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*J Immunol* 2012; 189:2309-2317; Prepublished online 27 July 2012; doi: 10.4049/jimmunol.1200453

http://www.jimmunol.org/content/189/5/2309

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Supplementary Material  
http://www.jimmunol.org/content/suppl/2012/07/27/jimmunol.1200453.DC1

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A TCR Affinity Threshold Regulates Memory CD4 T Cell Differentiation following Vaccination

Christina K. Baumgartner,* Hideo Yagita,† and Laurent P. Malherbe*

Diverse Ag-specific memory TCR repertoires are essential for protection against pathogens. Subunit vaccines that combine peptide or protein Ags with TLR agonists are very potent at inducing T cell immune responses, but their capacity to elicit stable and diverse memory CD4 T cell repertoires has not been evaluated. In this study, we examined the evolution of a complex Ag-specific population during the transition from primary effectors to memory T cells after peptide or protein vaccination. Both vaccination regimens induced equally diverse effector CD4 TCR repertoires, but peptide vaccines skewed the memory CD4 TCR repertoire toward high-affinity clonotypes whereas protein vaccines maintained low-affinity clonotypes in the memory compartment. CD27-mediated signaling was essential for the maintenance of low-affinity clonotypes after protein vaccination but was not sufficient to promote their survival following peptide vaccination. The rapid culling of the TCR repertoire in peptide-immunized mice coincided with a prolonged proliferation phase during which low-affinity clonotypes disappeared despite exhibiting no sign of enhanced apoptosis. Our study reveals a novel affinity threshold for memory CD4 T cell differentiation following vaccination and suggests a role for nonapoptotic cell death in the regulation of CD4 T cell clonal selection. *The Journal of Immunology, 2012, 189: 2309–2317.

Protective immunity against infectious diseases depends on Ag-specific memory T cells that survive for many years following initial exposure to Ag. Whereas many early vaccine studies were focused on the magnitude of the T cell response, recent studies suggest that more qualitative aspects of the response, such as T cell avidity and TCR repertoire diversity, may be crucial (1–3). Studies of infection with HSV in mice (4) and SIV in monkeys (5, 6) provide evidence that TCR diversity in a given epitope-specific response is important for effective immune control. Understanding the mechanisms that control the clonotypic diversity of memory T cells is critical for the design of future vaccines but remains poorly resolved in vivo.

Clonal diversity of the T cell compartment, which is established by random rearrangement of TCR gene segments during development, enables the immune system to respond to the vast pool of potential pathogens. Ag-specific T cells are selected from this vast pool of diverse naive cells based on the affinity of their surface TCR for peptide/MHC class I or class II complexes (7, 8). Below a TCR affinity threshold, T cell clones with demonstrable peptide/MHC class II (pMHCII) complexes binding start proliferating but are not propagated during the clonal expansion phase. Above this threshold, clones expressing higher affinity TCR have no proliferative advantage (7). We have shown that the choice of vaccine adjuvant, the Ag dose, and pMHCII stability all regulated this TCR-based selection and thereby modified the clonotypic diversity of the effector CD4 T cell compartment (9–11).

Following the resolution of a primary immune response, a large majority of activated T cell effectors die via apoptosis to leave a small but relatively stable population of memory cells (12). Whether TCR affinity plays a role during the transition from effector to memory T cells is unclear (13). Studies that have examined virus-specific CD8 T cell repertoires following infection have found essentially no differences in TCR repertoire usage between the effector and the memory pool (14–16). For CD4 T cells, there is evidence of avidity maturation during memory CD4 T cell differentiation (17, 18), and a narrowing of TCR repertoire diversity has been observed between the peak of the primary and the secondary responses (19, 20), but the precise role of TCR affinity in memory CD4 development remains to be elucidated.

The I-Ek-restricted murine response to cytochrome c provides an ideal experimental model to study Ag-specific memory CD4 T cell responses in vivo (21). Immunization of B10. BR mice with cytochrome c peptides in monophosphoryl lipid A (MPL) emulsion induces Vx11Vβ3-expressing CD4 T cells with restricted CDR3 regions. Previously we showed that vaccination with motif cytochrome c peptide (MCC88–103) gave rise to a clonally diverse effector CD4 T cell repertoire (11). In the present study we tracked cytochrome c-specific memory CD4 T cell development following peptide and protein vaccination. We demonstrate that although peptide and protein vaccination set the same TCR affinity threshold for effector CD4 T cell differentiation, clonal diversity was only maintained into the memory phase upon protein vaccination. In contrast, low-affinity clonotypes were rapidly lost during the contraction phase upon peptide vaccination. The maintenance of low-affinity T cells following protein vaccination was dependent on CD27/C70 costimulatory interaction, but administration of CD27 agonistic Abs did not rescue low-affinity T cells in peptide-immunized mice. The selective loss of low-affinity clonotypes upon peptide vaccination occurred rapidly after the peak of clonal expansion and was associated with a prolonged proliferation, but it did not correlate with enhanced apoptosis.
Materials and Methods

Mice

B10.BR, B10.BR-Thy1.1 congenic, and 5C.C7αβ and 2B4αβ transgenic mice were maintained under pathogen-free conditions at the Medical College of Wisconsin. The Medical College of Wisconsin Institutional Animal Care and Use Committee reviewed and approved all experiments.

Peptide synthesis

MCC88-103 and PCC103K. Peptide were synthesized by standard solid-phase methods, purified by HPLC, and confirmed by mass spectrometry.

MaeIE-MCC protein purification

MaeIE33 (vector from A. Sant) was digested with BamHI (Invitrogen). Synthetic 5′-phosphorylated oligonucleotides encoding MCC88-103 (IDT), oligonucleotides used: forward, 5′-phospho-GATCCGGCCAACCG-AACGCGCCGTCTCAGTCCATTCAGGAAGCCCAAAGCCCG-3′, reverse, 5′-phospho-GATCCGGCGTGTGCTCAGGAAGCCCG-GATGAGATCAGCGGCGTGTGCTCAGGAAGCCCG-3′ were annealed and ligated into MaeIE133 with T4 DNA ligase (Invitrogen). Sequenced clones were transformed into MaeIE-deficient Escherichia coli K12 ER2507 (New England BioLabs). MaeIE-MCC protein was purified from periplasmic extracts as described (22). Briefly, transformed K12 bacteria were grown in Luria-Bertani medium plus carbenicillin plus 0.2% maltose for 6.5 h at 37°C, pelleted, and resuspended in 30 mM Tris-HCl/20% sucrose/1 mM EDTA (pH 8.0). After shaking at room temperature for 10 min, bacteria were pelleted, resuspended in ice-cold 5 mM MgSO4, and shaken on ice for 10 min. After centrifugation, osmotic shock fluid was neutralized with 1 M Tris-HCl and filtered through 0.45-μm mesh. MaeIE proteins were purified on an amylose column (New England BioLabs) according to the manufacturer’s protocol.

Vaccination and adoptive transfer

Mice were vaccinated s.c. at the base of the tail with 60 μg or the indicated dose of MCC88-103 or PCC103K peptide, or with 100 μg MaeIE-MCC or wild-type MaeIE protein in MPL emulsion as indicated (23). For adoptive transfers, 10^7 total splenocytes from 2B4αβ or 5 x 10^7 total splenocytes from 5C.C7αβ transgenic mice containing 10^9 viable cytotoxic c-specific CD4 T cells were transferred i.v. into B10.BR-Thy1.1 congenic mice directly before s.c. vaccination with 60 μg MCC88-103 in MPL emulsion.

In vivo anti-CD70 and anti-CD27 Ab treatment

Anti-murine CD70 mAb (7B5, rat IgG2a) and anti-murine CD27 mAb (SB/199) were prepared as previously described (24, 25). For in vivo treatment, mice were i.p. vaccinated with 100 μg MalE-MCC in MPL emulsion as indicated (23). For adoptive transfer, mice were i.p. vaccinated with 100 μg MCC88-103 in MPL emulsion as indicated and injected i.p. with 100 μg anti-CD72 mAb or IgG2b isotype control Ab (BioXCell) every 3 d from day 0 to day 12.

Flow cytometry

Animals were killed on various days after immunization as indicated, and the draining lymph nodes (LN) were harvested for analysis. Inguinal and periaortic nodes were collected and teased through 80-μm mesh screens into cell suspensions in PBS with 5% FCS before estimation of cell count using a hemocytometer. Cell suspensions were labeled for 45 min at 4°C at a density of 2 x 10^6 cells/ml with predetermined optimal concentrations of the following fluorochrome-labeled mAbs: FITC-conjugated anti-Veg11 (RR8.1), PE-conjugated anti-Vβ3 (K25; all produced in the laboratory); Cy7-allophycocyanin–conjugated anti-CD44 (IM7), allophycocyanin–conjugated anti-CD90.2 (30-H12), Cy5–PE–conjugated anti-B220 (6B2), anti-CD8α (53-6.7), and anti-CD11b (M1/70), allophycocyanin-Cy7–conjugated anti-CD44 (Pgp-1), biotin-conjugated anti-IL7Rα (SB/199) (all BioLegend), and allophycocyanin-conjugated anti-CD26L (MEL-14; BD Biosciences). PE-MCC1/Eα tetramers (pMHCII tetramer), prepared as previously described (7), were incubated for 2 h at room temperature at a final concentration of 300 nM. After staining, cells were suspended in 1.5 μg/ml DAPI (Invitrogen) (for dead cells exclusion) for analysis. For annexin V staining, cells that had been incubated with anti–surface marker Abs were washed and incubated with annexin V-PE (BD Biosciences) in annexin V binding buffer and DAPI for 15 min at room temperature and analyzed immediately. Data were collected with FACSDiva slide software (BD Biosciences) and analyzed with FlowJo software (Tree Star). Profiles are presented as 5% probability contours with outliers.

Intracellular staining

Single-cell suspensions from lymphoid tissues in PBS with 5% FCS were labeled for surface markers as described above. Cells were fixed and permeabilized using BD Cytofix/Cytoperm (BD Biosciences) according to the manufacturer’s protocol. FITC-labeled anti-Ki67 (BD Biosciences), PE-conjugated anti-CD2 (D1C4), anti-Bcl-2 (54H6), and anti–Mcl-1 (Poly6136; BioLegend) were incubated in Perm/Wash Buffer (BD Biosciences) and incubated with fixed cells for 30 min at room temperature. PE-labeled anti-rabbit IgG Ab (Jackson ImmunoResearch Laboratories) was used for secondary detection of purified Abs. Cells were washed with Perm/Wash buffer followed by PBS with 5% FCS and analyzed. For the detection of activated caspases, a Vybrant FAM caspase-3 and -7 assay kit (Invitrogen) was used according to the manufacturer’s instructions. To analyze mitochondrial membrane potentials, cells that had been incubated with anti–surface marker Abs were washed and incubated in 10 nM 3,3′-dihexyloxycarbocyanine iodide (DiOC6; Molecular Probes) in PBS plus 5% FCS for 15 min at 37°C and analyzed immediately thereafter. To detect reactive oxygen intermediates, cells were first loaded with dihydrodorhodamine 123 (DHR123; Invitrogen) in PBS plus 5% FCS for 15 min at 37°C and, after washing, stained with anti–surface marker Abs before analyses.

Single-cell repertoire analysis

Single cells with the appropriate surface phenotype were sorted for repertoire analysis on a FACS_Aria and CloneCyt software (BD Biosciences). The synthesis of cDNA and amplification of the TCR Vβ3 region were carried out as previously described (26). Direct sequencing of the purified PCR products was carried out with a Vβ3-specific primer by a commercial vendor (IDT). Jβ-specific single-cell PCR was done in two rounds of nested PCR using V region- and C region-specific primers for the first round and V region- and Jβ1.2 or Jβ2.5 region-specific primers for the second round of PCR (9).

Quantitative real-time PCR

Cells were FACs sorted directly into TRIzol reagent (Invitrogen) and RNA was isolated according to the manufacturer’s instructions. cDNA was generated from RNA using M-MLV reverse transcriptase (Invitrogen) and subjected to real-time PCR using iQ SYBR Green Supermix (Bio-Rad). Reactions were run and recorded on an iCycler (Bio-Rad). β-actin was used for normalization. The following exon-spanning primer pairs were used: Puma, forward, 5′-AGCGAAGCAGGCAAGCCTA-3′, reverse 5′-GTGAGGACGTCGTTGTCGATG-3′, Noxa, forward, 5′-TGGCGAAAAGACGGACGAGTAG-3′, reverse 5′-CAGTCTTCTCAGAGTCCG-3′, Bim, forward, 5′-CGGACGAGGACGAGGTCGATCA-3′, reverse 5′-TT-CAGGCTCGCGGTAATCACTA-3′, Nor-1, forward, 5′-GATCCACAGCAGCAATGGTATTA-3′, reverse 5′-GAGGCTGTCCCTTTCCTG-3′, β-actin, forward, 5′-CTAGGGCACCACGGTGAAAG-3′, reverse 5′-AGCCCTGATGGCTACCTA-3′.

Statistical analysis

Statistical differences between experimental groups were determined by the Student t test or Mann–Whitney U test as indicated in the figure legends. A p value of <0.05 was considered statistically significant. For the analysis of public clonotype frequency, a Bonferroni correction was used for multiple comparisons, with a p value of <0.05 (per number of comparisons) considered significant. Statistical analysis was conducted with GraphPad Prism 4 software (GraphPad Software, La Jolla, CA).

Results

Low-affinity clonotypes fail to form memory after peptide vaccination

To examine the stability of memory CD4 T cell repertoire induced by vaccination, we first immunized mice with MCC88-103 in an adjuvant system based on MPL (a TLR4 agonist) and assessed the kinetics of the Ag-specific response. MCC88-103 peptide was selected because it formed high-stability pMHCII complexes with αEβ7- and induced Ag-specific Veg11+Vβ3+CD62L+CD44hi effector helper cells expressing diverse TCR (11). The Ag-specific CD4 T cell response to peptide vaccination peaked at day 7 and gradually declined thereafter, leaving a detectable population of memory CD4 T cells that was maintained over the time of analysis (90 d) (Fig. 1A, 1B). No significant accumulation of CD62Lhi
Ag-specific memory CD4 T cells was observed, leading us to focus
our analysis on CD62Llo memory CD4 T cells. To determine the
clonal composition of the responding T cells remaining in draining
LNs, we sorted single Ag-specific CD4 T cells 30 d after peptide
vaccination and sequenced their CDR3b regions using a single-cell
RT-PCR approach. As previously shown, the effector CD4 T cell
response induced by MCC88–103 peptide was composed at day 7 of
equal number of Jb1.2- and Jb2.5-expressing clonotypes and dom-
inated by Jb2.5 clonotypes expressing one specific public CDR3
rearrangement (SLNRGQDTQ). In contrast, at day 30, the prevail-
ance of clonotypes expressing Jb1.2 increased by 9-fold (Fig. 1C)
and the memory TCR repertoire was now dominated by Jb1.2 clo-
onotypes expressing a public CDR3 rearrangement (SLNNANSY
or 5C.7β-chain) (Fig. 1D). Next, we examined the stability of the
TCR repertoire induced by PCC103K, a peptide that forms more
stable pMHCII complexes than does MCC88–103 and induces a
more diverse Ag-specific effector TCR repertoire dominated by
clonotypes expressing unique or private CDR3β rearrangements
(11). Once again, we found that the memory TCR repertoire at
day 30 was dominated by public clonotypes expressing the 5C.
7β-chain.

We had previously shown that Jb2.5- and 5C.7β-expressing
clonotypes differ in their structural and functional avidities for
their common pMHCII ligands. Structurally, we have shown using
TCRβ transgenic mice that Ag-specific CD4 T cells expressing
the public 5C.7β-chain expressed high-affinity TCR (7). More-
over, in immunized B10.BR mice Ag-specific CD4 T cells that
bind the highest level of pMHCII tetramers rearranged exclusively
Jb1.2, whereas Jb2.5-expressing clonotypes were only found in Ag-
specific CD4 T cells that bind lower tetramer level (9). Functionally,
we have shown that decreasing the dose of peptides used for vac-
cination or decreasing the binding stability of the immunizing
peptides for MHC class II favored the expansion of clones ex-
pressing the public 5C.7β-chain and reduced the prevalence of
Jb2.5-expressing clonotypes (11). Thus, our present study sug-
ests that clonotypes with lower structural and functional avidities
for their pMHCII ligands failed to join the memory pool upon
peptide vaccination. Consistent with the TCR repertoire studies,
CD4 REPERTOIRE EVOLUTION FOLLOWING VACCINATION

a shift toward higher pMHCII tetramer-binding cells was observed between days 7 and 30 after peptide vaccination (Fig. 1E, IF, Supplemental Fig. 1) without any changes in TCR expression levels (Fig. 1G).

Low-affinity clonotypes did not participate in the recall response

To further determine the relative contribution of the different clonotypes to the memory CD4 T cell response, we examined the Ag-specific CD4 T cell repertoire during a secondary response. Immune B10.BR mice were challenged 30 d after the initial vaccination with MCC88–103 peptide in adjuvant, and TCR usage of Ag-specific CD4 T cells was analyzed at the peak of the secondary response (day 3, Fig. 2A). The overall Jβ usage of the Ag-specific secondary response was similar to the memory T cell pool with a predominance of Jβ1.2-expressing clonotypes (Fig. 2B). Similar to the day 30 memory repertoire, the secondary response was dominated by high-affinity 5C.C7β-chain–expressing clonotypes (Fig. 2C). We observed a significant increase in pMHCII tetramer staining MFI comparing the responding CD4 T cells at the peak of the primary and secondary responses (Fig. 2D), whereas TCR expression levels were not significantly altered (Fig. 2E). Hence, low-affinity clonotypes that dominate the primary effector CD4 T cell response to high-stability peptides fail to form memory and contribute minimally to secondary responses.

Low-affinity clonotypes form memory after protein vaccination

Most subunit vaccines used proteins instead of peptide Ags. To examine the stability of memory CD4 T cell repertoire induced by protein vaccination, we inserted MCC88–103 peptide into a subunit of the E. coli maltose-binding protein MalE that allowed heterologous peptide inserts of >20 aas without changing structure or function (27). Recombinant MalE-MCC protein induced a strong cytochrome c-specific effector CD4 T cell response (Fig. 3A, 3B). Analyses of the clonotypic diversity at day 7 revealed that the overall CD4 effector TCR repertoire induced by MalE-MCC protein determined either by Jβ gene segment usage or by frequency and distribution of public clonotypes was similar to the one elicited by MCC88–103 peptide (Fig. 3C, 3D), suggesting that a similar TCR affinity threshold regulates effector T cell expansion after peptide and protein vaccination.

Next, we analyzed the memory CD4 T cell compartment induced by MalE-MCC protein. Ag-specific CD4 T cell response contracted significantly faster in protein-primed than in peptide-primed mice. By day 30, 65% of the Ag-specific CD4 T cells were lost after protein vaccination whereas only ~25% were lost after peptide vaccination (Fig. 4A). When we analyzed the TCR usage of Ag-specific CD4 T cells remaining 30 d after protein vaccination, it was strikingly similar to the one observed at the peak of the primary response (day 7). Analyses of Jβ usage and CDR3 sequences indicated no differences in the frequency of Jβ1.2- and Jβ2.5-expressing clonotypes and cells expressing specific public rearrangements between days 7 and 30 after vaccination with MalE-MCC (Fig. 4B, 4C). This was also observed in detoxified protein preparation, excluding a role for LPS in long-term survival of low-affinity clonotypes (data not shown). Consistent with the maintenance of the TCR repertoire diversity, no increase in tetramer staining MFI was observed between days 7 and 30 after protein vaccination (Fig. 4D, 4E). Our data show that, in clear contrast to peptide vaccination, protein vaccination lowers the TCR affinity threshold for memory differentiation and maintains low-affinity effector clonotypes in the memory CD4 T cell compartment.

Loss of low-affinity clonotypes is independent of peptide dose and occurs rapidly during the contraction phase

Even though similarly high doses of peptide (60 μg) and protein (100 μg) were used in our vaccination, peptide vaccines contained a 16-fold molar excess Ag, which may have contributed to the TCR repertoire diversity. To investigate this possibility, we reduced the dose of peptide injected to a molar amount equivalent to our protein vaccines. Reducing by 16-fold the dose of peptide did not significantly affect the number of Ag-specific CD4 T cells in draining LNs present at day 30 (Fig. 5A), nor did it prevent the selective loss of Jβ2.5-expressing clonotypes at day 30 (Fig. 5B).
Moreover, we have previously shown that reducing further the dose of peptide injected dramatically reduced the prevalence of J\textsubscript{b}2.5-expressing clonotypes at day 7 (11). Overall, our results suggest that the loss of low-affinity clonotypes following peptide vaccination occurs independently from the Ag dose.

We next assessed when low-affinity clonotypes were lost after peptide vaccination. We extended the analysis of the Ag-specific TCR repertoire to the second week following immunization using J\textsubscript{b}-specific single-cell PCR reactions that resolve J\textsubscript{b}1.2 and J\textsubscript{b}2.5 usage (9). As indicated from the previous single-cell repertoire analysis, similar numbers of J\textsubscript{b}1.2- and J\textsubscript{b}2.5-expressing clonotypes were present 7–9 d after peptide vaccination. However, the frequency of J\textsubscript{b}2.5-expressing clonotypes was dramatically reduced by day 15 and already comparable to what was seen in

FIGURE 3. Protein and peptide vaccines set a similar TCR affinity threshold for effector T cell expansion. (A) Ag-specific CD4 T cells (DAPI\textsuperscript{+} B220\textsuperscript{−} CD8\textsuperscript{−} CD11b\textsuperscript{−} Vx11\textsuperscript{−} Vb\textsuperscript{3\textsuperscript{+}} CD4\textsuperscript{+} CD62L\textsuperscript{−}) at day 7 in draining LNs from B10.BR mice after s.c. priming with recombinant MalE-MCC or MalE proteins. (B) Total number of Ag-specific CD4 T cells in draining LNs from B10.BR mice 7 d after immunization with MPL emulsion containing PBS, MalE protein, MalE-MCC protein, or MCC\textsubscript{88–103} peptide. (C) Relative abundance of Ag-specific CD4 T cells expressing J\textsubscript{b}1.2 or J\textsubscript{b}2.5 gene segments 7 d after immunization with MalE-MCC protein or MCC\textsubscript{88–103} peptide. p ≤ 0.05, Mann–Whitney U test. (D) Amino acid sequences of CDR3\textsubscript{b} regions for Ag-specific CD4 T cells isolated days after immunization with MalE-MCC protein or MCC\textsubscript{88–103} peptide. Each filled circle represents the sequence information of a single cell. Data from three combined individual mice with each animal contributing 20 CDR3 sequences are displayed. The percentage of cells (±SEM) expressing public clonotypes within individual mice is shown. Statistical analyses were conducted using an unpaired Student t test with a Bonferroni correction. Means ± SEM for at least three animals per group are shown. Data shown are derived from at least three independent experiments (n ≥ 3 mice/group). p = 0.017.

FIGURE 4. Low-affinity T cells form memory after protein vaccination. (A) Total cell number of Ag-specific CD4 T cells (DAPI\textsuperscript{−} B220\textsuperscript{−} CD8\textsuperscript{−} CD11b\textsuperscript{−} Vx11\textsuperscript{−} Vb\textsuperscript{3\textsuperscript{+}} CD4\textsuperscript{+} CD62L\textsuperscript{−}) in draining LNs from B10.BR mice immunized with MalE-MCC protein, MCC88–103 peptide, or PBS during the course of the primary response. (B) Relative abundance of Ag-specific CD4 T cells expressing J\textsubscript{b}1.2 or J\textsubscript{b}2.5 gene segments 7 or 30 d after immunization with MalE-MCC protein. p ≤ 0.05, Mann–Whitney U test. (C) Amino acid sequences of CDR3\textsubscript{b} regions for Ag-specific CD4 T cells isolated 7 or 30 d after immunization with MalE-MCC protein. Each filled circle represents the sequence information of a single cell. Data from three combined individual mice with each animal contributing 20 CDR3 sequences are displayed. The percentage of cells (±SEM) expressing public clonotypes within individual mice is shown. Statistical analyses were conducted using an unpaired Student t test with a Bonferroni correction. p = 0.017. (D) MFI of pMHC\textsubscript{II} tetramer staining and (E) MFI of Va\textsubscript{11} staining for Ag-specific CD4 T cells (DAPI\textsuperscript{−} B220\textsuperscript{−} CD8\textsuperscript{−} CD11b\textsuperscript{−} Vx11\textsuperscript{−} pMH\textsubscript{C}CD4\textsuperscript{+}) 7 or 30 d after immunization with MalE-MCC protein. Means ± SEM for at least three animals per group are shown. Data shown are derived from at least three independent experiments (n ≥ 3 mice/group). p ≤ 0.05, unpaired Student t test.
the memory compartment at day 30 (Fig. 5C). Hence, the remodeling of Ag-specific CD4 T cell repertoire upon peptide vaccination occurred rapidly during the contraction phase.

To further assess the involvement of TCR affinity in the regulation of CD4 T cell survival following peptide vaccination, we adoptively transferred high (SC.C7αβ) or low-affinity (2B4αβ) TCR-transgenic monoclonal Ag-specific CD4 T cells (7, 28) into syngeneic hosts and mice were s.c. immunized with MCC88–103 peptide. Total number of transferred transgenic CD4 T cells (DAPI B220 − CD8 − CD11b − Vβ3 + CD44hiCD62Llo) expressing Jβ1.2 or Jβ2.5 gene segments in draining LNs from B10.BR mice immunized with MCC88–103 peptide during the course of the primary response. (D) Splenocytes from SC.C7αβ or 2B4αβ TCR transgenic mice were transferred into Thy1.1 syngeneic hosts and mice were s.c. immunized with MCC88–103 peptide. Total number of transferred transgenic CD4 T cells (DAPI B220 − CD8 − CD11b − Vβ3 + CD44hiCD62Llo) expressing Jβ1.2 or Jβ2.5 gene segments in draining LNs 7 or 11 d after peptide immunization. Means ± SEM for at least three animals per group are shown. Data shown are derived from at least two independent experiments (n ≥ 3 mice/group). *p ≤ 0.05, unpaired Student t test.

CD27/CD70 interaction is required but not sufficient for memory differentiation of low-affinity T cells

Thus far, our results suggested that low- and high-affinity CD4 T cells might differ in their requirement for their maintenance in the memory compartment. The TNF receptor family member CD27 has been shown to promote T cell survival (29) and support the maintenance of clonally diverse CD8 T cell responses (30). We next decided to analyze the effect of blocking CD27/CD70 signaling on the fate of low-affinity CD4 T cells following protein vaccination by injecting mice with CD70 blocking Ab and analyzing the cellular responses at day 15. The absolute numbers of Ag-specific CD4 T cells were comparable between mice treated with blocking anti-CD70 or isotype control (Fig. 6A, 6B). However, when we analyzed the Jβ usage by the Ag-specific CD4 T cells present at day 15, we found a 7-fold increase in Jβ1.2-expressing clonotypes in mice treated with anti-CD70 blocking Abs (Fig. 6C). Hence, CD27–CD70 interaction is required to promote memory CD4 T cell differentiation of low-affinity clonotypes during vaccination.

We next evaluated the effect of CD27 agonistic Abs on the fate of low-affinity CD4 T cells following peptide vaccination. Enhancing CD27 signal upon peptide priming did not alter the number of Ag-specific CD4 T cells present at day 15 (Fig. 6D, 6E) or the balance between high- and low-affinity clonotypes (Fig. 6F). Overall, our results suggest that the CD27–CD70 interaction is necessary but not sufficient for the maintenance of low-affinity CD4 T cells in the memory compartment.

Clonotype loss following peptide vaccination coincides with a protracted CD4 T cell proliferation

The decreased contraction of the CD4 T cell response and the concomitant loss of low-affinity clonotypes in peptide-vaccinated mice prompted us to examine phenotypic differences between peptide- and protein-induced CD4 T cells during the contraction phase. We first used the expression of Ki67, a nuclear protein expressed in cycling cells, to determine the proportion of proliferating cells at various times following vaccination. The frequency of Ag-specific CD4 T cells expressing Ki67 in peptide- and protein-vaccinated mice was similar early during the primary immune response (days 5–7). However, at later time points (days 9–11), the expression of Ki67 in Ag-specific CD4 T cells was significantly higher in peptide-vaccinated mice (Fig. 7A, 7B), suggesting that peptide vaccines induce a prolonged CD4 T cell proliferation.

We next investigated IL-7Rα expression by Ag-specific CD4 T cells in peptide- and protein-vaccinated mice. IL-7Rα is critical for survival of naive and memory CD4 T cells and is transiently downregulated on clonally expanding effector CD4 T cells (31). At the peak of the response (day 7), about half of the Ag-specific CD4 T cells were IL-7Rαhi in peptide- and protein-vaccinated mice. During the next week (days 7–15), the proportion of IL-7Rαhi Ag-specific CD4 T cells in protein-vaccinated mice decreased nearly 80% of cells were IL-7Rαhi by 30 d after vaccination. In contrast, the proportion of IL-7Rαhi Ag-specific CD4 T cells in peptide-vaccinated mice decreased between days 7 and 15 and remained thereafter lower than in protein-vaccinated mice (Fig. 7C, 7D). Overall, our data demonstrate that the selective loss of low-affinity clonotypes in peptide-vaccinated mice occurs at a time of a protracted CD4 T cell proliferation and IL-7Rα downregulation.

Low-affinity clonotypes show no signs of enhanced apoptosis during clonal contraction

Loss of low-affinity clonotypes has been associated with an imbalance in the expression of different proapoptotic and anti-apoptotic molecules (18, 32). We therefore examined in our adoptive transfer model whether a similar proportion of low-affinity 2B4αβ and high-affinity SC.C7αβ T cells underwent apoptosis during the contraction phase in peptide-immunized recipients. Apoptotic T cells are characterized by low mitochondrial potential, high levels of annexin V binding, and caspase activation. Additionally, apoptosis in T cells is often accompanied by bursts of reactive oxygen intermediates (ROI) (33). Surprisingly, significantly fewer 2B4αβ T cells were positive for annexin V (Fig. 8A) and activated caspase-3 and -7 (Fig. 8B) than their high-affinity counterparts 9 d after immunization. Additionally, 2B4αβ T cells maintained their mitochondrial potential during the contraction phase (Fig. 8C) and expressed fewer ROI than did
At the molecular level, we found that 2B4 and 5C.C7 cells expressed comparable levels of proapoptotic (Puma, Noxa, Bim, and Nor-1) and anti-apoptotic molecules (Bcl-2, Bcl-xL, and Mcl-1). Thus, the selective loss of low-affinity T cells during the contraction phase in peptide-immunized mice does not appear to be due to an enhanced apoptosis.

**Discussion**

Our studies revealed the evolution of a complex Ag-specific population during the transition from primary effectors to memory T cells after peptide or protein vaccination. Peptide and protein vaccines induced similarly diverse effector CD4 TCR repertoires, but peptide vaccines skewed the memory CD4 T cell repertoire toward high-affinity clonotypes whereas protein vaccines maintained diverse memory CD4 T cell repertoires. We have previously shown that the clonal expansion phase of the CD4 T cell response following vaccination is regulated by an affinity threshold. In this study, we propose that an additional TCR affinity threshold regulates memory CD4 T cell differentiation.

The progressive focusing of memory CD4 T cell repertoires toward higher affinity clonotypes has been suggested after peptide vaccination in CFA and infection with *Listeria monocytogenes*. In contrast, protein vaccination preserved low-affinity CD4 T cells in the memory compartment. Similarly, lymphocytic choriomeningitis virus, unlike *Listeria*, has been shown to promote the differentiation of low-affinity SMARTA CD4 T cells into memory cells (18). The signals required for the differentiation of memory CD4 T cells are not yet fully characterized.
of low-affinity clonotypes to memory T cells are still unclear. We showed in this study that CD27–CD70 interaction was necessary for memory differentiation of low-affinity CD4 T cells in protein-immunized mice. Interestingly, a recent study reported that the costimulatory molecule CD27 was required to maintain clonally diverse CD8 T cell responses following influenza virus infection (30), suggesting that CD27-derived signals play an essential role in the maintenance of both low-affinity CD4 and CD8 T cells during an immune response. Whether other TNFR ligands such as OX40 could provide similar help or whether CD27–CD70 is unique in its capacity to promote the memory differentiation of low-affinity CD4 T cells remains to be investigated.

We have found that treatment with anti-CD27 Abs did not promote the maintenance of low-affinity clonotypes into the memory compartment after peptide vaccination, suggesting that CD27 signaling is not sufficient to promote the survival of low-affinity clonotypes after peptide vaccination. One interesting difference between peptide and protein vaccines is the prolonged proliferation phase observed in peptide-immunized mice that coincided with a late downregulation of IL-7 receptor expression. The mechanism driving this prolonged proliferation is still unclear. We have previously shown that long-lived pMHCII depots persist in draining LNs for >2 mo after protein vaccination (21). Similar pMHCII depots can be found after peptide vaccination (data not shown) and could be driving this late proliferation. It is possible that the quality of the pMHCII depot generated after peptide and protein vaccination is different and responsible for the prolonged proliferation of Ag-specific CD4 T cells and the selective preservation of high-affinity clonotypes in the memory compartment.

The mechanisms responsible for the selective loss of the low-affinity clonotypes during the adaptive immune responses are still unclear. In peptide-immunized mice, the loss of low-affinity clonotypes occurred rapidly during the second week following immunization. The loss of low-affinity SMARTA CD4 T cells following Listeria infection also occurred early during the contraction phase (18). SMARTA T cells expressed higher levels of the proapoptotic factors Bim and in mice infected with Listeria versus lymphocytic choriomeningitis virus, suggesting that the strength of TCR signaling influences the ability of the effector CD4 cells to survive into the memory phase of the response by regulating Bim expression (18). A model based on Nor-1 expression levels has also been proposed to explain the survival of high-affinity effector T cells during the contraction phase (34). Finally, Wensveen et al. (32) have recently found that low-affinity T cells expressed lower levels of the antia apoptotic Mcl-1 and suggested that the selective preservation of high-affinity T cells could be driven by an apoptosis threshold determined by Mcl-1 and Nor-1. Our analysis of low- and high-affinity TCR transgenic T cells during the contraction phase in peptide-immunized mice does not support these models. Low-affinity 2B4αβ and high-affinity 5C7αβ T cells expressed similar levels of proapoptotic Bim, Noxa, Puma, and Nor-1 mRNA from transferred T cells sorted from syngeneic recipients 9 d after immunization. Results were normalized to β-actin. Means ± SEM for at least three animals per group. *p < 0.05, unpaired Student t test.
Acknowledgments
We thank Dr. Andrea Sant for the MaEl expression vector.

Disclosures
The authors have no financial conflicts of interest.

References