Induction of autoimmune disease in CTLA-4$^{-/-}$ mice depends on a specific CD28 motif that is required for *in vivo* costimulation

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CTLA-4-deficient mice develop a lethal autoimmune lymphoproliferative disorder that is strictly dependent on *in vivo* CD28 costimulation. Nevertheless, it is not known whether there is a specific site on the CD28 molecule that is required for induction of autoimmunity. Using CTLA-4-deficient mice expressing CD28 molecules with various point mutations in the CD28 cytosolic tail, the present study documents that *in vivo* costimulation for induction of autoimmune disease strictly requires an intact C-terminal proline motif that promotes lymphocyte-specific protein tyrosine kinase (Lck) binding to the CD28 cytosolic tail, because point mutations in C-terminal proline residues (Pro-187 and Pro-190) completely prevented disease induction. In contrast, *in vivo* costimulation for disease induction did not require either an intact YMNM motif or an intact N-terminal proline motif, which, respectively, promote phosphoinositide 3-kinase and IL2-inducible T cell kinase binding to the CD28 cytosolic tail. Thus, *in vivo* CD28 costimulation for induction of autoimmune disease is strictly and specifically dependent on an intact C-terminal proline motif that serves as a lymphocyte-specific protein tyrosine (Lck) kinase binding site in the CD28 cytosolic tail.

autoimmunity | lymphoproliferation

Initiation of T cell immune responses requires both T cell receptor (TCR) stimulation and CD28 costimulation. CD28 is the best-characterized and most important T cell costimulatory molecule and has two known ligands, B7–1 (CD80) and B7–2 (CD86), but it is still uncertain how surface CD28 molecules transduce costimulatory signals into T cells (1, 2). The cytosolic tail of CD28 consists of 41 aa and lacks intrinsic catalytic activity but contains distinct binding motifs that serve as docking sites for different intracellular kinases whose activity may be enhanced by binding to the CD28 cytosolic domain. *In vitro* molecular studies with recombinant proteins have demonstrated that the "YMNM motif" from residues 170–173 binds phosphoinositide 3-kinase (PI3K), Grb2, and Gads; the "N-terminal proline" motif from residues 175–178 binds IL2-inducible T cell kinase (Itk); and the "C-terminal proline motif" at residues 187–190 binds lymphocyte-specific protein tyrosine kinase (Lck), Fyn, and Grb2 (3–11). Despite much effort, it has been difficult to clearly identify which CD28 binding motifs are responsible for CD28 costimulatory functions, because most attempts at answering this question have yielded complex and contradictory results. A possible reason for this complexity is that structure–function analyses of CD28 have been performed under a variety of *in vitro* conditions and have assessed immune responses mediated either by T hybridoma cell lines or transgenic/retrovirally transduced primary T cells that likely overexpressed CD28. In contrast, structure–function analyses of CD28 costimulation that have been performed *in vivo* have yielded much more straightforward results. Using a closely matched set of CD28 transgenes that restored CD28 expression in CD28$^{-/-}$ mice to physiologic levels but that encoded CD28 molecules with disruptions in different cytosolic binding motifs, we have documented that the CD28 costimulatory signals required for *in vivo* IL-2 production and T regulatory (Treg) cell generation in the thymus were strictly dependent on an intact Lck binding site in the CD28 cytosolic tail (12). The importance of an intact Lck binding site for CD28 costimulation has since been confirmed for *in vivo* humoral immune responses by mice constructed with a gene knockin mutation of the C-terminal proline motif in the CD28 cytosolic tail (13).

The present study was undertaken to identify the specific CD28 binding motif(s) required for disease development in CTLA-4-deficient mice, because CTLA-4-deficient mice develop a lethal lymphoproliferative disorder that is strictly dependent on *in vivo* CD28 costimulation. We now report that disease induction in CTLA-4-deficient mice strictly requires *in vivo* CD28 costimulation by molecules with an intact Lck binding site in the CD28 cytosolic tail, but neither requires an intact PI3K or Itk binding site. Thus, the present study identifies a specific binding motif in the CD28 cytosolic tail that is required for autoimmune disease induction and *in vivo* costimulation.

Results

Disease in CTLA-4-Deficient Mice Requires *in Vivo* Costimulation by CD28 Molecules with an Intact Lck Binding Motif. CTLA-4$^{-/-}$ mice spontaneously develop a T cell-mediated lymphoproliferative disorder that is marked by splenomegaly, lymphadenopathy, growth retardation, and early death (14–16). As a result, CTLA-4-deficient mice are physically smaller than age-matched B6 mice and have significantly enlarged lymph nodes (LNs) (Fig. 1*A*). Indicative of *in vivo* autoimmune disease, CD4^{+} T cells in CTLA-4$^{-/-}$ mice express an activation/memory phenotype (CD25^{+}CD69^{+}CD44^{hi}CD62L^{lo}), whereas normal B6 CD4^{+} T cells express a resting/naïve phenotype (CD25^{-}CD69^{-}CD44^{lo}CD62L^{hi}) (Fig. 1*B*). Autoimmunity in CTLA-4$^{-/-}$ mice requires *in vivo* CD28 costimulation, because removal of *in vivo* CD28 expression from CTLA-4-deficient mice prevents disease (Fig. 1*A Left*) and prevents *in vivo* T cell activation (Fig. 1*B Middle*). In contrast, removal of *in vivo* expression of the adhesion molecule LFA-1 from CTLA-4-deficient mice fails to prevent disease (Fig. 1*A Right*) and fails to prevent *in vivo* T cell activation (Fig. 1*B Bottom*). Thus, CD28 costimulatory signals are

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Abbreviations: TCR, T cell receptor; Lck, lymphocyte-specific protein tyrosine kinase; PI3K, phosphoinositide 3-kinase; Itk, IL2-inducible T cell kinase; Treg, T regulatory; LN, lymph node; DKO, double knockout.

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Fig. 1. CD28 costimulation is required for disease induction in CTLA-4-deficient mice. (*A*) Size comparison of animals and LNs from B6, CTLA-4$^{-/-}$, and CD28$^{-/-}$CTLA-4$^{-/-}$ 4-week-old mice (*Left*) and from age-matched B6, CTLA-4$^{-/-}$, and LFA-1$^{-/-}$CTLA-4$^{-/-}$ 4-week-old mice (*Right*). (*B*) Surface phenotype of CD4^{+} LN T cells from the indicated mouse strains. Dashed lines represent negative control staining. CD4^{+} LN T cells from CTLA-4-deficient mice have an activated phenotype only if they express CD28 costimulatory molecules. Results are representative of at least three experiments. (*C*) CD4^{+}Foxp3^{+} Treg cells in CTLA-4-deficient mice. Intracellular Foxp3 staining in CD4^{+} LNT cells from 4-week-old mice is shown, and numbers indicate the frequency of CD4^{+}Foxp3^{+} T cells. Dashed lines represent negative control staining.

required for *in vivo* induction of T cell activation and lethal autoimmunity in CTLA-4-deficient mice.

Ironically, CD28 costimulation is also required for *in vivo* generation of Treg cells (12, 17). Consequently, we assessed CD28-replete and CD28-deficient CTLA-4-deficient mice for CD4^{+}Foxp3^{+} Treg cells (Fig. 1*C*). We found that CD28$^{+/+}$CTLA-4$^{-/-}$ mice contained normal frequencies of Tregs, whereas CD28$^{-/-}$CTLA-4$^{-/-}$ double knockout (DKO) mice contained few Treg cells (Fig. 1*C*), confirming the *in vivo* role of CD28 in Treg generation. In contrast, the presence of normal frequencies of Treg cells in CD28$^{+/+}$CTLA-4$^{-/-}$ mice demonstrated that CTLA-4 is not required for Treg cell generation, although it may be required for Treg cell function as CD28$^{+/+}$CTLA-4$^{-/-}$ mice develop autoimmune lymphoproliferative disease. Thus, these findings demonstrate that disease induction in CTLA-4-deficient mice requires *in vivo* CD28 costimulation but is independent of CD28's role in Treg cell generation.

To identify the CD28 structural motif(s) required for *in vivo* costimulation, we introduced into CD28$^{-/-}$CTLA-4$^{-/-}$ DKO mice a matched set of CD28 transgenes that are expressed *in vivo* at levels similar to that of endogenous CD28 (12) and that encode CD28 molecules with specific point mutations that disrupt binding of signaling kinases to the CD28 cytosolic tail

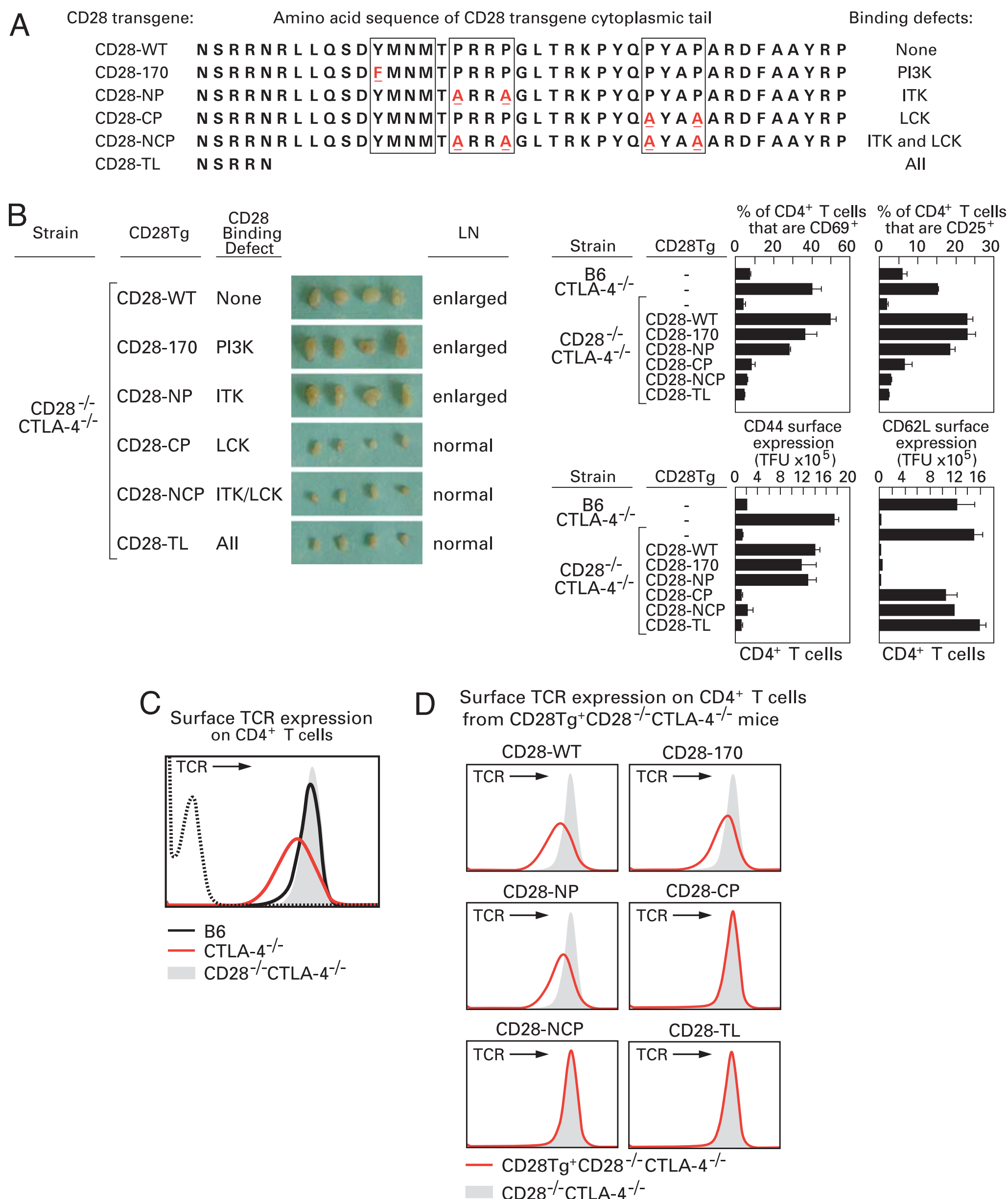

Fig. 2. Molecular mapping of the CD28 binding motif(s) required for *in vivo* activation of CTLA-4-deficient T cells. (*A*) Comparison of amino acid sequences of CD28 cytosolic tails. All CD28 transgenes encoded identical extracellular and transmembrane domains but differed in their cytosolic tails. Changes from the wild-type sequence are indicated in red, and the resulting binding defects are indicated (3–11). (*B*) CD28 transgenes were bred into CD28$^{-/-}$CTLA-4$^{-/-}$ DKO mice so that transgenic CD28 molecules were the only CD28 molecules expressed in these CTLA-4-deficient mice. Axillary LNs from mice at 4 weeks of age are shown (*Left*). CD4^{+} LNT cells from 4-week-old mice of the indicated strains were phenotyped for expression of CD69, CD25, CD44, and CD62L (*Right*). (*C*) *In vivo* CD28 costimulation reduces surface TCRβ expression. Shown is surface TCRβ expression on CD4^{+} LNT cells from 4-week-old mice of the indicated strains. (*D*) Molecular mapping of the effect of CD28 costimulation on surface TCRβ expression. A comparison of surface TCRβ expression on CD4^{+} LNT cells from CD28$^{-/-}$CTLA-4$^{-/-}$ mice and CTLA-4-deficient mice expressing different CD28 transgenic molecules is shown. Data are representative of three independent experiments.

(Fig. 2*A*). Notably, the CD28-WT transgene encodes CD28 molecules with a wild-type CD28 tail; the CD28–170 transgene encodes molecules with a Y→F mutation in tyrosine residue 170, which disrupts PI3K binding; the CD28-NP transgene encodes molecules with P→A mutations in N-terminal proline residues 175 and 178, which disrupt Itk binding; the CD28-CP transgene encodes molecules with P→A mutations in C-terminal proline residues 187 and 190, which disrupt Lck binding; the CD28-NCP transgene encodes molecules with P→A mutations in both N- and C-terminal proline residues (175, 178, 187, and 190), which disrupt both Itk and Lck binding; and the CD28-TL transgene encodes tailless CD28 molecules, which are unable to bind any intracellular molecules.

Importantly, lymphadenopathy was present only in DKO mice expressing CD28 molecules with intact Lck binding motifs (CD28-WT, CD28–170, and CD28-NP) but was absent from DKO mice expressing CD28 molecules lacking Lck binding motifs (CD28-CP, CD28-NCP, and CD28-TL) (Fig. 2*B Left*). Consistent with their lymphadenopathy, T cells from DKO mice expressing CD28 molecules with intact Lck binding motifs displayed an activation/memory phenotype, whereas T cells from DKO mice expressing CD28 molecules lacking Lck binding motifs displayed a resting/naïve phenotype [Fig. 2*B Right* and supporting information (SI) Fig. 4]. An activation/memory phenotype can reflect TCR-mediated T cell activation *in vivo*, but it

Fig. 3. Molecular mapping of the CD28 binding motif(s) required for disease induction in CTLA-4-deficient mice. (*A*) Microscopic views (×100) of pancreatic sections from 4-week-old mice of the indicated strains. (*B*) Microscopic views (×100) of pancreatic sections of 4-week-old mice from CTLA-4-deficient mice expressing transgenic CD28 molecules. (*C*) Macroscopic examination of the abdomen of CTLA-4-deficient mice expressing transgenic CD28 molecules. Muscle wasting, absence of pancreas, and accumulation in the gut of undigested food are evident in CTLA-4-deficient mice expressing CD28 molecules with an intact Lck binding motif (CD28-WT, CD28–170, and CD28-NP), whereas no pathology is detected in CTLA-4-deficient mice expressing CD28 molecules without an intact Lck binding motif (CD28-CP, CD28-NCP, and CD28-TL). (*D* and *E*) Size and weight comparison of 4-week-old CD28$^{-/-}$CTLA-4$^{-/-}$ mice expressing transgenic CD28 molecules. Displayed in the bar graph are the mean weight (± SEM) of no less than five mice from each transgenic line. Statistical analysis was by the two-tailed Student *t* test. NS, not significant ($P < 0.02$). (*F*) Survival of CTLA-4-deficient mice expressing transgenic CD28 molecules. Twenty to 30 mice of each transgenic line were observed daily (*Left*), and their median survival times were determined (*Right*). Mice alive at day 150 were also alive on day 300.

can also reflect cytokine-induced homeostatic T cell proliferation in the absence of *in vivo* TCR signaling. However, *in vivo* TCR-mediated T cell activation can be distinguished from *in vivo* cytokine-induced homeostatic proliferation by TCRβ surface expression levels, because TCR-mediated T cell activation activates Lck, which quantitatively reduces TCRβ surface expression levels on *in vivo* T cells (18, 19). Indeed, relative to TCRβ surface levels on CD4$^+$ T cells in normal B6 and in nonautoimmune DKO mice, TCRβ surface levels on CD4$^+$ T cells from autoimmune CTLA-4$^{-/-}$ mice were distinctly reduced (Fig. 2*C*). Importantly, TCRβ surface levels were also distinctly reduced on CTLA-4$^{-/-}$ T cells whose transgenic CD28 molecules contained

intact Lck binding motifs (CD28-WT, CD28–170, and CD28-NP), but were not reduced on DKO T cells whose transgenic CD28 molecules lacked Lck binding motifs (CD28-CP, CD28-NCP, and CD28-TL) (Fig. 2*D*), demonstrating that *in vivo* TCR-mediated T cell activation requires CD28 molecules with intact Lck binding motifs.

Taken together, these data demonstrate that induction of lymphadenopathy and autoimmune T cell activation in CTLA-4-deficient mice strictly requires *in vivo* costimulation by CD28 molecules with intact Lck binding motifs but does not require CD28 molecules with intact PI3K or Itk binding motifs.

Molecular Mapping of CD28 Costimulatory Signals Required for Lymphocytic Infiltration in CTLA-4-Deficient Mice. CTLA-4-deficient mice develop destructive lymphocytic infiltrates in multiple organs that depend on *in vivo* CD28 costimulation, because such lymphocytic infiltrates do not occur in $CD28^{-/-}CTLA\text{-}4^{-/-}$ DKO mice (SI Fig. 5*A*). Indeed, we found destructive lymphocytic infiltrates in pancreatic islets of $CTLA\text{-}4^{-/-}$ and $LFA\text{-}1^{-/-}$ $CTLA\text{-}4^{-/-}$ mice but not in pancreatic islets of $CD28^{-/-}CTLA\text{-}4^{-/-}$ DKO mice (Fig. 3*A*). We then used our panel of CD28 transgenic DKO mice to identify the CD28 signaling motif(s) required for induction of tissue destructive lymphocytic infiltrations (Fig. 3*B* and SI Fig. 5*B*). Destructive lymphocytic infiltrations were found in pancreatic islets and other nonlymphoid tissues from DKO mice expressing CD28 molecules with intact Lck binding motifs (CD28-WT, CD28–170, and CD28-NP) but were not found in DKO mice expressing CD28 molecules lacking Lck binding motifs (CD28-CP, CD28-NCP, and CD28-TL) (Fig. 3*B* and SI Fig. 5*B*). Gross examination of the abdomen of these DKO mice revealed that mice expressing CD28 with intact Lck binding motifs (CD28-WT, CD28–170, and CD28-NP) destroyed their pancreases, had difficulty digesting food, were severely malnourished, and had significant muscle wasting (Fig. 3*C*). In fact, reflecting the severity of their *in vivo* autoimmune disease, DKO mice expressing CD28 molecules with intact Lck binding motifs were smaller (Fig. 3*D*), weighed significantly less (Fig. 3*E*), and had markedly shortened lifespans (Fig. 3*F*). Such severe CD28-dependent autoimmune pathology strictly required an intact Lck binding motif in the CD28 cytosolic tail, because CTLA-4-deficient mice lacking an Lck binding motif in the CD28 cytosolic tail (CD28-CP, CD28-NCP, and CD28-TL) remained disease-free. But such severe CD28-dependent autoimmune pathology was not dependent on an intact PI3K or Itk binding motif in the CD28 cytosolic tail. Thus, the Lck binding motif in the CD28 cytosolic tail is specifically required to generate the *in vivo* costimulatory signals that induce autoimmune disease in CTLA-4-deficient mice.

Discussion

CTLA-4-deficient mice develop a severe, ultimately lethal lymphoproliferative disorder that depends on *in vivo* CD28 costimulation. The present study documents that such *in vivo* costimulation strictly requires CD28 molecules with an intact C-terminal proline motif that promotes Lck binding to the CD28 cytosolic tail. Indeed, point mutations of the two C-terminal proline residues (Pro-187 and Pro-190) in the CD28 cytosolic tail completely prevented disease induction in CTLA-4-deficient mice. In contrast, disease induction was not affected either by mutation of Tyr-170, which disrupts the PI3K binding site, or by mutations of the N-terminal Pro-175 and Pro-178, which disrupt the Itk binding site. Thus, the present study documents that *in vivo* costimulation by CD28 for induction of autoimmunity is specifically dependent on an intact C-terminal proline motif that serves as an Lck binding site in the CD28 cytosolic tail.

It is interesting that the same C-terminal proline motif in the CD28 cytosolic tail that we have now shown to be required for *in vivo* autoimmune disease induction in CTLA-4-deficient mice was previously shown to be critical for *in vivo* generation of Treg cells, *in vivo* stimulation of IL-2 production, and *in vitro* T cell proliferative responses (7, 12, 13, 20). As a result we think that the Lck binding motif in the CD28 cytosolic tail is especially important for CD28-mediated costimulation of TCR-signaled thymocytes and T cells. Identification of the binding motifs in the CD28 cytosolic tail that are functionally important for CD28 costimulation in T cells has been a subject of dispute. Most previous studies have assayed CD28 costimulation in different *in vitro* responses and have used T cells that overexpressed CD28. The results of experiments with retrovirally transduced T cells concluded that CD28 costimulation was not dependent on any single CD28 binding motif, a result that was attributed to extensive redundancy in function of the signaling molecules that are recruited to the CD28 cytosolic tail (21). However, it was possible that functional redundancy resulted from overexpression of the retrovirally transduced mutant CD28 molecules. In contrast, our experiments revealed a strict requirement for an intact C-terminal proline motif for *in vivo* induction of autoimmunity, although other CD28 binding sites may well be important for other CD28 functions. Indeed, binding of PI3K to an intact YMNM motif in the CD28 cytosolic tail is dispensable for *in vivo* germinal center development (22) and *in vivo* Treg cell development (12), but it has been shown to be important for *in vitro* T cell activation and survival (20, 22) as well as for *in vivo* induction of graft-versus-host disease (23).

We think that the importance for *in vivo* costimulation of the C-terminal proline motif in the CD28 cytosolic tail results from the fact that, by binding Lck, CD28 prolongs the residency time of Lck in the immunological synapse, thereby increasing the intensity and duration of TCR signaling as originally suggested by Shaw and colleagues (7, 24). Indeed, our present findings that *in vivo* CD28 costimulation induced both TCRβ down-regulation and CD69 up-regulation supports the importance of Lck binding to CD28 for costimulatory function, because both TCRβ down-regulation and CD69 up-regulation require Lck activation (18, 19, 25), and we found that signaling of both functions required an intact Lck binding motif in the CD28 cytosolic tail. In CTLA-4-deficient mice, disease induction requires CD28 enhancement of TCR signaling by autoreactive TCR specificities with presumably high affinity for self-ligands, because disease induction is delayed by *in vivo* expression of transgenic TCRs with low affinity for self ligands (26, 27). Thus, we think that the importance of an intact Lck binding motif in the CD28 cytosolic tail for disease induction in CTLA-4-deficient mice reflects the fact that, by increasing the residency time of Lck in the immunological synapse, CD28 costimulation specifically increases the intensity and duration of *in vivo* TCR signaling by T cells with autoreactive TCR specificities.

In summary, the present study documents that disease induction in CTLA-4-deficient mice strictly requires costimulation by CD28 molecules with an intact C-terminal proline motif that promotes Lck binding to the CD28 cytosolic tail, but neither requires an intact YMNM motif for PI3K binding or an intact N-terminal proline motif for Itk binding. These results molecularly map an *in vivo* autoimmune disease to a single motif in the CD28 cytosolic tail and contribute to the emerging perspective that *in vivo* CD28 costimulation of many different T cell functions depends on this same motif.

Materials and Methods

Animals. $CTLA\text{-}4^{-/-}$ (14), $CD28^{-/-}$ (28), $CD28^{-/-}CTLA\text{-}4^{-/-}$ (29), and $LFA\text{-}1^{-/-}$ (30) were obtained and maintained in our own animal colony. $LFA\text{-}1^{-/-}CTLA\text{-}4^{-/-}$ mice were generated in our own colony. C57BL/6 (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME), and B10.A mice were obtained from the National Cancer Institute (Frederick, MD). Transgenes encoding wild-type and mutant CD28 molecules (12) were bred into $CD28^{-/-}CTLA\text{-}4^{-/-}$ DKO mice. All mice were

cared for in accordance with National Institutes of Health guidelines and were used at 4 weeks of age unless indicated otherwise.

Immunofluorescence and Flow Cytometry. Antibodies with the following specificities were obtained from Pharmingen (San Diego, CA) and eBioscience (San Diego, CA) and used in the present study: TCR (H57-597), CD4 (RM4.5), CD25 (PC61 and 7D4), CD69 (H1.2F3), CD44 (1M7), CD62L (MEL-14), and Foxp3 (FJK-16s). Cells were analyzed on FACSVantage SE (Becton Dickinson, Franklin Lakes, NJ) with four-decade logarithmic amplification. Dead cells were excluded by forward scatter and propidium iodide staining. Where indicated, surface fluorescence was quantified into linear fluorescence units by use of an empirically derived calibration curve constructed for each logarithmic amplifier used. For Foxp3 staining, freshly isolated LN cells were first surface-stained with anti-CD4 and anti-CD8 and then stained for intracellular Foxp3.

Histology. Tissues from experimental mice were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin.

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