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CD28 Promotes CD4+ T Cell Clonal Expansion during Infection Independently of Its YMNM and PYAP Motifs

Antonio J. Pagán,*† Marion Pepper,*†,1 H. Hamlet Chu,*†,2 Jonathan M. Green,‡ and Marc K. Jenkins*†

CD28 is required for maximal proliferation of CD4+ T cells stimulated through their TCRs. Two sites within the cytoplasmic tail of CD28, a YMNM sequence that recruits PI3K and activates NF-κB and a PYAP sequence that recruits Lck, are candidates as transducers of the signals responsible for these biological effects. We tested this proposition by tracking polyclonal peptide:MHCII-specific CD4+ T cells in vivo in mice with mutations in these sites. Mice lacking CD28 or its cytoplasmic tail had the same number of naïve T cells specific for a peptide:MHCII ligand as wild-type mice. However, the mutant cells produced one tenth as many effector and memory cells as wild-type T cells after infection with bacteria expressing the antigenic peptide. Remarkably, T cells with a mutated PI3K binding site, a mutated PYAP site, or both mutations proliferated to the same extent as wild-type T cells. The only observed defect was that T cells with a mutated PYAP or Y170F site proliferated even more weakly in response to peptide without adjuvant than wild-type T cells. These results show that CD28 enhances T cell proliferation during bacterial infection by signals emanating from undiscovered sites in the cytoplasmic tail.


The generation of a primary CD4+ T cell response to an infection depends on the display of MHCII-bound pathogen-derived peptides (p:MHCII) on the surface of APCs (1). Naïve CD4+ T cells that express TCRs specific for these p:MHCII undergo multiple rounds of division and differentiate into effector cells capable of secreting cytokines that promote the microbicidal activity of other cells (2). Some of these effector T cells then survive the contraction phase to become long-lived quiescent memory cells capable of protecting the host from a second infection (3).

Although necessary, TCR signaling is not sufficient for maximal clonal expansion; concomitant CD28 signaling in response to its APC-displayed ligands CD80 and CD86 also is required. The importance of CD28 is demonstrated by the failure of CD28-deficient mice to produce germinal centers and T cell-dependent Ab responses and to clear certain infections (4, 5). At the cellular level, it has been proposed that CD28 acts by enhancing cell division by augmenting IL-2 mRNA production or stability (6, 7), whereas other reports indicate that CD28 has no effect on proliferation but promotes cell survival by increasing glucose metabolism (8) or inducing Bcl-xL (9, 10).

CD28 signal transduction is also unclear. Some studies indicate that the biological effects of CD28 depend on a signal cascade emanating from the YMNM site in the CD28 cytoplasmic tail. PI3K has been reported to bind to the YMNM phosphotyrosine (11), resulting in the recruitment of 3-phosphoinositide-dependent protein kinase, Akt, and protein kinase Cα (PKCα) to the immunological synapse (12, 13). 3-Phosphoinositide-dependent protein kinase and Akt cooperate with PKCα to activate the Bcl110/Carmal/Malt1 signalosome and subsequently induce the translocation of NF-κB to the nucleus and the transcription of NF-κB target genes encoding IL-2 (12, 14–16) and Bcl-xL (17–19). These results suggest a model in which CD28 ligation in the presence of TCR signaling activates NF-κB through PI3K. Akt also activates mammalian target of rapamycin, resulting in increased cell cycle activity and glucose metabolism (20–22). The observation that pharmacological inhibition of PI3K limits T cell proliferation and glucose metabolism in vitro (8) is consistent with this scenario. This model is challenged, however, by the determination that genetic disruption of the YMNM motif prevented PI3K binding and phosphorylation of Akt with no effect on IL-2 production or T cell proliferation (23, 24).

Other reports indicate that CD28 signals through the C-terminal PYAP motif (25). Disruption of this site eliminates PKCα, filamin A, and Lck recruitment to the CD28 cytoplasmic tail and prevents CD28-dependent enhancement of the immunological synapse (13, 26–33). These results suggest a model in which CD28 signaling causes cytoskeletal changes that indirectly improve TCR signaling by promoting the formation of the immunological synapse. This model is supported by the report that genetic disruption of the PYAP site reduced phosphorylation of PKCα and IL-2 secretion and impaired T cell proliferation (24).

It is possible that many of the conflicting reports about CD28 signaling relate to the experimental systems used. Many of the aforementioned studies used transformed cell lines with aberrant TCR signaling machinery, long-lived cultured T cell lines, non-p:
CD28 SIGNALING IN CD4+ T CELL EXPANSION IN VIVO

Materials and Methods

Mice
Six- to 8-week-old C57BL/6 (B6), B6.PL-Thy1a/CyJ mice, B6.129S7-Prkcytm1Jgr/J (C57), B6.129S2-Cd28tm1Mak/J (Cd28tm1Mak) (35), and B6.129S7-Prkcytm1Jgr/J (Cd28tm1Mak) (36) mice were purchased from The Jackson Laboratory or the National Cancer Institute. Cd28tm1/1, Cd45.1/1,.Cd45.1/2, heterozygotes, Thy1.1/1,2 heterozygotes, Cd28tm1AAA/AAA (AYAA), and Cd28Y170F/170F (AYAA) (25) were bred in-house. Bone marrow from Cd28tm1AAA/AAA (AYAA/F170F) mice was provided by J. Green, and bone marrow from Cd28tm1/1 full-length Cd28 transgenic and tail-less Cd28 transgenic mice (37) was provided by A. Singer. Bone marrow from Prkcytm1Jgr/J (39, 40) mice was provided by M. Farrar. Mice were housed in specific pathogen-free conditions according to guidelines from the University of Minnesota and the National Institutes of Health. The Institutional Animal Care and Use Committee of the University of Minnesota approved all of the animal experiments.

Listeria monocytogenes infection
Mice were injected i.v. with 10^7 CFUs of ΔactA L. monocytogenes bacteria expressing a recombinant protein containing the 2PE (EAWGALANWAVDSA) fused to chicken OVA (42).

p:MHCI tetramer production
Soluble 2W-1A^b and listeriolysin O peptide 190–201 (LLOp)-A^b molecules were produced and biotinylated in Drosophila melanogaster S2 cells and then combined with streptavidin-allophycocyanin or streptavidin-PE (Prozyme) to make tetramers, as described previously (43).

p:MHCI tetramer staining and magnetic enrichment
2W-1A^b and LLOp-1A^b staining and magnetic enrichment were performed as described previously (44). In brief, single-cell suspensions of spleen and lymph nodes were stained with 10 nM allophycocyanin- or PE-labeled 2W-1A^b or LLOp-1A^b tetramers for 1 h at room temperature. Samples then were incubated with magnetic anti-fluorochrome microbeads and run through a magnetized LS column (Miltenyi Biotec).

Abs and flow cytometry
All of the Abs were from eBioscience unless noted. Samples were stained at 4°C with Pacific Blue- or eFluor450-conjugated anti-B220 (RA3-6B2), anti-CD11b (MI-70), anti-CD11c (N418), and anti-F4/80 (BM8; Invitrogen). Pacific Orange-conjugated anti-CD8α (5H10; Invitrogen), FITC-conjugated anti-CD3e (145-2C11), PerCP-cyanine 5.5-conjugated anti-CD3e (145-2C11), anti-lyo (B20.1), or anti-CD4 (RM4-5). Alexa Fluor-conjugated anti-CD44 (B7), or allophycocyanin-Alexa Fluor 750- or allophycocyanin-Fluor 780-conjugated anti-CD4 (RM4-5). Abs. Samples were run on an LSRII flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star).

DNA staining and detection
Tetramer-enriched samples were stained with Abs against surface Ags and subsequently fixed and permeabilized with eBioscience fixation and permeabilization buffers. Cells then were stained with DAPI (1 μg/mL; Invitrogen) diluted in eBioscience permeabilization buffer for 1 h at 4°C. The DAPI signal was visualized in linear mode on an LSRII equipped with a UV laser. The DAPI-A and DAPI-W parameters were used to exclude cell aggregates.

Adoptive transfer
Polyclonal CD4+ T cells from the spleen and lymph nodes of wild-type and Cd28tm1/1 mice were isolated with a CD4+ T cell isolation kit (Miltenyi Biotec). These then were labeled with CFSE (5 μM; Invitrogen) as described previously (45). A total of 4 × 10^5 CD4+ T cells were transferred i.v. into individual Cd90.1 sex-matched recipients. A day after transfer, some mice were infected with Lm-2W bacteria, and 2W-1A^b-specific T cells from the spleen and lymph nodes were magnetically enriched and detected as described above. PE-conjugated anti-Cd90.2 (53-2.1) was used to identify donor-derived cells.

Bone marrow irradiation chimeras
Bone marrow cells were harvested from crushed femurs, tibias, and humeri. T cells were depleted from bone marrow cell suspensions with anti-Cd90.2 (30-H12) and low-toxicity rabbit complement (Cedarlane Laboratories). To generate 50:50 mixed bone marrow chimeras, equal amounts of bone marrow from each donor strain were combined. Recipient mice were irradiated with 1000 rad and injected i.v. with 5–10 × 10^6 bone marrow cells. Chimerism in the blood was assessed 8 wk after reconstitution by determining the percentages of donor-derived B cells or T cells of each strain. FITC-conjugated anti-Cd45.2 (104), PerCP-cyanine 5.5-conjugated anti-Cd45.1 (A20), PE-cyanine 7-conjugated anti-Cd90.1 (H1S51), and allophycocyanin-conjugated anti-Cd90.2 (53-2.1) Abs were used to identify donor-derived cells. Variations in the absolute numbers of 2W-1A^b-specific T cells due to minor differences in chimerism were corrected with the formula, c = (trip × 50%) / p, where p is the percentage of cells among donor-derived cells obtained experimentally, r is the experimentally determined absolute number of 2W-1A^b-specific T cells, and c is the absolute number after the correction (46).

Peptide immunization
For the analysis of clonal expansion without adjuvant, mice were immunized i.v. with 50 μg of 2PE (EAWGALANWAVDSA) (GenScript) diluted in PBS.

ReA translocation assay
OT-II CD90.1^+124^ T cells were isolated from spleen and lymph nodes with a CD4+ T cell isolation kit (Miltenyi Biotec), and 0.5–1 × 10^6 cells were adoptively transferred into CD90.2^+ wild-type recipients. The next day, recipient mice were injected i.v. with 5 μg of Escherichia coli LPS (List Biological Laboratories) diluted in PBS, and then a day later, some mice were injected i.v. with 100 μg of chicken OVA peptide 323–339 (Invitrogen) diluted in PBS. Splenocytes were harvested 20 min after peptide injection, and single-cell suspensions of splenocytes were made immediately in 1.5% paraformaldehyde (Electron Microscopy Sciences). CD90.1^+ cells were magnetically enriched as described previously (44). Enriched cells were permeabilized in 0.5% saponin (Sigma-Aldrich) and stained with biotin-conjugated anti-Cd90.1 (H1S51), PerCP-cyanine 5.5-conjugated streptavidin, PE-conjugated anti-Vβ5 (MR9-4; BD Pharmingen), Alexa Fluor 488-conjugated anti-ReA (F-6; Santa Cruz Biotechnology), and 7-aminomethocynycin D (7AAD) (5 μM; Invitrogen). ReA localization was determined with an ImageStream 300 (Amnis Corp.) scanning flow cytometer as described previously (47).

Statistical analyses
Statistical tests were performed with Microsoft Excel or GraphPad Prism software. p values less than 0.05 were considered statistically significant. Comparisons of absolute cell numbers were done on the log10 of each value (i.e., linearized) to minimize differences in statistical variance of the raw values caused by exponential growth. The two-tailed Student t test was used when comparing two groups, and a one-way ANOVA with Bonferroni’s posttest was performed when comparing three groups. The one-phase exponential decay was used to calculate the half-life of 2W-1A^b T cells during the contraction (days 5–20) and memory (days 20–160) phases. Mean absolute numbers of 2W-1A^b^ T cells at each time point within a specified time span were used to determine the best-fit values.

Results
CD28 enhances the expansion of polyclonal CD4+ T cells responding to an L. monocytogenes-derived p:MHCI in vivo
We used p:MHCI tetramers and a magnetic bead-based enrichment method to detect endogenous p:MHCI-specific CD4+ T cells (43, 44) responding to a bacterial infection in mice expressing the I-A^b MHCI molecule. The L. monocytogenes strain used for these experiments was attenuated due to a lack of the actA gene product needed by the bacteria to spread from one host cell to

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another (48) and was engineered to secrete plasmid-encoded chicken OVA fused to a peptide called 2W (AWGALANWA) under the control of the hhy promoter (Lm-2W) (42). These bacteria also secrete listeriolysin O expressed from the endogenous hly gene on the bacterial chromosome. The 2W peptide and LLOp bind to 1-A\textsuperscript{b} MHCII molecules and stimulate CD4\textsuperscript{+} T cells in spleen and lymph nodes of naive and infected mice.

We sought initially to determine whether the preimmune CD4\textsuperscript{+} T cell repertoires of wild-type and CD28-deficient mice were similar. Uninfected wild-type and CD28-deficient mice contained ∼200 2W:I-Ab\textsuperscript{-} and 80 LLOp:I-Ab\textsuperscript{-}-specific CD4\textsuperscript{+} T cells in the spleen and lymph nodes, most of which were CD44\textsuperscript{low} as expected for naive cells (Fig. 1A). No CD4\textsuperscript{+} T cells bound both tetramers, and fewer than 5 CD8\textsuperscript{+} T cells per mouse bound either tetramer, demonstrating that tetramer binding was TCR-specific. TCR V\textsuperscript{a} cells were underrepresented in the 2W:I-Ab\textsuperscript{-}-specific naive populations but overrepresented in the LLOp:I-Ab\textsuperscript{-}-specific naive populations in wild-type and CD28-deficient mice (Fig. 1B). The fact that 2W:I-Ab\textsuperscript{-} and LLOp:I-Ab\textsuperscript{-}-specific naive populations had similar sizes and compositions in wild-type and CD28-deficient mice indicated that CD28 deficiency did not grossly alter the T cell repertoire.

Wild-type and CD28-deficient mice then were infected intravenously with Lm-2W bacteria to assess the role of CD28 in the activation of naive T cells in vivo. The 2W:I-Ab\textsuperscript{-}-specific cells in each group increased comparably over the first 3 d after Lm-2W infection (Fig. 2A). However, by day 4 this population was 10 times smaller in CD28-deficient mice than that in wild-type mice, a difference that was maintained at the peak on day 5. The 2W:I-Ab\textsuperscript{-}-specific populations in CD28-deficient and wild-type mice then contracted between days 5 and 20 with a half-life of ∼2 d and fell to 10% of their respective maximum values. The 10-fold difference between the two groups then was maintained after day 20 as both populations declined slowly during the memory phase with a half-life of ∼50 d (Fig. 2A). LLOp:I-Ab\textsuperscript{-} specific T cells also expanded ~10-fold less well in CD28-deficient than in B6 mice, a difference that was maintained 20 d postinfection (Fig. 2B). Thus, CD28 deficiency impaired the extent of CD4\textsuperscript{+} T cell expansion but did not alter survival during the contraction or memory phases.

**CD28 is necessary to sustain cell cycle activity of p:MHCII-specific CD4\textsuperscript{+} T cells in vivo**

CD28 signals enhance cell cycle entry and G\textsubscript{1}-to-S phase cell cycle progression of CD4\textsuperscript{+} T cells in vitro (51–55) and sustain proliferation of monoclonal T cells in vivo (56, 57). DNA replication was measured to determine if CD28 deficiency impaired cell cycle activity in polyclonal p:MHCII-specific CD4\textsuperscript{+} T cells during infection (Fig. 3A). All of the naive CD44\textsuperscript{low} 2W:I-Ab\textsuperscript{-}-specific T cells from uninfected wild-type and CD28-deficient mice were in G\textsubscript{0}/G\textsubscript{1}. Similar numbers of 2W:I-Ab\textsuperscript{-} specific T cells progressed to S and G\textsubscript{2}/M in both groups on day 2. The proportion of cells in S and G\textsubscript{2}/M in both groups peaked on day 3, but CD28-deficient mice had fewer cells in S and G\textsubscript{2}/M than controls. By day 4, both groups were returning to G\textsubscript{0}/G\textsubscript{1} with CD28-deficient mice having fewer cycling cells. Thus, CD28 was not needed for entry into S and G\textsubscript{2}/M in the early stages of clonal expansion but was required for maintaining cell cycle activity.

To further test whether the requirement of CD28 for sustained cell cycling was cell intrinsic, polyclonal CD4\textsuperscript{+} T cells from the spleen and lymph nodes of wild-type or CD28-deficient mice were labeled with CFSE and transferred into congenic recipients, which

![FIGURE 1. Detection of naive p:MHCII-specific CD4\textsuperscript{+} T cells in wild-type and CD28-deficient mice. 2W:I-Ab\textsuperscript{-} and LLOp:I-Ab\textsuperscript{-} T cells were magnetically enriched from pooled spleen and lymph nodes and visualized by flow cytometry.](http://www.jimmunol.org/)

(A) Flow cytometry plots of 2W:I-Ab\textsuperscript{-} and LLOp:I-Ab\textsuperscript{-} tetramer staining of CD3\textsuperscript{-} non-T lineage marker\textsuperscript{-} CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells (left panels), and enumeration of tetramer-stained CD4\textsuperscript{+} T cells in wild-type and CD28-deficient mice (right panel). Numbers on the plots depict the percentages of 2W:I-Ab\textsuperscript{-} and LLOp:I-Ab\textsuperscript{-} within the enriched CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells. Lines on the graph connect the number of 2W:I-Ab\textsuperscript{-} and LLOp:I-Ab\textsuperscript{-} T cells in individual mice. (B) Histograms (left panels) of V\textsubscript{a2} staining on 2W:I-Ab\textsuperscript{-}, LLOp:I-Ab\textsuperscript{-}, and tetramer\textsuperscript{-} T cells and the percentage of V\textsubscript{a2} cells among these three cell types (right panel). Numbers in the histograms show the percentage of V\textsubscript{a2} cells in tetramer\textsuperscript{-} T cells (top), 2W:I-Ab\textsuperscript{-} T cells (middle), and LLOp:I-Ab\textsuperscript{-} T cells (bottom). Horizontal lines on the plot indicate mean values, and each symbol depicts a value from an individual mouse. Groups were compared with a two-tailed Student t test. Pooled data from two (A) or four (B) independent experiments are shown. n.s., Not significant; p > 0.05.
then were infected with Lm-2W bacteria. Approximately 100 donor-derived wild-type and CD28-deficient 2W:I-Ab-specific T cells were detected in uninfected recipients, and these were CFSE$^{\text{high}}$ (Fig. 3B), as expected for quiescent naive cells. By day 3, both 2W:I-Ab-specific wild-type and CD28-deficient donor T cells had diluted CFSE similarly and expanded $\sim 4$-fold (Fig. 3B). By day 5, most of the wild-type T cells had diluted CFSE beyond the limit of detection. In contrast, most CD28-deficient T cells had not diluted CFSE beyond the levels achieved by day 3. The transferred wild-type T cells expanded $\sim 130$-fold above the starting number by day 5, whereas the CD28-deficient T cells increased only 7-fold (Fig. 3B). Thus, CD28 was necessary to sustain, but not initiate, CD4$^+$ T cell proliferation in response to L. monocytogenes infection.

**FIGURE 2.** Kinetics of primary CD4$^+$ T cell response to L. monocytogenes in wild-type and CD28-deficient mice. (A and B) 2W:I-A$^{b+}$ or LLOp:I-A$^{b+}$ T cells were magnetically enriched from wild-type and CD28-deficient mice infected i.v. with Lm-2W. Flow cytometry plots of (A) 2W:I-A$^+$ or (B) LLOp:I-A$^+$ versus CD44 staining on CD4$^+$ T cells from wild-type or CD28-deficient mice (top panels) with graphs depicting the mean number ($n \geq 3$) of (A) 2W:I-A$^{b+}$ or (B) LLOp:I-A$^{b+}$ T cells (bottom panels). A two-tailed Student $t$ test on the log$_{10}$ values of each group at the indicated time points (arrow) was used to determine statistical significance. Pooled data from six (A) or two (B) independent experiments are shown. n.s., Not significant, $p > 0.05$, **$p < 0.01$, ***$p < 0.001$.

**FIGURE 3.** CD28 is required to sustain but not initiate CD4$^+$ T cell proliferation in vivo. (A) Histograms of DAPI staining on 2W:I-A$^{b+}$ T cells from wild-type or CD28-deficient mice infected with Lm-2W bacteria (left panels). Gates show the percentages of cells in S plus G2/M phases of the cell cycle. Mean percentages ($n \geq 3$) of 2W:I-A$^{b+}$ T cells in S plus G2/M phases (right panel). A two-tailed Student $t$ test was used to identify statistical differences between groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (B) Histograms of CFSE in 2W:I-A$^{b+}$ T cells from CD90.2$^+$ wild-type or CD28-deficient CD4$^+$ T cells in CD90.1$^+$ wild-type recipients that were infected subsequently with Lm-2W (left panels) with the mean number (two to four mice per time point) of donor-derived wild-type or CD28-deficient 2W:I-A$^{b+}$ T cells (right panel). Each graph shows pooled data from three independent experiments.
The PYAP and Y170F motifs of CD28 are dispensable for CD4+ T cell clonal expansion in response to *L. monocytogenes* infection

We next attempted to identify the regions of the CD28 cytoplasmic tail that produced these biological effects. This was done using mixed hematopoietic cell chimeras produced by transplanting lethally irradiated mice with equal numbers of wild-type and CD28-deficient bone marrow cells (Fig. 4A). These chimeras contained similar numbers of wild-type and CD28-deficient 2W:I-Ab-specific naive CD4+ T cells (Fig. 4B). In contrast, the wild-type 2W:I-Ab-specific CD4+ T cells expanded 50-fold more than the CD28-deficient cells after Lm-2W infection (Fig. 4B).

The role of the cytoplasmic tail was tested in mixed chimeras containing wild-type T cells and \( \text{Cd28}^{2/2} \) T cells expressing full-length or cytoplasmic tail-deficient (tail-less) Cd28 transgenes. The 2W:I-Ab-specific CD4+ T cells with tail-less CD28 expanded ~10 times less than the cells with full-length CD28 (Fig. 4C). Remarkably, however, 2W:I-Ab-specific CD4+ T cells with a mutated C-terminal PYAP motif (AYAA), an SH2 domain-binding YMNM motif (Y170F), or both mutations expanded to the same extent as the wild-type CD4+ T cells after Lm-2W infection (Fig. 4D–F). Thus, the cytoplasmic tail, but neither the PYAP nor the YMNM motifs within the tail, were required for the in vivo effects of CD28 on clonal expansion after this infection.

**FIGURE 4.** The PYAP and YMNM motifs of CD28 are dispensable for CD28-dependent CD4+ T cell clonal expansion in response to *L. monocytogenes* infection. (A) Flow cytometry plot showing gates used to identify wild-type and CD28-deficient CD4+ T cells in a 2W:I-Ab tetramer-enriched sample from a radiation bone marrow chimeric mouse. Numbers indicate the percentage of donor wild-type or CD28-deficient CD4+ T cells in the enriched sample. (B) Flow cytometry plots of 2W:I-Ab versus CD44 on donor-derived wild-type or CD28-deficient CD4+ T cells from uninfected or day 5 Lm-2W–infected mice (left panel). (B, right panel, through F) Numbers of wild-type and CD28 mutant 2W:I-Ab+ T cells in uninfected and day 5 Lm-2W–infected mice. (G) Flow cytometry plots of 2W:I-Ab versus CD44 on CD4+ T cells from the spleens of wild-type or CD28 mutant mice (left panels) and absolute numbers of 2W:I-Ab+ T cells in unimmunized mice (open circles) or mice that had been injected i.v. 5 d earlier with 2W peptide without the addition of any adjuvant (filled circles) (right panel). A two-tailed Student t test was used to compare the log_{10} values of Lm-2W–infected groups, and a one-way ANOVA with Bonferroni’s posttest was used to compare the log_{10} values of peptide-immunized groups. All of the groups were compared with each other. Asterisks above wild-type and \( \text{Cd28}^{2/2} \) samples indicate that all of the comparisons involving these groups revealed statistically significant differences. Data are from one to five independent experiments. n.s., \( p > 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \).
It was possible that these negative results were related to the involvement of costimulatory receptors other than CD28, the ligands for which are induced by infection. This possibility was tested by assessing the responsiveness of the CD28-deficient T cells after the injection of peptide without an adjuvant. As shown in Fig. 4G, injection of 2W peptide alone induced the expansion of wild-type 2W-I-A\(^b\)-specific T cells but at a level that was ∼40-fold lower than that induced by Lm-2W infection. The expansion of CD28-deficient 2W-I-A\(^b\)-specific T cells after the injection of 2W peptide alone was 8-fold lower than that of wild-type cells. The expansions of 2W-I-A\(^b\)-specific CD4\(^+\) T cells with mutated PYAP or YMNM motifs also were lower than that of wild-type cells but only by 2-fold. These results indicate that the PYAP and YMNM sites in the cytoplasmic tail of CD28 transduce signals in polyclonal p:MHCII-specific CD4\(^+\) T cells under conditions of minimal inflammation.

**CD28 deficiency phenotypically resembles T cell-intrinsic NF-κB signaling deficiency during the response to *L. monocytogenes* infection**

The perplexing finding that neither of the suspected motifs within the tail was required for the in vivo effects of CD28 led us to investigate whether NF-κB signaling was involved. This was the case as demonstrated by the finding that CD28-deficient 2W-I-A\(^b\)- and LLOp-I-A\(^b\)-specific T cells did not induce Bcl-\(x_L\), an NF-κB-regulated gene product (17, 18), 3 d after Lm-2W infection like control cells (Fig. 5A). These findings suggested that signals from CD28 contribute to the activation of NF-κB in this setting. If so, then loss of NF-κB signaling would be expected to produce the same defects in T cell activation as CD28 deficiency. This possibility was tested in radiation bone marrow chimeras containing wild-type and *Card11*\(^\Delta/\Delta\) T cells lacking the CARD-containing MAGUK protein 1 (CARMA1) component of the NF-κB signaling pathway. Like CD28-deficient cells, CARMA1-deficient 2W-I-A\(^b\)-specific T cells began to expand normally on day 3 postinfection but did not continue to expand to day 5 when these T cells exhibited a 40-fold defect compared with wild-type cells (Fig. 5B). Likewise, CARMA1-deficient cells failed to induce Bcl-\(x_L\) (Fig. 5B) like CD28-deficient cells. PKC\(\mu\)- and NF-κB1/c-Rel-deficient 2W-I-A\(^b\)-specific CD4\(^+\) T cells had similar clonal expansion defects (Fig. 5C, 5D). These results show that the expansion defect of CD28-deficient cells resembles that of NF-κB signaling-deficient cells.

**FIGURE 5.** CD28 deficiency phenotypically resembles NF-κB signaling deficiency in vivo. (A) Histograms of isotype control or Bcl-\(x_L\) staining on tetramer\(^-\) CD44\(^{low}\) or 2W:I-Ab\(^+\) plus LLOp:I-Ab\(^+\) wild-type or CD28-deficient mice 3 d after Lm-2W infection. Values for median fluorescence intensity of Bcl-\(x_L\) staining minus that of isotype control are shown in the right panel of (A). (B–D) Mixed radiation chimeras made with wild-type and CARMA1-deficient (B), wild-type and PKC\(\mu\)-deficient (C), or wild-type and p50/c-Rel-deficient (D) bone marrow cells. Mean numbers (n = 2–4) of wild-type or CARMA-deficient 2W-I-A\(^b\)-specific T cells in uninfected mice and mice at the indicated times after Lm-2W infection are shown in the left panel of (B). The right panel of (B) shows differences in median fluorescence intensity of Bcl-\(x_L\) staining minus that of isotype control for wild-type and CARMA1-deficient cells. Horizontal lines indicate the mean values, and each symbol shows data from an individual mouse. A two-tailed Student \(t\) test was used to compare the log\(_{10}\) values of tetramer\(^+\) cells in Lm-2W-infected groups. **\(p < 0.01\), ***\(p < 0.001\).
CD28 signaling increases Ag-dependent nuclear translocation of RelA in vivo independently of the PYAP and YMNM motifs.

To determine whether CD28 signaling indeed enhanced NF-κB activation in vivo, we used an image scanning flow cytometer to measure nuclear translocation of RelA in CD4+ T cells responding to Ag in vivo. We chose to focus on RelA because this NF-κB isoform can dimerize with p50 or c-Rel in T cells (58), translocates into the nucleus upon TCR plus CD28 stimulation, and is a component of a protein complex that can bind the CD28 response element consensus sequence (59). CD4+ T cells from wild-type or CD28-deficient OVA peptide:I-Ab-specific OT-II TCR transgenic mice were transferred into normal mice, which then were injected i.v. with LPS to induce CD80 and CD86 on APCs, then with OVA peptide (60, 61) (Fig. 6A). RelA was in the cytoplasm of wild-type and CD28-deficient OT-II cells in recipient mice injected with LPS alone (Fig. 6). In contrast, RelA translocated to the nucleus in OT-II cells 20 min after the injection of OVA peptide, and this translocation was significantly greater in wild-type cells than that in CD28-deficient cells (Fig. 6C). In addition, OT-II cells expressing CD28 molecules with mutated PYAP or YMNM sites showed the same amount of peptide-induced RelA translocation as wild-type cells (Fig. 6C). These data demonstrate that CD28 signaling is required for optimal TCR-induced activation of RelA in vivo through a pathway that does not involve the YMNM or PYAP sites.

Discussion

Our results show that the major effect of CD28 signaling on polyclonal p:MHCII-specific T cells responding to an infection was sustained effector cell proliferation. It was surprising to find that CD28-deficient T cells began proliferating in vivo at the same rate as wild-type T cells given the many reports that CD28 enhances the production of the early T cell growth factor IL-2. However, this result is less surprising considering evidence that IL-2 is not required for CD4+ T cell proliferation in vivo (60). It is possible that CD28 maintains effector cell proliferation indirectly by sustaining the production of a lymphokine other than IL-2 (62) or by directly transducing signals that promote cell cycle progression (51, 53).

It has been reported that CD28 does not influence in vitro T cell proliferation but rather promotes clonal expansion by opposing apoptosis through the induction of Bcl-xL (10). This was proposed to be an indirect effect of OX40, which promotes memory T cell formation and expression of Bcl-xL and is induced in a CD28-
dependent fashion (63). In contrast, we found that the polyclonal p:MHCI-specific effector cells that were formed in CD28-deficient mice did not undergo an exaggerated contraction phase and produced memory cells at the expected frequency. This conclusion is supported by the observation of Dahl et al. (64) that overexpression of Bcl-\(x_\text{l}\) failed to restore the clonal expansion of CD28-deficient T cells in vivo. Therefore, the in vivo results indicate that CD28 enhances memory cell generation by sustaining the proliferation of the effector cell population from which memory cells are derived.

It was surprising to find that mutations of the YMMN site had no effect on the production of effector and memory T cells after bacterial infection. The YMMN site has been shown to bind to PI3K, a key molecule in the activation of multiple signaling pathways that regulate cellular metabolism (Akt/mammalian target of rapamycin) (12, 13), the immunological synapse (PKC\(\theta\)) (12, 14–16), and the transcription of genes involved in T cell proliferation and survival (Bcl10/Carmal/Malt1/NF-kB) (17–19). Our findings extend this work by documenting that the YMMN site is dispensable for the infection-induced proliferation of polyclonal p:MHCI-specific polyclonal CD4\(^+\) T cells. Notably, both the PAFY and YMMN sites played a role in T cell expansion induced by peptide without adjuvant, perhaps because fewer costimulatory ligands other than CD80 and CD86 are induced in this case than by infection. It was even more surprising to find that mutations in the PAFY site had no effect on the production of effector and memory T cells after bacterial infection. This site is involved in the activation of PDK1 and PKC\(\theta\) (24), and its disruption impairs in vivo T cell functions such as promotion of Ab production (25) and induction of nuclear and cytoplasmic RNAs are regulated with complex kinetics. Mol. Cell. Biol. 15: 3197–3205.

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