-specific CD8+ T cells during persistent infection; however, blockade of CD27 and CD28 costimulation in persistently infected mice caused a reduction in PyV-specific CD8+ T cells. Taken together, these data indicate that CD8+ T cells primed within the distinct microenvironments of acute vs persistent virus infection differ in their costimulation requirements. The Journal of Immunology, 2006, 176: 1814–1824.

Complete Ag-driven T cell differentiation generally requires the contribution of multiple costimulatory signals that amplify the signal initiated by TCR engagement with a cognate peptide-MHC ligand. Optimal CD8+ T cell responses to acute viral infections require costimulation through at least one of several pathways, including B7-CD28, CD40-CD40 ligand (CD40L),4 41BB-41BB ligand, and CD27-CD70 (1, 2). The relative importance of each of these costimulatory signals is often virus dependent. The generation and maintenance of lymphocytic choriomeningitis virus (LCMV)-specific CD8+ T cell effectors and memory T cells is largely CD28 dependent (3–5), whereas vesicular stomatitis virus, vaccinia virus, and influenza virus (IV)-specific CD8+ T cell responses are CD28 independent (5–9). Acute, but not memory, LCMV-specific CD8+ T cell responses are also CD40-CD40L independent, whereas vesicular stomatitis virus-specific CD8+ T cell responses are CD40-CD40L dependent (10–12). Furthermore, 41BB-41BB ligand costimulation is necessary for optimal primary, memory, and secondary CD8+ T cell responses to LCMV and IV infections (4, 13–16). CD27 costimulation promotes the survival and accumulation of IV-specific CD8+ T cells at effector sites (17, 18).

Less is known about the requirement for costimulation of an ongoing CD8+ T cell response to a persistent virus infection. In the setting where CD8+ T cells are buffered by Ag long-term, costimulation may be required to sustain protective immunity and keep the virus in check. The most thoroughly characterized mouse models of persistent virus infection include gammaherpesvirus 68 (γHV-68) and highly replicating strains of LCMV (e.g., clone 13 or 11b). The magnitude of the γHV-68-specific CD8+ T cell response is normal in CD40L−/− mice, although CD40−/− and CD40L−/− mice lack long-term control of the virus (19–21). CD28−/− mice, in contrast, maintain long-term viral control but mount a delayed CD8+ T cell IFN-γ response, despite normal CTL activity (20). Virus-specific CD8+ T cell responses in CD40L−/− mice infected by LCMV clone 13 or 11b undergo progressive functional deterioration (22). In addition, Ab-mediated blockade of the B7-CD28 and CD40-CD40L pathways during persistent infection by LCMV clone 13 significantly decreases virus-specific CD8+ T cell IFN-γ production and prolongs viremia (23). These data suggest that antiviral CD8+ T cells require continual CD28 and/or CD40L costimulation for protective immunity in the settings of latent (e.g., γHV-68) and high-level (e.g., LCMV clone 13) persistent infection. In humans infected by HIV and CMV, a large fraction of virus-specific CD8+ T cells express CD27 (24, 25). Little information is available on the contribution of CD27 costimulation to maintaining antiviral CD8+ T cells in persistent infection.

The requirements for costimulation to elicit and maintain CD8+ T cell responses against viruses that establish low-level, systemic persistent infection are not well defined. Mouse polyoma virus

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establishes a low-level persistent infection in immunocompetent inbred strains of mice, which require a PyV-specific CD8\(^+\) T cell response for control of viral persistence and protection against tumor development (26–28). PyV epitope-specific CD8\(^+\) T cell populations vary in rates of expansion and contraction, hierarchy, phenotype, and cytokine profile in acute and persistent infection (29). Importantly, we found that new effector PyV-specific CD8\(^+\) T cells are primed during the persistent phase of infection (29). Antiviral CD8\(^+\) T cells newly recruited during persistent infection may be particularly dependent on costimulation for optimal priming because reduced or absent virus-induced inflammatory signals during persistent infection might elevate the need for costimulation. In addition, CD40-CD40L interaction is a major component of CD4\(^+\) T cell help provided to B cells (30). Thus, blockade of the CD40-CD40L pathway decreases virus-specific IgG responses to a number of virus infections, such as LCMV, vaccinia virus, and PyV, but it does not completely abolish them (10, 31). The contribution of the B7-CD28 costimulatory pathway to IgG responses in PyV infection has not been previously examined. In this study, we used costimulation blockade and homozygous gene knockout mice to determine how obstruction of several costimulatory pathways impacts the magnitude and functionality of the PyV-specific CD8\(^+\) T cell response, humoral response, and viral clearance. Costimulation blockade or gene knockout of either CD28 or CD40L substantially dampened the magnitude of the acute PyV-specific response. Simultaneous blockade of these pathways severely depressed the acute PyV-specific CD8\(^+\) T cell response, altered the cell surface phenotype of PyV-specific CD8\(^+\) T cells, decreased PyV V1-specific serum IgG titers, and was associated with an increase in viral DNA levels in multiple organs. Combined CD28 and CD40L blockade during acute infection also dampened the memory PyV-specific CD8\(^+\) T cell response and serum IgG titer, but control of viral persistence varied between mouse strains and among organs. We further demonstrate that PyV-specific CD8\(^+\) T cells generated de novo during persistent infection do not require CD28 and CD40L costimulation. PyV-specific CD8\(^+\) T cells declined in number when CD27 and CD28 were expressed, followed by two washes in FACS buffer. Samples were immediately assayed by flow cytometry.

**Materials and Methods**

**Mice**

C57BL/6Ncr (B6) and C3H/HeNcr female mice were purchased from the Frederick Cancer Research and Development Center of the National Cancer Institute. B6.129S2-Traf5tm1Ins (CD40L\(^+\)) on a B6 background and B6.129S2-Cd28tm1MK (CD28\(^-\)) on a B6 background were purchased from The Jackson Laboratory. B6.SJL-Piprctm1/BoAitac (CD45.1) mice were purchased from Taconic. Mice were housed and bred in accordance with the guidelines of the Institutional Animal Care and Use Committee and the Department of Animal Resources at Emory University.

**Viruses and virus inoculation**

PyV strain A2 was molecularly cloned and plaque purified, and virus stocks were prepared on primary baby mouse kidney cells as previously described (28). Each mouse was inoculated s.c. in each hind footpad with 2 × 10\(^6\) PFU of virus. Mice were infected at 6–12 wk of age.

**Peptides and class I MHC tetramers**

MT245-253, LT638-646, and MT389-397 peptides were synthesized and stored as described (29). LT359-368C7Abu (SAVKNY[Abu]JSDKL), in which the cysteine residue at position 7 was replaced with \(\alpha\)-aminobutyric acid, a thiol-less cysteine analog residue, was synthesized by the Emory University Microchemical Core Facility. Recently, we determined that H-2D\(^b\) monomers refolded with LT359-368C7Abu with greater efficiency than did monomers with the LT360-368C6Abu peptide (AVKNY[Abu]JSDKL) (data not shown), which was previously used to construct tetramers (29). At peptide concentrations ≤1 \(\mu\)M, LT359-368C7Abu stimulated IFN-\(\gamma\) production by PyV-specific CD8\(^+\) T cells from the spleens of infected mice equivalent to that from the LT360-368C6Abu peptide (data not shown). In addition, RMA/S class I MHC peptide stabilization assays revealed that LT359-368C7Abu bound H-2\(D^d\) with 10-fold greater affinity than did LT360-368C6Abu (data not shown). These data confirm that an asparagine residue at position 5 optimizes peptide binding to D\(d\), as previously described for D\(d\)-restricted LCMV CD8\(^+\) T cell epitopes (32). For simplicity, we refer to this population of Ag-specific T cells as LT359-specific CD8\(^+\) T cells. D\(d\) LT359C7Abu and D\(d\) MT389 tetramers were generated as described (29).

**Flow cytometry**

Lymphocyte populations were isolated from the blood, spleen, and lungs as previously described (29). Cells were stained in PBS containing 2% FBS and 0.1% sodium azide (FACS buffer) for 45 min at 4°C or room temperature, followed by two washes in FACS buffer. Samples were immediately acquired on a FACS Calibur (BD Biosciences) or were fixed in PBS containing 1% paraformaldehyde overnight. Cells were stained with allophycocyanin-conjugated tetramers. CD8\(a\), CD45.1, CD122, NKGA2A/C (clone 29A5), and Armenian hamster isotype control Abs were purchased from BD Pharmingen. CD27, CD28, CD122, NKGA2A (clone 16a11), and Golden Syrian hamster isotype control Abs were purchased from eBioscience. CD11a and L-selectin (CD62L) Abs were purchased from Caltag Laboratories. Data were analyzed using CellQuest software (BD Biosciences).

**Intracellular IFN-\(\gamma\) staining**

Cells were stimulated directly ex vivo with 10 \(\mu\)M synthetic peptides and stained for CD8\(a\) and IFN-\(\gamma\) as described elsewhere (29). The absolute number of Ag-specific IFN-\(\gamma\) CD8\(^+\) T cells was determined by subtracting unstimulated IFN-\(\gamma\)-CD8\(^+\) cells (no peptide) from peptide-stimulated IFN-\(\gamma\)-CD8\(^+\) cells.

**CD28 and CD40L costimulation blockade and bone marrow chimeras**

The costimulation blockade protocol was performed as previously described (33). For acute CD28 and CD40L costimulation blockade, mice received 500 \(\mu\)g of hamster anti-mouse CD40L Ab (clone MR1; BioExpress) and human CTLA4-Ig (Bristol-Myers Squibb), separately or combined, i.p. on days −2, 0, and +2 relative to the day of PyV infection. The generation of persistently infected naive CD45 congenic bone marrow chimeras has been previously described (29). Persistently infected, basulafan-treated B6 (CD45.2\(^-\)) mice received bone marrow from naive B6.SJL (CD45.1\(^+\)) mice, with or without 500 \(\mu\)g of MR1 Ab and CTLA4-Ig on days 0, 2, and 4 post-transplant and weekly thereafter for a 7-wk duration. Naive basulafan-treated B6 (CD45.2\(^-\)) mice given bone marrow from naive B6.SJL (CD45.1\(^+\)) mice were infected by PyV 48 days later with or without 500 \(\mu\)g of MR1 Ab and CTLA4-Ig on days −2, 0, and +2 of infection.

**CD70 blockade**

For blockade during acute infection, mice received 500 \(\mu\)g of rat anti-mouse CD70 (clone FR70; Bio Express) ± 500 \(\mu\)g of CTLA4-Ig (or 500 \(\mu\)g of ChromPure rat IgG (Jackson ImmunoResearch) alone) i.p. 2 days before PyV infection and 250 \(\mu\)g of FR70 ± 500 \(\mu\)g of CTLA4-Ig (or 250 \(\mu\)g of rat IgG alone) on days 0 and +2 of infection. For blockade during persistent infection, mice received 250 \(\mu\)g of FR70 ± 500 \(\mu\)g of CTLA4-Ig (or 250 \(\mu\)g of rat IgG alone) i.p. on days 40, 42, 44, 50, 57, 64, and 71 postinfection (p.i.).

**Tagman real-time PCR**

DNA isolation and Tagman PCR were performed as described (29). The PyV DNA quantity is expressed in genome copies per milligram of tissue or per milliliter of blood and is calculated based on a standard curve of known PyV genome copy number vs threshold cycle of detection. The detection limit with this assay is 10 copies of genomic viral DNA.

**ELISAs**

PyV major capsid protein VP1-specific ELISAs were performed in 96-well microtiter plates coated with purified recombinant VP1 protein (50 ng/well in carbonate buffer) that was produced in Escherichia coli (34) and kindly provided by R. L. Garcea (University of Colorado Health Sciences.

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Center, Denver, CO). The serum samples were serially diluted (2-fold) and tested in duplicates. Biotinylated goat anti-mouse IgG and streptavidin-HRP were used (Vector Laboratories) to detect IgG with 3,3'5,5'-tetramethyl-benzidine tablet substrates (Sigma-Aldrich). The plates were read at 450 nm by a THERMOMAX plate reader and Softmax software (Molecular Devices). Two-fold serial dilutions of a positive control serum sample harvested from PyV-infected C57BL/6 mice on day 21 p.i. were used to obtain a standard reference curve on each 96-well plate, and the VP1-specific IgG concentrations of the test samples were expressed in arbitrary units based on comparison with this standard curve.

**Statistics**

Statistical significance was determined by the unpaired Student’s t test, assuming unequal variances. A p value of <0.05 was considered statistically significant.

**Results**

CD28 and CD40L costimulation blockade diminishes acute PyV-specific cellular and humoral responses and is associated with elevated viral levels

C57BL/6 (B6) mice, but not C3H mice, have been reported to generate allospecific T cells in the absence of both B7-CD28 and CD40-CD40L costimulation (33). Therefore, we asked whether these mouse strains differ in the PyV-specific immune responses after blockade of each or both of these costimulation pathways. We previously showed that B6 mice mount a potent CD8+ T cell response that peaks at day 8 p.i., with a viral epitope-specific dominance hierarchy of LT359>MT245>LT638 (29). In C3H mice, PyV-specific CD8+ T cells are predominantly focused on a single epitope, MT389, and peak in magnitude on day 7–8 p.i (28, 35). Ab-mediated costimulation blockade of either the B7-CD28 or CD40-CD40L pathways substantially dampened the magnitude of the acute PyV-specific response in both B6 and C3H mouse strains, resulting in a decrease in the absolute number of splenic DnLT359- and DnMT389-specific CD8+ T cells, respectively, as detected by intracellular IFN-γ production (Fig. 1, A and B). Similar results were obtained by enumerating DnLT359 or DnMT389 tetramerCD8+ T cells in the spleen directly ex vivo (data not shown). In B6 mice, the subdominant MT245- and LT638-specific CD8+ T cell responses were likewise reduced (Fig. 1A). Simultaneous blockade of both pathways almost entirely eliminated the acute PyV-specific CD8+ T cell response and was associated with a ≥1 log increase in PyV genomes in the blood and multiple organs, including the cartilage, heart, kidney, lungs, salivary gland, and spleen (Fig. 1, A–D). Blockade of either the B7-CD28 or CD40-CD40L pathway individually led to only modest increase in viral DNA levels (data not shown), presumably because sufficient numbers of antiviral CD8+ T cells were elicited to control PyV infection.

We next asked whether the PyV-specific CD8+ T cells primed in the face of costimulation blockade were phenotypically altered. Of the small population of tetramerCD8+ T cells primed during acute infection in the presence of CD28 and CD40L blockade, there was a substantially reduced percentage of CD62Llow cells (compared with controls) in both B6 and C3H mice (Fig. 1E). In addition, a smaller percentage of tetramerCD8+ T cells expressed the NK cell inhibitory receptor CD94/NKG2A+ (Fig. 1E), which is up-regulated on PyV-specific CD8+ T cells during the course of infection (36). Despite these phenotypic differences, PyV-specific CD8+ T cells primed in the presence or absence of CD28 and CD40L costimulation blockade were uniformly CD11a(high) (data not shown). Blockade of either the B7-CD28 or CD40-CD40L pathway individually did not lead to phenotypic differences of antiviral CD8+ T cells (data not shown). These data suggest that combined CD28 and CD40L costimulation blockade impeded differentiation of PyV-specific CD8+ T cells.

Unlike many viruses that require T cell help to generate protective, isotype-switched antiviral Ab responses, PyV infection elicits a virus-neutralizing, isotype-switched, T-cell-independent type 2 Ab response (37, 38). A reduced PyV-specific Ab response against the major PyV capsid protein, VP1, could account for the elevated PyV DNA levels in mice that received costimulation blockade. As shown in Fig. 1F, VP1-specific serum IgG levels were reduced 2-fold in B6 and C3H mice that received costimulation blockade compared with wild-type mice at day 8 p.i., similar to that reported at day 14 p.i. in B6 mice given CD40L Ab (31).

**Acute PyV-specific CD8+ T cell and humoral responses in CD40L−/− and CD28−/− mice**

We sought to confirm the above results using homozygous CD28 and CD40L gene knockout mice. When compared with wild-type B6 mice, both CD28−/− and CD40L−/− mice generated reduced acute dominant and subdominant PyV-specific CD8+ T cell responses, which were further diminished with Ab blockade of the alternate pathway (Fig. 2, A and B). In CD28−/− mice, the LT359-specific response was reduced to a greater extent than was the MT245-specific response, suggesting that the dominant population was more dependent on CD28 signaling than a subdominant population (Fig. 2B). This reduction in the magnitude of the CD8+ T cell response was associated with significantly higher levels of PyV DNA in the blood and in nearly all organs examined (Fig. 2, C and D). Furthermore, in CD40L−/− mice that received CTLA4-Ig and in CD28−/− mice that received MR1 Ab, there was a substantial reduction in the percentage of tetramerCD8+ T cells that were CD62Llow, NKG2A+, and CD122high, compared with wild-type mice (Fig. 2, E and F); LT359-specific CD8+ T cells from all groups of mice were uniformly CD11a(high) (data not shown). In agreement with previous data (Fig. 1F), VP1-specific serum IgG titers were modestly reduced in CD40L−/− mice (3-fold, compared with wild-type; Fig. 2G). CD28KO mice mounted an equivalent VP1-specific response to wild-type mice, which was reduced 2-fold if CD40-CD40L interactions were blocked with MR1 Ab (Fig. 2H).

**Acute CD28 and CD40L costimulation blockade dampens PyV-specific cellular and humoral responses during the persistent phase of infection**

Given the profound effect of CD28 and CD40L costimulation blockade on the acute PyV-specific CD8+ T cell response, we next examined the long-term consequences of costimulation blockade (administered only during acute infection) on the magnitude and function of the PyV-specific CD8+ T cell response. The magnitude of LT359-specific (B6 mice) and MT389-specific (C3H) CD8+ T cell responses in persistently infected mice were each reduced after acute CD28 and CD40L costimulation blockade but were depressed to a greater extent in C3H mice (Fig. 3A). PyV DNA levels were not significantly different between control B6 and costimulation blockade groups by 41 days p.i. in multiple organs, although a greater proportion of costimulation blockade-treated mice displayed viremia (Fig. 3B). In contrast, C3H mice that had received acute costimulation blockade remained viremic by 40 days p.i. and averaged ≥1 log increase in viral DNA in the cartilage, heart, kidney, lungs, and salivary gland (with a significant increase (p = 0.01) observed in the cartilage), compared with control mice (Fig. 3C). In summary, acute CD28 and CD40L costimulation blockade dampened the PyV-specific CD8+ T cell response long-term, with control of viral persistence after acute costimulation blockade varying between strains of mice and among organs.

Finally, we asked whether acute costimulation blockade also dampened the humoral VP1-specific response during the persistent
phase of infection. B6 mice that received MR1 and CTLA4-Ig during acute infection had a >1 log lower serum VP1-specific IgG titer by day 41 p.i., compared with control mice (Fig. 3D). This decrease was due primarily to blockade of the CD40-CD40L pathway, because titers were reduced to the same extent in B6 mice that had received only MR1, but not CTLA4-Ig, during acute infection. Similar results were obtained on day 40 p.i. in C3H mice that had received combined MR1 and CTLA4-Ig treatment, although this mouse strain exhibited only a 2-fold lower serum Ab titer with MR1 treatment alone, compared with the B6 strain (Fig. 3D). Taken together, B6 mice that received CD28 and CD40L costimulation blockade during acute infection were able to control persistent virus infection to a greater extent than were C3H mice, despite having a lower antiviral humoral response.

**PyV-specific CD8$^+$ T cells that are primed during persistent infection do not require CD28 and CD40L costimulation**

Using a persistently infected bone marrow chimera model, we previously demonstrated that naive LT359-specific CD8$^+$ T cells are primed and expand during the persistent phase of PyV infection...
New antiviral CD8<sup>+</sup> T cells primed in this distinct environment of low-level persistent virus infection may be more dependent on costimulation for proper maturation, as virus-induced inflammatory signals that might bypass conventional costimulatory signals are likely absent. Two approaches were used to test this hypothesis. First, we administered 500 μg of MR1 Ab and CTLA4-Ig to persistently infected mice on days 20, 22, 24, and 26 p.i. (the same regimen that impaired acute phase anti-PyV
CD8$^+$ T cell responses (Figs. 1 and 2) and assessed the integrity of the PyV-specific CD8$^+$ T cell response at day 35 p.i. Costimulation blockade at this time did not alter the magnitude of dominant and subdominant PyV-specific CD8$^+$ T cell responses (Fig. 4).

Furthermore, PyV DNA levels were similar between control and CD28 and CD40L blockade-treated mice in the blood, cartilage, heart, kidney, lungs, salivary gland, and spleen (data not shown). In the second approach, we administered costimulation blockade repeatedly to persistently infected CD45.1 bone marrow chimeras (beginning at the time of donor transplant) for 7 wk. By 49 days post-transplant, microchimerism was established and donor-derived LT359-specific CD8$^+$ T cells were detectable in the blood, spleen, and lungs (29). The magnitude of host-derived (CD45.1$^+$) LT359-specific CD8$^+$ T cells in the lungs and spleen was unchanged in the presence of CD28 and CD40L costimulation blockade (Fig. 5A), as was seen in persistently infected B6 mice given MR1 and CTLA4-Ig (Fig. 4). Remarkably, the total numbers of donor-derived (CD45.1$^+$) LT359-specific CD8$^+$ T cells, which represent a pool of cells primed only during persistent infection, were not reduced in the presence of costimulation blockade compared with the donor-derived response in control chimeric mice (Fig. 5A). Furthermore, the majority of newly primed donor-derived Db LT359 tetramer$^+$CD8$^+$ T cells in the spleen and lungs were CD11ahighCD62Lhigh, irrespective of administration of costimulation blockade (data not shown). In addition, costimulation blockade did not affect viral control, as PyV DNA levels were unchanged in organs in which we could routinely detect PyV DNA by this time p.i., including the cartilage, heart, and kidney (data not shown).

To rule out the possibilities that the costimulation blockade regimen was insufficient to block B7-CD28 and CD40-CD40L interactions completely in the chimeric mice or that the bone marrow CD8$^+$ T cell responses (Figs. 1 and 2) and assessed the integrity of the PyV-specific CD8$^+$ T cell response at day 35 p.i. Costimulation blockade at this time did not alter the magnitude of dominant and subdominant PyV-specific CD8$^+$ T cell responses (Fig. 4). Furthermore, PyV DNA levels were similar between control and CD28 and CD40L blockade-treated mice in the blood, cartilage, heart, kidney, lungs, salivary gland, and spleen (data not shown). In the second approach, we administered costimulation blockade repeatedly to persistently infected CD45.1 bone marrow chimeras (beginning at the time of donor transplant) for 7 wk. By 49 days post-transplant, microchimerism was established and donor-derived LT359-specific CD8$^+$ T cells were detectable in the blood, spleen, and lungs (29). The magnitude of host-derived (CD45.1$^+$) LT359-specific CD8$^+$ T cells in the lungs and spleen was unchanged in the presence of CD28 and CD40L costimulation blockade (Fig. 5A), as was seen in persistently infected B6 mice given MR1 and CTLA4-Ig (Fig. 4). Remarkably, the total numbers of donor-derived (CD45.1$^+$) LT359-specific CD8$^+$ T cells, which represent a pool of cells primed only during persistent infection, were not reduced in the presence of costimulation blockade compared with the donor-derived response in control chimeric mice (Fig. 5A). Furthermore, the majority of newly primed donor-derived Db LT359 tetramer$^+$CD8$^+$ T cells in the spleen and lungs were CD11ahighCD62Lhigh, irrespective of administration of costimulation blockade (data not shown). In addition, costimulation blockade did not affect viral control, as PyV DNA levels were unchanged in organs in which we could routinely detect PyV DNA by this time p.i., including the cartilage, heart, and kidney (data not shown).

To rule out the possibilities that the costimulation blockade regimen was insufficient to block B7-CD28 and CD40-CD40L interactions completely in the chimeric mice or that the bone marrow

**FIGURE 4.** CD28 and CD40L costimulation blockade administered during persistent infection does not alter the magnitude of the memory PyV-specific CD8$^+$ T cell response. PyV immune B6 mice (day 20 p.i.) were given four injections of MR1 and CTLA4-Ig on days 20, 22, 24, and 26 p.i. The absolute numbers of LT359-, MT245-, and LT638-specific CD8$^+$ T cells at day 35 p.i. in the spleen of untreated or costimulation blockade-treated mice were determined by short-term ex vivo peptide stimulation and IFN-γ intracellular cytokine staining. Values represent the average of three mice ± SD. Similar results were obtained by quantitating D$^b$ LT359 tetramer$^+$CD8$^+$ T cells in the spleen directly ex vivo (data not shown).

**FIGURE 3.** CD28 and CD40L costimulation blockade during acute infection diminishes the memory PyV-specific CD8$^+$ T cell response. A, Absolute number of LT359-specific CD8$^+$ T cells (day 41 p.i.) and MT389-specific CD8$^+$ T cells (day 40 p.i.) in the spleen of untreated mice and mice given MR1 and CTLA4-Ig during acute PyV infection. Mean values of two mice ± SD are shown, and data are representative of two independent experiments. Similar results were obtained by quantitating D$^b$ LT359 or D$^b$ MT389 tetramer$^+$CD8$^+$ T cells in the spleen directly ex vivo (data not shown). Levels of PyV DNA in whole blood and organs of B6 (B; day 41 p.i.) or C3H mice (C; day 40 p.i.) were quantitated by Taqman real-time PCR. Individual mice (four per group) are shown. Mice without detectable PyV DNA are assigned a value of 1. D, VP1-specific IgG serum titers of B6 (day 41 p.i.) and C3H (day 40 p.i.) mice, compared with mice that received MR1, CTLA4-Ig, or MR1 and CTLA4-Ig. The mean values ± SD of four mice per group are shown.
microchimerism protocol created an environment in which T cells were refractory to CD28 and CD40L costimulation, we analyzed the effect of costimulation blockade on the LT359-specific CD8\(^+\) T cell response in mice rendered chimeric before PyV infection. Congenic bone marrow chimeras created using the same busulfan and bone marrow regimen were infected 7 wk post-transplant, when microchimerism was fully established (data not shown), and received 500 \(\mu\)g of MR1 and CTLA4-Ig on days −2, 0, and +2 of infection. Both the host and donor-derived LT359-specific CD8\(^+\) T cell responses at day 8 p.i. in the lungs and spleen were severely reduced in the presence of costimulation blockade treatment compared with controls (Fig. 5B), just as seen in B6 mice given costimulation blockade during acute infection (Fig. 1A). In summary, these data indicate that the donor-derived, PyV-specific CD8\(^+\) T cell population is primed in a CD28- and CD40L-dependent fashion during acute, but not persistent, infection.

**Blockade of the CD27 and CD28 costimulatory pathways diminishes the PyV-specific CD8\(^+\) T cell response during acute and persistent infection**

Although these data may indicate a reduced/absent requirement for CD8\(^+\) T cell costimulation during persistent infection, an alternative possibility is that other costimulatory pathways may be preferentially used at different phases of a viral infection. Recent studies using an IV infection mouse model show that CD27 signaling is necessary to promote CD8\(^+\) T cell survival and accumulation in the periphery and that there is a switch in dependence from CD28 to 4-1BB costimulation between the early and late phases of the antiviral CD8\(^+\) T cell response (15–18, 39). In addition, signaling through the CD27-CD70 and OX40-OX40L pathways have been shown to be crucial for CD28 and CD40L-independent allograft rejection (40, 41). The majority of LT359-specific CD8\(^+\) T cells express CD27 in the spleen and lungs during persistent infection, and a subset of LT359-specific CD8\(^+\) T cells also expresses CD28 (Fig. 6, A and B). To determine whether CD27-CD70 signaling is important in maintaining the anti-PyV CD8\(^+\) T cell response during persistent infection, we administered a CD70 blocking Ab (FR70) to persistently infected mice for 1 mo. Because CD27 and CD28 signals complement each other and CD70 blockade is most effective in the absence of CD28 signaling (18, 41), we also co-administered FR70 and CTLA4-Ig to block CD27 and CD28 costimulation simultaneously. The number of D\(^b\) LT359 tetramer CD8\(^+\) T cells in the spleen and lungs was unchanged after CD70 blockade during persistent infection (Fig. 6, C and D). In contrast, the number of LT359-specific CD8\(^+\) T cells in the lungs was significantly reduced (\(p < 0.05\)) after blockade of the CD27 and CD28 pathways (Fig. 6D). Similar results were obtained by enumerating LT359-specific IFN-\(\gamma\) CD8\(^+\) T cells after short-term ex vivo peptide stimulation of spleen or lung lymphocytes (data not shown). Simultaneous blockade did not significantly alter the number of LT359-specific CD8\(^+\) T cells in the spleen (Fig. 6C). In summary, PyV-specific CD8\(^+\) T cell numbers decline if both CD27 and CD28 costimulation are blocked, which suggests that the ongoing PyV-specific CD8\(^+\) T cell response depends on CD27 costimulation in the absence of CD28 signaling.

We also examined the requirement for CD27 costimulation during the acute phase of PyV infection. Nearly all LT359-specific CD8\(^+\) T cells in the spleen and lungs express CD27 and CD28 at day 8 p.i., and the majority express higher levels of CD27 in acute infection, compared with persistent infection (Fig. 6, A and B). In contrast with persistent infection, CD70 blockade substantially reduced the number of D\(^b\) LT359 tetramer CD8\(^+\) T cells in the spleen, lungs, and popliteal lymph nodes during acute infection.
taining T cells primed during persistent and acute infection, we tentatively infected congenic bone marrow chimeras as a means to distinguish PyV-specific CD8+ T cells in the spleen (A) and lungs (B) was analyzed by flow cytometry during persistent (day 47 p.i.) and acute (day 8 p.i.) infection. Representative dot plots (gated on CD8+ cells) are shown and values represent the percentage of D8 LT359 tetramer+ T cells that are CD27+ and CD28+; gates are set from isotype controls. Acutely and persistently infected mice received FR70 ± CTLA4-Ig or rat IgG alone (on days −2, 0, and +2 p.i. for acute infection and on days 40, 42, 44, 50, 57, 64, and 71 p.i. for persistent infection), and the LT359-specific CD8+ T cell response in the spleen (C) and lungs (D) was quantitated by D8 LT359 tetramer staining on day 74 or day 8 p.i. Mean values of three mice ± SD and a significant difference, compared with controls (*, p < 0.05) are shown. Similar results were obtained by enumerating LT359-specific IFN-γ+ CD8+ T cells after short-term ex vivo peptide stimulation of spleen or lung lymphocytes (data not shown).

Our data confirm that the sensitivity of antiviral CD8+ T cells to CD28 and CD40L costimulation blockade during the persistent phase of infection varies with the type of virus infection. Williams et al. (23) showed that costimulation blockade administered to mice persistently infected (~20 days p.i.) by LCMV clone 13 was associated with a 3- to 4-fold decrease in LCMV-specific CD8+ T cells and prolonged viremia. In contrast, we found that the same costimulation blockade regimen administered to PyV-infected mice beginning on day 20 p.i. had no impact on the magnitude of the PyV-specific CD8+ T cell response or on viral load. These findings indicate that LCMV clone 13-specific CD8+ T cells are more dependent on CD28 and CD40L costimulation than are PyV-specific CD8+ T cells during this timeframe. Unlike LCMV clone 13 infection, where infectious virus is readily detected up to 80 days p.i (45), PyV cannot be detected by plaque assay after day 12 p.i (35); moreover, PyV elicits a potent neutralizing Ab response during acute infection that precludes PyV challenge infection (Refs. 26 and 42 and data not shown). In the setting of high systemic viral load (and presumably high T cell epitope display), coupled with absence of virus-neutralizing Ab (46), costimulation via one or both of these costimulation pathways may counter exhaustion of LCMV-specific CD8+ T cells. In this connection, CD28 costimulation has been reported to promote T cell survival upon Ag-driven activation via up-regulation of the anti-apoptotic molecule Bcl-xL (47–49).

Examination of the PyV-specific CD8+ T cell response during persistent infection in B6 and C3H mice revealed strain-specific differences in viral control after CD28 and CD40L costimulation blockade. Although both mouse strains had similarly diminished virus-specific CD8+ T cell responses and depressed VP1-specific serum IgG levels after costimulation blockade administered at the time of PyV inoculation, B6 and C3H mice differed in their capacity to control systemic virus long-term. A number of factors may be invoked to account for this discrepancy, including differences in anti-PyV CD8+ T cell TCR avidity and differences in modulation of antiviral effector functions by cell surface inhibitory
receptors (50) or by regulatory T cells (51). Recent studies on CD8\(^+\) T cell responses and viremia in resolved and chronic hepatitis C virus and cytomegalovirus infection in humans have given rise to the concept that a broad multispecific CD8\(^+\) T cell response, rather than one narrowly focused on a limited set of epitopes, favors control of persistent viral infection (52–54). Such a multi-pronged CD8\(^+\) T cell response would facilitate clearance of infected cells that vary in density of different epitopes (55) or express viral proteins at different points in the viral lifecycle (56). In the PyV mouse model, extensive epitope mapping using overlapping synthetic peptides uncovered three CD8\(^+\) T cell epitopes derived by the viral MT and LT proteins in B6 mice, but only a strong dominant and a very weak subdominant epitope from MT in C3H mice (Refs. 29 and 35 and our unpublished observations). The importance of early control for persistently infecting viruses is highlighted by a recent study showing that yHV-68 latency is established during acute infection (57). Finally, the superior control of persistent PyV infection in B6 than in C3H mice may stem from more efficient priming of naive PyV-specific CD8\(^+\) T cells during persistent infection in B6 mice. Given the potent oncogenic potential of PyV and the association of high genome copy number with PyV-induced tumors, the higher systemic viral burden in C3H mice given costimulation blockade during acute infection should predispose them to PyV tumorigenesis.

CD28 and CD40L costimulation blockade altered the surface phenotype of PyV-specific CD8\(^+\) T cells during acute infection, specifically by decreasing the proportion of LT359- and MT389-specific CD8\(^+\) T cells that were CD62L\(^{low}\), CD94/NKG2A\(^+\), and CD122\(^{high}\). These phenotypic changes may reflect a priming environment that provides suboptimal signals for full T cell activation and differentiation. This possibility is consistent with a report demonstrating that maintenance of CD94/NKG2A expression by CD8\(^+\) T cells may require chronic TCR stimulation (59). Reduced T cell stimulation due to costimulation blockade could also translate into either a short-lived down-regulation of surface CD62L or preferential differentiation into a CD62L\(^{high}\) subset (60–62); enhanced CD62L levels may, in turn, alter the trafficking patterns of costimulation-blocked anti-PyV CD8\(^+\) T cells.

A central finding of this study is that the requirement for costimulation differs between acute and persistent phases of infection. Within the priming microenvironment of persistent infection and CD28 and CD40L costimulation blockade, PyV-specific CD8\(^+\) T cells receive sufficient signals for expansion and acquisition of effector function, despite lower levels of Ag and virus-induced inflammation than in acute infection. Engagement of other costimulatory molecules on PyV-specific CD8\(^+\) T cells, such as CD27, could provide survival signals sufficient to maintain the antiviral CD8\(^+\) T cell response in the absence of CD28 and CD40L signaling. Blockade of either CD27 or CD28 costimulation alone during the persistent phase of infection did not lead to a deterioration of the PyV-specific T cell response (Figs. 4 and 6). Interestingly, combined CD27 and CD28 costimulation blockade during persistent infection led to a significant decline in PyV-specific CD8\(^+\) T cell numbers in the lungs, but not in the spleen (Fig. 6, C and D). In this connection, CD27 supports IV-specific CD8\(^+\) T cell survival and accumulation in the lungs, particularly in the absence of CD28 (17, 18). Taken together, these data suggest that the costimulation requirements for PyV-specific CD8\(^+\) T cells during persistent infection are less dependent on any single pathway; the ongoing antiviral T cell response wanes only after multiple costimulatory signals are obstructed.

Multiple factors may contribute to the differential requirement for CD27 and CD28 costimulation to maintain PyV-specific CD8\(^+\) T cells in the lungs vs spleen during persistent infection, including APC diversity among tissues and variability in their presentation of T cell epitopes, as well as tissue-specific dependence on particular costimulatory signals. PyV DNA levels are 10- to 20-fold lower in the lungs than in the spleen during persistent infection (Fig. 3B), which could translate into reduced presentation of viral T cell epitopes by resident APCs in the lungs. It has been appreciated that acute viral replication in vivo sustains a high level of Ag display that heightens TCR signaling and reduces the requirement for T cell costimulation (63, 64). With decreased TCR engagement by cognate Ag, PyV-specific CD8\(^+\) T cells may require more costimulation to achieve sufficient TCR signal strength to generate and/or maintain antiviral CD8\(^+\) T cells in persistently infected hosts.

The finding that PyV-specific CD8\(^+\) T cells are primed independently of CD28 and CD40L signaling during persistent infection has clinical implications for development of costimulation blockade therapies to promote transplantation tolerance. Our observations address a continuing concern that CD28 and/or CD40 blockade-based strategies, while promoting graft acceptance, may disrupt protective immunity to preexisting infections. Reactivation of BK virus, a human PyV, is a major cause of renal allograft dysfunction and failure (65). Primary BK virus infection of renal allografts or reactivation of latent BK virus in the host may be due to the use of strong immunosuppressants after transplantation (66). Tolerance induction via targeting of the B7-CD28 and CD40-CD40L pathways is a potential alternative therapy, but the effects of costimulation blockade have not been completely characterized during persistent infection. Our findings suggest that kidney graft tolerance induction via CD28 and CD40L costimulation blockade may be considered as a therapy that does not dampen the ongoing and emerging anti-PyV-specific CD8\(^+\) T cell response, thereby preventing viral resurgence and allograft failure.

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References


