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Constitutive Activation of Wnt Signaling Favors Generation of Memory CD8 T Cells

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T cell factor-1 (TCF-1) and lymphoid enhancer-binding factor 1, the effector transcription factors of the canonical Wnt pathway, are known to be critical for normal thymocyte development. However, it is largely unknown if it has a role in regulating mature T cell activation and T cell-mediated immune responses. In this study, we demonstrate that, like IL-7Rα and CD62L, TCF-1 and lymphoid enhancer-binding factor 1 exhibit dynamic expression changes during T cell responses, being highly expressed in naive T cells, downregulated in effector T cells, and upregulated again in memory T cells. Enforced expression of a p45 TCF-1 isoform limited the expansion of Ag-specific CD8 T cells in response to Listeria monocytogenes infection. However, when the p45 transgene was coupled with ectopic expression of stabilized β-catenin, more Ag-specific memory CD8 T cells were generated, with enhanced ability to produce IL-2. Moreover, these memory CD8 T cells expanded to a larger number of secondary effectors and cleared bacteria faster when the immunized mice were rechallenged with virulent L. monocytogenes. Furthermore, in response to vaccinia virus or lymphocytic choriomeningitis virus infection, more Ag-specific memory CD8 T cells were generated in the presence of p45 and stabilized β-catenin transgenes. Although activated Wnt signaling also resulted in larger numbers of Ag-specific memory CD4 T cells, their functional attributes and expansion after the secondary infection were not improved. Thus, constitutive activation of the canonical Wnt pathway favors memory CD8 T cell formation during initial immunization, resulting in enhanced immunity upon second encounter with the same pathogen. The Journal of Immunology, 2010, 184: 1191–1199.

P rotective cellular immunity relies on Ag-specific CD4 and CD8 T cells, which exist at very low frequencies in naive hosts (1–3). Postinfection or immunization, naive T cells undergo massive clonal expansion, generating large numbers of Ag-specific effector T cells equipped with cytokines and/or cytolytic molecules to combat pathogens. The proliferative expansion phase is followed by a rapid decline of Ag-specific T cells, and such contraction of the effector T cells does not seem to be correlated with clearance of the pathogens (4). A fraction of Ag-specific T cells (~5–10%) survive the contraction phase and form a pool of memory T cells, which will respond with accelerated expansion rate upon re-encounter with the same Ag. This immunological memory is the basis for vaccination, and much effort has been devoted to improve vaccine design through manipulating T cell responses to maximize memory T cell formation (1–5). Three major classes of signals are known to be critical for T cell activation and transition to memory T cells: signal 1 from Ag stimulation of TCR, signal 2 derived from costimulatory molecules such as CD28, and signal 3 from proinflammatory cytokines including IL-12 and type I IFNs (1, 2). Recent studies demonstrated that the effect of inflammatory cytokines such as IL-12 on effector and memory CD8+ T cells may be at least partly mediated by differential expression of the T-box transcription factor, T-bet (8, 9). It is not known whether additional signals such as morphogenic Wnt signaling and its downstream transcriptional programs have a role in modulating T cell responses.

Wnt proteins are secreted, lipid-modified glycoproteins that activate multiple signal transduction pathways to regulate a variety of cellular activities, including cell fate determination, proliferation, and gene expression (10, 11). The canonical Wnt pathway transduces signals via the intracellular mediator β-catenin. In the absence of interaction between Wnt and its Frizzled receptors, β-catenin is kept at a low level by a multimolecular destruction complex containing casein kinase 1 and glycogen synthase kinase 3. These two kinases sequentially phosphorylate a set of conserved serine and threonine residues in the N terminus of β-catenin, and the resulting phosphorylated footprint marks β-catenin for constant degradation by the proteosome. Under this condition, the Wnt effector transcription factors T cell factor-1 (TCF-1) and lymphoid enhancer-binding factor 1 (LEF-1) are associated with Groucho/transducin-like enhancer of split (TLE) corepressor proteins and act as transcriptional repressors. When Wnt ligand binds to Frizzled receptors and coreceptors, glycogen synthase kinase 3 activity is inhibited and the destruction complex is inactivated, resulting in accumulation of β-catenin in the cytoplasm. Upon entering the nucleus, β-catenin replaces Groucho/TLE, forms complexes with...
TCF-1/LEF-1, and activates the transcription of Wnt target genes. Although LEF-1 null mice did not display abnormalities in T cell development, inactivation of TCF-1 resulted in incomplete blocks at multiple early T cell developmental stages (12). TCF-1 and LEF-1 double deficiency completely arrested T cell development at the immature single-positive thymocyte stage (13), indicating functional redundancy between TCF-1 and LEF-1 and a relatively dominant role of TCF-1 during T cell development. Ablation of β-catenin was initially found to perturb β selection during thymocyte development (14); however, recent studies indicate that both β-catenin and γ-catenin are dispensable for thymopoiesis (15–17). Nevertheless, deletion of phosphorylation sites in the N terminus of β-catenin led to accumulation and constitutive activation of the canonical Wnt pathway, and, when driven by a T cell specific promoter, the stabilized β-catenin transgene enhances thymocyte production by extending thymocyte survival (18, 19).

Despite its critical roles during T cell development, TCF-1 appeared to be dispensable for T cell proliferation and cytotoxic function upon activation in vitro (20), albeit the canonical Wnt pathway was shown to be operative in naive or activated CD8+ T cells (17). A recent study demonstrated that overexpression of stabilized β-catenin can increase the survival of CD4+CD25+ regulatory T cells and induce anergic phenotype in CD4+CD25− effector T cells (21). In the current study, we investigated the effect of constitutive activation of Wnt signaling on CD4+ and CD8+ T cell responses to infection.

Materials and Methods
Mice and infectious agents
The β-catenin transgenic mice (β-Cat-Tg) and p45 transgenic mice (p45-Tg) were as described (19, 22). p45-Tg and p45 single transgenic (Tg) mice were used as breeders, and littermates of all four genotypes (i.e., single Tg mice, double Tg [dTg], and no transgene controls) were used in this study for parallel comparisons. Listeria monocytogenes-, lymphocytic choriomeningitis virus (LCMV)-, and vaccinia virus (VacV)-infected mice were housed in accordance with the manufacturer’s directions. Labeled Thy1.1+ OT-I Tg mice were then washed and used in intracellular staining for IFN-γ.

Isolation of Ag-specific T cells and quantitative RT-PCR
Naïve CD8 T cells were purified from uninfected OT-I Tg mice via negative selection to ≥95% purity (StemCell Technologies, Vancouver, British Columbia, Canada). To obtain Ag-specific effector and memory T cells, naïve C57BL/6 (Thy1.2+) mice were adoptively transferred with 500 CD8+ T cells from OT-I TCR Tg mice (Thy1.1+), and 1 d later infected with actA− LM-OVA. Ag-specific T cells were purified on day 5 (as effector T cells) and day 135 (as memory T cells) postinfection by labeling splenocytes with PE-conjugated antibodies (Miltenyi Biotec, Auburn, CA), and quantitative PCR was performed on an ABI 7300 Real Time PCR System (Applied Biosystems). β-actin was used to normalize the expression of other genes of interest. For each individual gene, its relative expression in naïve T cells was arbitrarily set to one, and its expression changes in effector and memory T cells were calculated as fold repression or induction.

Results
Dynamic gene expression changes of TCF-1 and LEF-1 during T cell responses
To explore if the Wnt signaling pathway has a role in modulating mature T cell responses, we first measured relevant gene expression levels in naive, effector, and memory T cells. Effector and memory T cells were obtained 5 and 135 d postinfection from mice that had initially received low numbers (≈500) of OT-I CD8 T cells (31) and were then infected with an attenuated strain of L. monocytogenes (actA− LM) expressing OVA (23). IL-7Rα is known for its dynamic gene expression changes during T cell responses, being expressed in naive T cells, downregulated in effectors, and restored to an even higher levels in memory T cells (32, 33). Consistent with previous observations, IL-7Rα transcripts were considerably lower in day 5 effector T cells than those in naive T cells, whereas day 135 memory T cells showed higher IL-7Rα expression than naive T cells (Fig. 1). We previously demonstrated that GA-binding

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protein (GABP) is required for transcriptional activation of the IL-7Rα gene (34); however, the expression of GABPs (the DNA binding subunit) and GABPβ1 (the transactivation subunit) were relatively stable during T cell responses (Fig. 1). In contrast, both TCF-1 and LEF-1 were dramatically downregulated in day 5 effector T cells compared with naive T cells, but were partially restored in day 135 memory T cells. The expression levels of both transcription factors in memory T cells were still lower than naive T cells, consistent with previous findings that Ag-experienced human CD8 T cells expressed less TCF-1 and LEF-1 than naive cells (35). The corepressor Groucho/TELE family consists of four proteins of similar molecular weight and structure in mammals (36), and another corepressor C-terminal binding protein 1 has been shown to interact with TCF family transcription factors (37). Interestingly, all four TLE and C-terminal binding protein 1 transcripts were decreased in effector T cells and partially upregulated in memory phase again, similar to the expression changes in TCF-1 and LEF-1 (Fig. 1). These findings collectively suggest that TCF-1/LEF-1–mediated activation and repression of Wnt downstream genes are both attenuated in effectors and restored in memory T cells, albeit to a lesser extent compared with naive T cells.

Constitutively active Wnt signaling limits CD8 T cell expansion and contraction but favors formation of memory CD8 T cells

The dynamic expression changes of TCF-1 and LEF-1 along with IL-7Rα are suggestive of their potential functional importance during T cell responses. It has been shown that enforced expression of IL-7Rα alone was not sufficient to protect effector T cells from contraction (28, 38). Because stabilized β-catenin and expression of TCF-1 extended thymocyte survival (19, 22), we hypothesized that constitutive activation of Wnt signaling may provide survival signals to effector T cells and thus result in a larger memory T cell pool. To test this, we used two lines of Tg mice, expressing a stabilized β-catenin and a p45 isoform of TCF-1, respectively (19, 22). The stabilized β-catenin has an internal deletion of 20 aas containing four critical phosphorylation sites, preventing proteosome-mediated degradation. The stabilized β-catenin transgene (referred as βCat-Tg) is driven by the CD4 promoter, which allows transgene expression in both CD4 and CD8 T cells (19, 39). Among multiple TCF-1 isoforms, the p45 TCF-1 protein contains an N-terminal β-catenin interaction domain and can at least partially restore normal thyMIC cellularity and thymocyte development in TCF-1–deficient mice (22). The p45 transgene was driven by an H-2Kb promoter coupled with IgH enhancer, resulting in persistent expression in lymphocytes including T lineage cells (referred to as p45-Tg) (22). We crossed βCat-Tg and p45-Tg to generate DTg, and the expression of both transgenes may help ensure constitutively active Wnt signaling in effector T cells in which the endogenous TCF-1 proteins are downregulated.

To make sure that the expression of βCat-Tg and p45-Tg does not affect the TCR repertoire, we first used a panel of Abs to detect TCR Vβ subtypes on splenic CD8 and CD4 T cells and observed no significant changes (Supplemental Fig. 1A, 1B for CD8 T cells; data not shown for CD4 T cells). It was shown previously that the expression of constitutive active β-catenin extended thymocyte survival by upregulating Bcl-2 (19, 22), and that Tg expression of Bcl-2 resulted in a bias toward 3’ Jα usage in the TCR-α subtypes (40). To determine if βCat-Tg functionally altered the repertoire of Ag-specific T cells, we measured their functional avidity, which would likely be affected by skewed TCR-α subtypes (41, 42). We infected βCat-Tg, p45-Tg, DTg, and wild-type (WT) littermate controls with the attenuated actA−LM-OVA (28). On day 7 postinfection, we isolated splenocytes, incubated them with 10-fold serial dilutions of OVA257–264 peptide, and measured the fraction of cells producing IFN-γ. As shown in Supplemental Fig. 1C, no apparent differences in T cell functional avidity were observed among the various strains, suggesting that the TCR repertoire was not detectably skewed by the βCat-Tg and/or p45-Tg.

To measure T cell responses in vivo, we infected WT, βCat-Tg, p45-Tg, and DTg mice with actA−LM-OVA (28), and then measured the OVA257–264-specific CD8 T cell responses using intracellular cytokine staining for peptide-stimulated IFN-γ on days 5, 7, 9, 14, and 42 postinfection. During the clonal expansion phase (5–9 d postinfection), all groups of mice showed expansion of OVA-specific CD8 T cells, as manifested by IFN-γ production upon OVA peptide stimulation, with the peak response appearing on day 7 in all mouse strains (Fig. 2A, 2G). Effector CD8 T cells in each group showed similarly diminished expression of CD62L and IL-7Rα subtypes (41, 42). We infected βCat-Tg, p45-Tg, DTg, and wild-type (WT) littermate controls with the attenuated actA−LM-OVA (28). On day 7 postinfection, we isolated splenocytes, incubated them with 10-fold serial dilutions of OVA257–264 peptide, and measured the fraction of cells producing IFN-γ. As shown in Supplemental Fig. 1C, no apparent differences in T cell functional avidity were observed among the various strains, suggesting that the TCR repertoire was not detectably skewed by the βCat-Tg and/or p45-Tg.

During the contraction phase (day 14 postinfection), ~90% of the Ag-specific CD8 T cells was eliminated in WT mice as expected. When normalized to their respective peak responses, a similar contraction was seen in βCat-Tg, whereas a higher percentage of cells survived in p45-Tg (25%) and DTg (60%) mice.
When the mice were examined at the memory phase (day 42 postinfection), dTg mice had higher frequencies and increased numbers of Ag-specific CD8 cells compared with WT or single Tg mice (Fig. 2D,2E,2G), despite a similar CD8 frequency in splenocytes among all Tg and control strains (Fig. 2F). When normalized to the peak response, more Ag-specific CD8 T cells survived and became memory cells in dTg mice (29% in dTg versus 3–6% in WT and single Tg mice; Fig. 2H). The memory CD8 T cells in all of the mouse strains examined exhibited similar CD27high subsets and similar upregulation of CD62L and IL-7Rα (Fig. 2D). These observations collectively indicate that constitutive activation of Wnt signaling in T cells favors the generation of memory CD8 T cells.

FIGURE 2. Constitutive activation of Wnt signaling favors formation of memory CD8 T cells. A and D, WT, βCat-Tg, p45-Tg, and dTg mice were infected with actA+LM-OVA and the CD8 T cell response to OVA257–264 was monitored in the spleen at day 7 (A) and day 42 (D) postinfection using intracellular cytokine staining for IFN-γ. The percentages of IFN-γ+ CD8 cells are shown in the contour plots in the absence (shown for A) or presence of OVA peptide stimulation. IFN-γ+ cells were further analyzed for IL-2+, CD62Lhigh, IL-7Rα+, or CD27high subsets, and their percentages are shown. Staining with isotype control for CD62L and IL-7Rα is displayed as a dotted line in histograms showing IL-7Rα staining. Data are representative of two independent experiments with at least three mice examined in each experiment. Frequency of IFN-γ+ cells in CD8 (B), and frequency of CD8 cells in splenocytes (C) on day 7 postinfection. Frequency of IFN-γ+ cells in CD8 (E) and frequency of CD8 cells in splenocytes (F) on day 42 postinfection. Fold changes of mean values for each Tg strain versus WT mice are shown for B, C, E, and F. G, Kinetics of OVA-specific CD8 T cell responses shown as total numbers at indicated time points. Data are means ± SEM in one of the two independent experiments with similar results (n = 3). H, Survival rate of OVA-specific CD8 T cells at contraction and memory phases. The mean value of OVA-specific CD8 T cell numbers at days 14 and 42 (as in G) was normalized to respective peak response on day 7. The percentage of survived cells is shown. *p < 0.05; **p < 0.01 by Student t test for each group of Tg mice versus WT controls.

Heightened secondary expansion and bacteria clearance in double Tg mice

Effective immunological memory is characterized by an accelerated expansion of memory cells upon re-encounter of the same Ag. Interestingly, the Ag-specific memory CD8 T cells showed increased capacity to produce IL-2 upon in vitro restimulation with OVA peptide in βCat-Tg or p45-Tg mice (Figs. 2D, 3A), and IL-2+ IFN-γ+ CD8 T cells in dTg mice were significantly elevated in absolute numbers (Fig. 3B). To further determine if the increased number and IL-2 production by dTg memory CD8 T cells confers functional advantages, we challenged the immune mice (45 d after the primary infection) with virulent LM-OVA. After the secondary infection, OVA-specific CD8 T cells in all mouse strains expanded
T cells per spleen in each group are shown in means indicated genotypes were first immunized with secondary Ag-specific CD8 T cell responses. Mice of Thy1.2+IFN- γ Tg mice versus WT controls. 0.05; pp represents one mouse. All data are from one of two spleen (CFUs) in livers and spleens on day 3 after rechallenging naive or controls (1.5- and 2.4-fold increase in cell number in dTg mice compared with controls at days 3 and 5, respectively), albeit the increase did not reach a statistical significance. It is noteworthy that the secondary expansion of Ag-specific CD8 T cells was not limited by the enforced expression of the p45 TCF-1 protein, which is different from the primary CD8 response. The contraction following the secondary CD8 response was more moderate as compared with that after the primary response (1), and dTg mice continued to exhibit a 3.2-fold increase of OVA-specific CD8 T cells over WT mice (Fig. 3D). Significantly more secondary memory CD8 T cells were detected on day 42 after secondary infection in dTg (3.6-fold increase over WT littermates; p < 0.05) (Fig. 3D). Thus, in the presence of constitutively active Wnt signaling, the increased primary memory CD8 T cells, when rechallenged, gave rise to larger numbers of secondary effector and memory T cells. An important feature of memory T cells is to provide protection upon encounter with the same pathogen. We next determined protection from virulent LM-OVA by measuring bacterial numbers (CFUs) in livers and spleens on day 3 after rechallenging naive or immunized mice. Whereas the bacteria expanded drastically in both liver and spleen of naive mice, the immunized mice effectively controlled the infection, as many fewer CFUs were seen in WT, single Tg, and dTg mice, especially in the spleen (Fig. 3E, 3F). Interestingly, virulent LM-OVA was almost completely cleared in the liver of dTg mice, which is likely accounted for by a larger expansion of Ag-specific CD8 T cells observed in these mice. 

Constitutively active Wnt signaling suppresses caspase-3/7 activation

Our observations above indicate that forced expression of the p45 TCF-1 isoform and a constitutively active β-catenin limited the contraction of Ag-specific CD8 effectors and resulted in increased numbers of memory T cells. These Ag-specific memory CD8 T cells generated more secondary effector T cells upon rechallenge, leading to a more effective reduction in bacterial burden. The reduced contraction of effector T cells in dTg mice may be a result of increased proliferation and/or survival of Ag-specific T cells. To distinguish these possibilities, we i.p. injected BrdU into dTg and WT littermate controls on day 7 postinfection with actA- ×-OVA and measured BrdU incorporation in OVA-specific CD8 T cells on day 9, the early stage of contraction phase. As shown in Fig. 4A, the proliferation rates of OVA-specific CD8 T cells were similar in both WT and dTg mice. We next determined the expression levels of prosurvival Bcl-2 family members in OVA-specific T cells. Consistent with previous findings that Bcl-2 expression is downregulated in Ag-specific effector T cells in response to LCMV infection (43), the OVA-specific T cells in both WT and dTg mice expressed similarly low levels of Bcl-2 (Supplemental Fig. 2A). In thymocytes, activation of β-catenin upregulates Bcl-XL and increases their survival (19, 22). We purified OVA-specific CD8 T cells by cell sorting of HLA-× SIINFEKL tetramer-positive cells from both WT and dTg mice and by quantitative RT-PCR measured transcript levels of Bcl-XL and Mcl-1, other prosurvival Bcl-2 family members. Both genes showed a minimal increase in expression (<1.5-fold on average) in dTg OVA-specific T cells compared with those in WT littermates (Supplemental Fig. 2B), consistent with previous observations that β-Cat-Tg did not increase Bcl-XL expression in mature T cells (19).

Apoptotic signals converge on activation of effector caspases, via either intrinsic (such as withdrawal of growth factors) or extrinsic pathways (such as Fas engagement by Fas ligand) (44, 45). To further discern the apoptotic status of Ag-specific CD8 T cells during the contraction phase, we detected activated caspase-3 and -7 using a fluorescent inhibitor of caspases (FLICA) methodology.

**FIGURE 3.** Memory CD8 T cells generated in the presence of constitutively active Wnt signaling manifested enhanced functionality. Frequency (A) and numbers (B) of IL-2-producing OVA-specific memory CD8 T cells. The numbers were calculated from the frequency of IL-2+IFN-γ+ cells (A) and the absolute number of OVA-specific CD8 T cells on day 42 postinfection as in Fig. 2. C and D. Secondary Ag-specific CD8 T cell responses. Mice of indicated genotypes were first immunized with acetA- ×-OVA as in Fig. 2, and detection of OVA-specific CD8 T cells on day 42 postimmunization was confirmed in periphery blood leukocytes by intracellular staining for IFN-γ (data not shown). The immunized mice were then infected with virulent LM-OVA, and CD8 responses to OVA were determined. OVA-257-264-specific CD8 T cells at day 3 and day 42 after secondary infection were detected as Thy1.2+IFN-γ+ CD8 cells, with the percentages shown in C. Total numbers of Ag-specific CD8 T cells per spleen in each group are shown in D as means ± SEM. E and F. Clearance of secondary bacterial infection by primary CD8 memory T cells. Naive or immunized mice were infected with virulent LM-OVA, and 3 d later, livers and spleens were harvested and CFUs were determined. Data are reported as CFU numbers per gram of liver (E) or per spleen (F). LOD, limit of detection. Each symbol represents one mouse. All data are from one of two independent experiments with similar results. *p < 0.05; **p < 0.01 by Student t test for each group of Tg mice versus WT controls.
in which the active center of activated caspases is bound by the fluoromethyl ketone moiety of fluorochrome-labeled FLICA (46). As shown in Fig. 4B, a smaller portion of OVA-specific CD8 effector cells from dTg mice had active caspase-3/7 compared with those from WT controls. Collectively, the reduced contraction of effector T cells in the presence of active Wnt signaling is at least partly explained by reduced caspase activation and hence alleviated apoptosis, which is probably independent of Bcl-2 and other prosurvival Bcl-2 family members.

**Constitutively active Wnt signaling favors memory CD8 T cell formation in response to viral infection**

We next investigated if constitutively active Wnt signaling can promote memory CD8 T cell formation in response to other forms of infectious agents in addition to bacterial infection. VacV and LCMV are known to elicit cellular immune responses mainly determined by CD8 T cells. We first infected WT and dTg mice with VacV-OVA (26, 47). On day 42 postinfection, we measured OVA-specific memory CD8 T cells in the spleen by intracellular staining for IFN-γ after the OVA peptide stimulation. The OVA-specific memory CD8 T cells in dTg mice exhibited on average a ~2.5-fold increase compared with WT littermate controls (Fig. 5A). We next infected the mice with LCMV-Armstrong and determined memory phase CD8 responses in the spleen to multiple epitopes including the immunodominant epitopes located in glycoproteins GP33–41 and GP276–286, nucleoprotein NP396–404, and subdominant epitopes NP205–212 (48). By measuring peptide-stimulated IFN-γ production, dTg mice were found to have more memory CD8 T cells to GP33–41, NP396–404, and NP205–212 epitopes than the WT control mice (Fig. 5B), and all these increases are statistically significant. On the other hand, GP276–286-specific memory CD8 T cells in dTg mice showed only a small increase (Fig. 5B). Taken together, these results suggested that activation of Wnt signaling positively impacted the generation of memory CD8 T cells in response to both bacterial and viral infections.

**Constitutively active Wnt signaling favors memory CD4 T cell formation but does not improve secondary expansion**

Finally, we determined whether the enforced Wnt signaling similarly affected CD4 T cell responses by detecting LLO190–201 peptide-specific CD4 cells at various stages after actA-MLM-OVA infection. The peak expansion of LLO190–201-specific effector CD4 T cells in WT and βCat-Tg mice occurred at day 7 after LM infection (Fig. 6A, 6G), with βCat-Tg mice exhibiting moderately increased frequency of the Ag-specific CD4 cells (Fig. 6B). On the other hand, the frequency of Ag-specific CD4 T cells in p45-Tg and dTg mice was similar to the WT controls (Fig. 6B). The frequency of CD4 T cells in the spleens was also similar among WT, βCat-Tg, and p45-Tg mice, but slightly decreased in dTg mice (Fig. 6C). The absolute numbers of Ag-specific CD4 T cells were similar among all Tg strains and WT littermate controls on day 5 postinfection; however, those in p45-Tg and dTg mice had already started to decline on day 7 postinfection (Fig. 6G). Despite the change in kinetics, Ag-specific CD4 T cells in all mouse strains showed similar capacity of IL-2 production, similar downregulation of CD62L and IL-7Rα, and comparable portions of CD27high subsets (Fig. 6A).

Although p45-Tg and dTg mice exhibited an early decline in the frequency of Ag-specific CD4 T cells, their numbers were similar in all mouse strains at day 14 postinfection (Fig. 6G). Whereas LLO190201-specific CD4 T cells in WT and single Tg mice further declined by day 42 postinfection, those in dTg mice persisted at a higher frequency and total number (Fig. 6D, 6E, 6G). The splenic CD4 frequency in all mouse strains at the memory phase was similar (Fig. 6F). In contrast to their numbers, memory CD4 T cells in all Tg strains and WT controls exhibited similar re-expression of CD62L and IL-7Rα on the cell surface. Unlike memory CD8 T cells, dTg memory CD4 T cells from dTg mice did not show an improved
ability to produce IL-2 upon peptide stimulation (Fig. 6D). These data suggest that enforced expression of p45 and a stabilized \(\beta\)-catenin enhances the generation of memory CD4 T cells, which does not seem to be accompanied by obvious functional changes.

We also assessed the secondary responses of memory CD4 T cells in all mouse strains expanded rapidly (Fig. 6H and Supplemental Fig. 3). Although there were more primary memory CD4 T cells to start with in dTg mice (Fig. 6G), they expanded to a similar ceiling level as seen in WT and single Tg mice (Fig. 6H). The LLO-specific CD4 cells contracted similarly and stabilized on a similar level as secondary memory CD4 cells in WT and dTg mice. On the other hand, if any, fewer LLO-specific CD4 cells were detected on days 14 and 42 after secondary infection in \(\beta\)Cat-Tg and p45-Tg mice (Fig. 6H and Supplemental Fig. 3). Collectively, these data suggest that activation of the Wnt pathway does not improve the secondary response of CD4 cells, even though more memory CD4 cells were generated during the initial immunization.

**Discussion**

The canonical Wnt pathway, with TCF-1 and LEF-1 as the effector transcription factors, is known to play critical roles in T cell development, but its function in peripheral T cells remains relatively unknown. In this study, we report that constitutive activation of the Wnt pathway, by the enforced expression of a stabilized \(\beta\)-catenin together with a \(\beta\)-catenin receptive p45 TCF-1 isoform, resulted in an increased frequency and number of primary memory CD8 T cells accompanied by an increased ability to produce IL-2. Moreover, upon rechallenge, the enlarged memory CD8 T cell pool expanded to larger numbers of secondary effector T cells and the pathogen was cleared faster. Future studies should determine if primary memory dTg CD8 cells have enhanced per cell protective capacity. Nevertheless, our results indicated that constitutive activation of the canonical Wnt pathway favors formation of memory CD8 T cells, leading to a “win by numbers” after re-encountering the same pathogen. On the other hand, although CD4 memory T cells were also increased, enforced Wnt signaling did not improve the
secondary expansion of Ag-specific CD4 cells and their IL-2 production. The distinct effects of Wnt signaling on CD4 and CD8 cells are not surprising because several intrinsic differences between CD4 and CD8 responses have been noted. For examples, CD4 expansion upon activation is less pronounced than that of CD8 cells, and formation of memory CD4 T cells requires prolonged Ag contact (3, 49, 50). Our observations thus suggest a specific role of the canonical Wnt signaling pathway in promoting memory CD8 T cell formation and enhancing their functionality.

One implication of our study is the potential use of Wnt ligands as adjuvants in vaccine to enhance memory CD8 T responses. Current strategies to improve vaccination efficiency include the use of epitopes with enhanced affinity of binding to the TCR and the use of costimulatory molecules and of cytokines as adjuvants (5, 51). The addition of noncytokine factors, such as Wnt ligands, may further improve the T cell responses. We found that p45 overexpression limited initial CD8 T cell expansion; however, when coupled with stabilized β-catenin expression, the contraction of Ag-specific CD8 T cells was also reduced, resulting in increased numbers of memory CD8 cells. Our observations are in line with a recent finding that in vitro treatment of CD8 T cells with pharmacological inhibitors of glycosynthase kinase 3, hence stabilizing β-catenin and activating Wnt pathway, blocked their differentiation to effector T cells but promoted them to adopt a CD8 memory stem cell phenotype (52). There are at least 19 known Wnt proteins in mammals and 10 known Fzd receptors for Wnt ligands (10, 11). Using reporter assays, it has been shown that Wnt signaling is active in both naïve and activated T cells (17). Future studies need to be directed toward identification of Wnt ligand and Fzd receptor pairs that are critical in mature T cell function for potential adjuvant applications. It should be noted that the expression of stabilized β-catenin alone was not sufficient to improve CD8 T cell memory. This may be explained by the downregulation of TCF-1 and LEF-1 in effector T cells, which may render them unresponsive to activation of Wnt signaling. We previously demonstrated that downregulation of IL-7Rα in effector T cells was mediated by the PI3K and Akt pathway and that inhibition of the PI3K/Akt pathway in activated T cells allowed retention of higher IL-7Rα expression (53). Elucidation of the signaling pathway(s) leading to TCF-1/LEF-1 downregulation and intervention of this pathway may ultimately help maximize the beneficial effects of active Wnt signaling.

Activation of the canonical Wnt signaling has diverse effects on hematopoietic cells, and the effects are dependent on the cell context and the way Wnt signals are delivered. For example, purified Wnt ligands can expand hematopoietic stem cells (HSCs) in vitro and enhance their self-renewal and reconstitution activities (54, 55); however, constitutive or induced expression of stabilized β-catenin in mouse HSCs had adverse effects on HSC maintenance and blocked multilineage differentiation (56, 57). The stabilized βCat-Tg used in our study was previously shown to extend thymocyte survival (19). In agreement with this observation, we found that coupled with p45 expression, stabilized β-catenin partly protected effector CD8 cells from massive apoptosis during the contraction phase. The reduced apoptosis/contraction in Tg mice can be at least in part explained by suppression of caspase activation. In contrast to upregulation of Bcl-XL by stabilized β-catenin in thymocytes, we did not find substantial upregulation of prosurvival Bcl-2 family members including Bcl-2, Bcl-XL, and Mcl-1, which is suggestive of differential regulatory roles of Wnt pathway in developing and mature T cells. A recent study described the induction of anergy in nonregulatory T cells (CD4+ CD25−) following retroviral transduction with a stable β-catenin (21). We observed a reduced expansion of Ag-specific CD4 and CD8 cells in the presence of the p45 transgene alone or in combination with the stabilized βCat-Tg during the primary responses. We do not believe that these cells are anergic because memory cells derived from these cells expanded robustly and cleared bacterial infection upon secondary challenge. The discrepancy may lie in the difference in the experimental system and how Wnt activation is achieved. Nevertheless, it should be kept in mind that when and how Wnt signaling is activated may determine the outcomes of prophylactic or therapeutic interventions.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Figure 1. βCat and p45 transgenes did not cause apparent changes in TCR repertoire. (A) and (B) Detection of TCR Vβ subtypes on splenic CD8 T cells. Splenocytes from dTg and WT littermate controls were stained for CD4 and CD8 along with TCR Vβ screening panel antibodies (BD Biosciences). Representative flow cytometric data for selected Vβ subtypes are shown in (A). Note that the complete Vβ subtype antibodies are only available in FITC-conjugated format, and that the low
expression of GFP in mature CD8 T cells accompanying β-catenin transgene did not affect the detection of most of Vβ subtypes that are expressed at high levels. The results from 2 independent experiments are collectively shown in (B). Vβ2, Vβ3, Vβ14, and Vβ17a subtypes are less abundant and expressed weaker, and no apparent differences have been noted. (C) Functional avidity of antigen-specific T cells in the transgenic strains. Splenocytes from act4LM-Ova-infected mice were isolated on day 7 post-infection and incubated with the Ova257-264 peptide at indicated concentrations for 6 hours. Fractions of IFN-γ-producing cells were determined by intracellular staining. The response to 200 nM Ova peptide in each mouse strain was arbitrarily set to 100%, and responses to other concentrations of Ova peptide in the same strain were normalized. Data are average of 2 independent measurements.
Supplemental Figure 2. Similar expression levels of pro-survival Bcl-2 family members in antigen-specific effector CD8 T cells in WT and dTg mice.

(A) Detection of Bcl-2 protein in Ova-specific effector CD8 T cells. WT and dTg mice were infected with actA LM-Ova, and on day 7 post-infection, expression of Bcl-2 in Ova-specific CD8 T cells were detected by simultaneous intracellular staining for Bcl-2 and IFN-γ. Gray lines denote staining with isotype control for Bcl-2, and red lines denote Bcl-2 staining. The percentages of Bcl-2+ cells are marked.

(B) Detection of Bcl-X<sub>L</sub> and Mcl-1 transcripts in Ova-specific effector CD8 T cells. Antigen-specific CD8 T cells were detected and labeled with MHC I/SIINFEKL tetramer, and the tetramer-positive cells were purified by FACS sorting on days 7 and 42, respectively. Total RNAs were prepared and relative expression of Bcl-X<sub>L</sub> and Mcl-1 was determined by quantitative reverse transcription PCR. Results are means ± s.e.m. of duplicate measurement of 2 independent samples.
Supplemental Figure 3. Secondary expansion of antigen-specific CD4 T cells in WT and transgenic strains. Mice of indicated genotypes were first infected with actA LM-Ova, and 45 days later infected with virulent LM-Ova. On days 3 and 42 after the second challenge, antigen-specific CD4 T cells in splenocytes were detected by ICS for IFN-γ after 6-hour incubation of the splenocytes with LLO_{190-201} peptides. Percentages of IFN-γ^+CD4^+ cells are shown. Data are representative of 2 independent experiments with at least 6 mice analyzed for each genotype.