CD28 and ITK signals regulate autoreactive T cell trafficking

Nitya Jain1, Bing Miu1, Jian-kang Jiang2, Kai K McKinstry1, Amanda Prince1, Susan L Swain1, Dale L Greiner3, Craig J Thomas2, Michael J Sanderson4, Leslie J Berg1 & Joonsoo Kang1

Activation of self-reactive T cells and their trafficking to target tissues leads to autoimmune organ destruction. Mice lacking the co-inhibitory receptor cytotoxic T lymphocyte antigen-4 (CTLA-4) develop fatal autoimmunity characterized by lymphocytic infiltration into nonlymphoid tissues. Here, we demonstrate that the CD28 co-stimulatory pathway regulates the trafficking of self-reactive Ctla4−/− T cells to tissues. Concurrent ablation of the CD28-activated Tec family kinase ITK does not block spontaneous T cell activation but instead causes self-reactive Ctla4−/− T cells to accumulate in secondary lymphoid organs. Despite excessive spontaneous T cell activation and proliferation in lymphoid organs, Itk−/−; Ctla4−/− mice are otherwise healthy, mount antiviral immune responses and exhibit a long lifespan. We propose that ITK specifically licenses autoreactive T cells to enter tissues to mount destructive immune responses. Notably, ITK inhibitors mimic the null mutant phenotype and also prevent pancreatic islet infiltration by diabetogenic T cells in mouse models of type 1 diabetes, highlighting their potential utility for the treatment of human autoimmune disorders.

CD28 is the primary co-stimulatory molecule for naive CD4+ conventional T cell activation1. Binding of CD28 to B7 ligands leads to increased duration and magnitude of T cell responses2, enhanced survival and glucose metabolism3,4 and acquisition of migratory properties5. CD28 activates integrin-mediated adhesion of T cells6 and promotes actin polymerization7,8. Cd28−/− mice have impaired delayed-type hypersensitivity responses9 and fail to develop experimental autoimmune encephalitis10,11. In nonobese diabetic (NOD) mice, loss of CD28 exacerbates type 1 diabetes (T1D)12, probably owing to decreased frequency of forkhead box P3 (FOXP3)-positive regulatory T cells (Treg cells)13. However, NOD mice treated with CTLA4-Ig (abatacept), a receptor-immunoglobulin fusion protein that binds to and sequesters B7, potentially inhibiting CD28 signals, are protected from diabetes14. Interpretations of these studies are complicated by the function of the CD28 antagonist, CTLA-4, which is upregulated on activated T cells and binds B7 with a much higher affinity than CD28 (refs. 15,16).

CTLA-4 maintains T cell tolerance to self15, and polymorphisms in Ctla4 have been linked to human autoimmune diseases17. Ctla4−/− mice die of a lymphoproliferative disorder driven by CD28-dependent self-reactive CD4+ T cell activation and infiltration into tissues18,19. This loss in tolerance is initiated by the inability of CTLA-4–deficient Treg cells to function19–22, resulting in increased CD80 and CD86 expression on antigen-presenting cells and increased proliferation of T cells20,21. CTLA-4 also has conventional T cell–intrinsic functions and regulates trafficking of self-reactive T cells19,22. Expression of a truncated CTLA-4 containing only the B7-binding domain protects Ctla4−/− mice from organ infiltration by T cells23. These results suggest that modulation of CD28 signals by competitive sequestration of B7 ligands can regulate tissue infiltration by autoreactive T cells.

Studies have suggested the involvement of CD28-activated phosphatidylinositol 3-kinase (PI3K) in the trafficking of effector T cells to tissues24,25. The interleukin-2 (IL-2)–inducible Tec kinase ITK is recruited to both the T cell receptor (TCR) and CD28 upon stimulation in a PI3K-dependent manner26. Phosphorylated ITK activates phospholipase C-γ1, leading to calcium (Ca2+) mobilization and actin polarization to the site of TCR stimulation27. ITK is also activated by β2 integrins and is involved in Cdc42- and Rac-mediated chemokine-induced migration28,29. However, CD28 and ITK appear to be dispensable for T cell infiltration to target tissues in inflammatory settings, for example, in models of infection with Leishmania major, nematodes and influenza A virus16,30. Here, we show that CD28-ITK signals specifically regulate self-reactive T cell migration in tissues. Notably, small-molecule inhibitors of ITK reduced T cell infiltration and destruction of islet cells in T1D models, providing proof of principle that targeting ITK may be beneficial for treating T cell–mediated, organ-specific autoimmune diseases.

RESULTS

Ctla4−/− T cell migration to tissues requires CD28-B7 signals

Ctla4−/− CD4+ T cells recognize tissue self antigens and represent a model of multigorgan autoimmunity. Mice deficient in both Cd28 and Ctla4 are protected from lethal autoimmunity, as their T cells cannot be activated31. Further, CD28 signals are necessary for tissue infiltration by self-reactive T cells, as transfer of Ctla4−/− lymph node (LN) T cells into B7-sufficient Rag1−/− mice (lacking B and T cells) induced an aggressive autoimmune disease similar to that seen in intact Ctla4−/− mice, but transfer into B7-deficient Rag1−/− mice did not (Fig. 1a). Transfer of Ctla4−/− T cells into major histocompatibility complex (MHC) class II–deficient Rag1−/− mice...
resulted in an intermediate disease course, with 75% of mice displaying tissue infiltrates (Supplementary Fig. 1a). These results suggest a more stringent requirement for CD28 than TCR–MHC class II signals for activated Cita4−/− T cell accumulation in tissues.

Endothelial cells (ECs) in LNs express some B7 and MHC class II molecules.32–35 We determined the expression of B7 on stromal subsets in the lungs (Supplementary Fig. 1b). CD86 was expressed on CD45+ hematopoietic cells and at low but appreciable amounts on CD45+ stromal cells (Supplementary Fig. 1c). Imaging studies also identified a CX3CR1+ dendritic cell population on vessel walls of lungs that projected dendrites into the lumen (ref. 36 and data not shown). These results suggest that B7 molecules in tissues are accessible to blood-borne T cells. In vitro, B7-CD28 signals were required for the migration of activated Cita4−/− T cells across B7+ SVEC4-10 ECs (refs. 34,37) (SVEC4-10 is a mouse endothelial cell line derived by SV40 transformation of ECs from axillary lymph node vessels), as neutralizing antibodies specific for B7 reduced their migration (Supplementary Fig. 1d).

We next performed two-photon imaging of fluorescently labeled Cita4−/− T cells in the lung vasculature38,39 of wild-type (WT) and B7-deficient mice to characterize their motility. Cita4−/− T cells in WT mice were highly motile within blood vessels in lung slices and made frequent stable contacts with, and often

![Image](https://example.com/image1)

**Figure 1** B7 signals regulate Cita4−/− T cell migration. (a) H&E sections of tissues from Rag1−/− and B7-deficient Rag1−/− mice 3 weeks after transfer of Cita4−/− T cells. Data are representative of three or more experiments with 4–6 mice in each group. Scale bars, 50 μm. (b–e) Imaging of carboxyfluorescein succinimidyl ester (CFSE)-labeled Cita4−/− T cells in lung vasculature of WT or B7-deficient mice. (b) Representative frames (0–20 min) from a Video Savant recording showing T cell movement (green) in blood vessels (red) of lung slices. Scale, 0.18 μm per pixel. (c) Two dimensional (2D) tracks of ten representative T cells within blood vessels (10 min) superimposed after normalizing their starting coordinates to the origin. A minimum of 30 cells was analyzed for each genotype. (d) Displacement of individual Cita4−/− T cells in WT or B7-deficient lungs from the point of origin in 10 min. (e) Roundness of cells as calculated by ImageJ software. 1, circular object; <1, decreasing circularity. Data in d and e are expressed as means ± s.e.m. P values in d and e are based on the Mann-Whitney and Student’s t-test, respectively.

![Image](https://example.com/image2)

**Figure 2** ITK deficiency prolongs the lifespan of Cita4−/− mice. (a) Survival curves of Cita4−/− (n = 10) and Itk−/−/Cita4−/− (DKO) (n = 10) mice. P < 0.0001 (log-rank Mantel-Cox test). (b) Left, size of peripheral (inguinal, axial and brachial) LNs. Scale bar, 1 cm. Right, cell numbers in peripheral LNs, mesenteric LNs (MLNs) and spleen of 3-week-old Cita4−/− and 6- to 8-week-old WT, Itk−/− and DKO mice. Data are expressed as means ± s.e.m. (c) Left to right: frequencies of CD4+CD8+ T cells, CD4+ conventional T (Tconv) cells expressing activation markers CD44 and CD62L, CD4+ Tconv cells expressing Ki67 and Treg cells expressing FOXP3 in LNs of indicated mice as in b. The numbers in each quadrant and above the horizontal bars indicate frequency of cells. Data in b and c are representative of three experiments with four or more mice in each group.
migrated across vessel walls (Supplementary Video 1 and Fig. 1b). They showed significant mean displacement within the time frame of recording (Fig. 1c,d) and had the characteristic elongated morphology of migrating cells (Fig. 1b,e). In contrast, 

Ctla4−/− T cells in ex vivo B7-deficient lung tissues did not make stable contact with ECs, lost directionality and assumed a circular morphology (Fig. 1b–e and Supplementary Video 2). These results indicate that CD28-B7 interactions modulate T cell trafficking in tissues.

ITK deficiency reduces lethality of Ctla4−/− mice

To identify a CD28 signaling molecule required for T cell trafficking, we focused on ITK. To confirm that ITK functions downstream of CD28, we stimulated naive WT and 

Itk−/− CD4+ T cells with a CD28-specific superagonist (SAC-CD28) antibody that can trigger T cell activation without overt TCR signaling. WT naive T cells responded to SAC-CD28 crosslinking by upregulating the activation markers CD25 and CD69, but 

Itk−/− CD4+ T cells failed to do so (Supplementary Fig. 1e).

We then generated 

Itk−/−; Ctla4−/− double-knockout (DKO) mice to test whether ITK functions in self-reactive T cell trafficking. Unlike 

Ctla4−/− mice, which died by 3–4 weeks of age due to multiorgan autoimmune disease, DKO mice were healthy and had a significantly extended life span (Fig. 2a). However, DKO mice had enlarged LNs (Fig. 2b) 20 times larger than those of normal mice, containing proliferating (Ki67+) activated (CD44hiCD62Lhi) T cells (Fig. 2b,c) that upon stimulation with PMA-ionomycin, produced IL-2, IL-17, IFN-γ, TNF-α, IL-4 and IL-10 (Supplementary Fig. 2a) and that were comparable to activated 

Ctla4−/− T cells, with respect to frequency of cells undergoing apoptosis (Supplementary Fig. 2b). These results indicate that ITK signaling is not required for the activation of 

Ctla4−/− T cells. Consistent with this, naive CD4+ T cells from 

5C.C7 TCR-transgenic 

Itk−/−; Ctla4−/−; Rag1−/− mice responded normally to the cognate antigen moth cytochrome c peptide (Supplementary Fig. 2c). Lastly, global gene expression profiling of CD4+ T cells from 

Ctla4−/− and DKO mice showed that these two populations were similar, with less than 0.5% of genes differentially expressed (Supplementary Fig. 2d), supporting the notion that the activation and functional status of 

Ctla4−/− T cells were not impaired by the loss of ITK.

Lack of autoimmune tissue pathology in DKO mice

Although DKO mice exhibited an increased frequency of Treg cells in secondary lymphoid organs (SLOs) (Fig. 2c), the lack of CTLA-4 rendered these Treg cells functionally impaired, as they were unable to suppress colitis induced by colitogenic naive WT T cells in vivo (Supplementary Fig. 2e). Further, in the presence of WT Treg cells in mixed bone marrow chimeras, DKO T cells were excluded from activation, as indicated by the predominantly naive phenotype (Supplementary Fig. 2f), showing that the rampant T cell expansion in DKO mice was caused by the lack of CTLA-4 on Treg cells.

Despite defective Treg cell function and lymphoproliferation, DKO mice did not exhibit autoimmune infiltration into the heart, lung, liver or kidney, as determined by histological analysis (Fig. 3a). The few T cells present in tissues of older DKO mice did not exhibit differences in cell survival as compared to those in 3-week-old 

Ctla4−/− mice (Supplementary Fig. 2b). Reduced infiltration of DKO T cells into tissues was a cell-intrinsic defect, as reconstitution of 

Ctla4−/− T cells caused lethal autoimmunity (Fig. 3b). Pertussis toxin (PTx) facilitates the infiltration of autoreactive T cells into brain tissue and the induction of experimental autoimmune encephalitis in mice.

Treatment of DKO mice with PTx led to a rapid wasting disease, with death starting at 1 month after treatment (data not shown). Consistent with this, PTx administration increased lymphocytic infiltrates into organs and induced IL-2 and IL-17 production by CD4+ T cells (Fig. 3c). Notably, DKO mice infected with the A/PR8 strain of influenza A or lymphocytic choriomeningitis virus (LCMV) Armstrong viruses were able to mount effective antiviral T cell responses in tissues and clear infections similar to controls (Supplementary Fig. 2g,h). These results indicate that DKO T cells are not irreversibly excluded from tissues and that ITK appears to license only aberrantly activated self-reactive T cells to accumulate in tissues.

Chemokine responses and tissue homing of DKO T cells

Gene expression and flow cytometry assays revealed no substantial differences in the expression of adhesion molecules and most chemokine
receptors between CItla4−/− and DKO T cells (Supplementary Fig. 3a,b). RNA analysis showed similar expression of the sphingosine 1-phosphate (SIP) receptor, which is important for LN egress, and comparable migration to S1P ligand by CItla4−/− and DKO T cells (Supplementary Fig. 3c). Increased numbers of CD4+ T cells in blood of DKO mice further indicated that DKO T cells can exit the LNs (Supplementary Fig. 3d). Expression of CXCR3 was decreased (ref. 42) on activated DKO CD4+ T cells, and these cells failed to migrate towards CXCL-11 in vitro (Supplementary Fig. 4a,b). However, although Cxcr3−/−; CItla4−/− mice displayed a small but significant (P = 0.015) extension in lifespan relative to CItla4−/− mice, lymphocytic infiltrates were still evident in most organs (Supplementary Fig. 4c,d). Thus, reduced expression of CXCR3 on DKO CD4+ T cells probably contributes to, but cannot fully account for, the paucity of DKO T cells in tissues.

To determine whether DKO T cells are selectively impaired in migration to tissues, we performed a competitive short-term homing assay of DKO and CItla4−/− T cells in Rag1−/− mice. At 6–8 h after transfer, the cells remained undivided, and there was no difference in the expression of the apoptosis marker annexin V on T cells in tissues (Supplementary Fig. 4e). In the lungs and liver, the normalized ratios of DKO to CItla4−/− CD4+ and DKO to CItla4−/− CD8+ T cells (homing index, HI) were <1.0 (Fig. 4a and Supplementary Fig. 4e), indicating a considerable advantage of CItla4−/− T cells in repopulating these tissues. Reciprocally, an HI of >1.5 in the LNs indicated an enhanced accumulation of DKO T cells in lymphoid tissues. The difference was observed regardless of initial input ratios ranging from 2:1 to 1:4 of CItla4−/− to DKO cells (data not shown). These results indicate that in this short time frame, DKO T cells do not migrate to nonlymphoid tissues as efficiently as CItla4−/− T cells.

**DKO T cells are defective in transendothelial migration**

Morphological changes enable T cells to traffic across ECs from the blood to tissues43. Activated DKO CD4+ T cells had impaired F-actin polarization relative to CItla4−/− T cells ex vivo (Fig. 4b and Supplementary Fig. 3e). Unlike CItla4−/− T cells, fewer DKO CD4+ T cells were able to migrate across an EC layer in vitro (Fig. 4c). Pretreatment of DKO T cells with the Ca2+ ionophore ionomycin, which can partly substitute for CD28 signaling, increased the frequency of DKO T cells that were able to migrate across the endothelium (Supplementary Fig. 3f). Similarly, migration of CItla4−/− T cells across ECs in vitro was blocked by an ITK inhibitor44 (Supplementary Fig. 3g).

Two-photon imaging of DKO lymphocytes in lung slices showed that, in contrast to CItla4−/− T cells, DKO T cells exhibited random movement within blood vessels in lung tissue and were morphologically distinct (Fig. 4d–g and Supplementary Videos 3 and 4). The migratory properties of DKO cells were similar to those of CItla4−/− T cells in B7-deficient lung slices (Fig. 1), suggesting that impaired CD28 signaling in DKO T cells may be responsible for the observed defects.

**ITK inhibitors moderate autoimmunity**

To examine whether pharmacological inhibition of ITK might attenuate autoimmune disease pathogenesis, we treated CItla4−/−
mice with two ITK inhibitors, BMS509744 (ref. 45) and 10n (ref. 46), and found that they significantly increased their lifespan (Fig. 5a). Inhibition of ITK did not alter the activation state or proliferative capacity of Cita4−/− T cells (Supplementary Fig. 5a) but increased the cellularity of the LNs (Supplementary Fig. 5b), which is consistent with earlier genetic studies (Fig. 2b,c). However, despite the increased longevity, lymphocytic infiltration was observed in most tissues (data not shown). This result was not unexpected given the relatively poor pharmacokinetics of the ITK inhibitors combined with the rapid-onset destructive disease in Cita4−/− mice.

We next tested the ability of ITK inhibitors to prevent beta islet infiltration in NOD mice. Administration of 10n to female NOD mice caused an increase in the cellularity of the LNs (Supplementary Fig. 5c). 10n treatment reduced the migration of self-reactive T cells into beta islets of NOD mice by 50% (Fig. 5b,c). Given the large amount of 10n required for long-term studies in NOD mice, we instead chose to examine whether 10n could block diabetogenic BDC2.5-NOO CD4+T cells from causing islet cell destruction. Transfer of BDC2.5-NOO CD4+CD25− T cells into young NOD–severe combined immunodeficient (SCID) mice followed by 10n treatment at 1 mg per kg body weight. Insulitis index (d) and frequency (e) of diabetic mice at 14 days after transfer of T cells. Data are from three experiments with 10 and 5 control and 10n-treated mice, respectively. Data are expressed as means ± s.e.m.

DISCUSSION

CTLA-4 and CD28 are critical for regulating autoimmunity, such as that seen in T1D, but are often dispensable for responses against foreign pathogens. 12,21,48,49. Most in vivo studies support the role of ligand competition as the primary mode of CTLA-4 inhibition of CD28 signals.33,50–53. We show here that CD28-B7 interactions and ITK regulate the trafficking of self-reactive T cells to tissues but are not essential for pathogen clearance.54. Given the established biochemical connection between CD28 and ITK, these data support the model that one conventional T cell–intrinsic function of CTLA-4 is to modulate the CD28–ITK pathway controlling self-reactive T cell motility in tissues.

We showed that ITK inhibitors are effective in treating T1D in mice. Co-stimulatory blockade is a major therapeutic strategy for autoimmune diseases such as rheumatoid arthritis and T1D. Abatacept, a CD28 antagonist, has been used with moderate success in patients with T1D, leading to the preservation of beta cell mass for >2 years.55. However, this approach ultimately fails owing to the increased antigen sensitivity of autoreactive T cells and their relative independence from co-stimulation for their activation. Another major drawback is the failure to specifically target auto-reactive T cells, as the B7 blockade can interfere with Treg cell function.13 In contrast, focused approaches aimed at regulating self-reactive T cell migration to organs to limit immune pathology are beneficial in treating severe autoimmunity.56, ITK has been shown to function similarly in human and mouse T cells.45,57,58. Our data suggest that ITK inhibitors could be used as an alternate strategy to treat diverse human T cell–mediated organ-specific autoimmune diseases without affecting pathogen-elicted immune responses.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. Microarray data were deposited in the Gene Expression Omnibus with accession code GSE51099.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS

We thank E. Huseby, B. Seed and R. Friedline for discussion, S. Turley for advice on stromal cells, T. Hunig (University of Wurzburg) for the SACD28 antibody, M. Coles for assistance with microscopy, M. Krummel for advice on imaging, D. Serreze for studies with diabetogenic CD8+ T cells, E. Huseby (University of Massachusetts Medical School) for MHC class II–deficient Rag2−/− mice and R. Welsh for the LCMV infection protocol. Core resources supported by the University of Massachusetts Medical School Diabetes Endocrinology Research Center grant DK32520 were used. This work was supported by US National Institutes of Health (NIH) grants to D.L.G. (AI46629, AI050864), S.L.S. (AI046530), L.J.B. (AI083505) and J.K. (RC1 DK086474 and AI083505). US NIH Chemical Genomics Center was supported by the Molecular Libraries Initiative and the Intramural Research Program of the NIH National Human Genome Research Institute.

AUTHOR CONTRIBUTIONS

N.J., L.J.B. and J.K. designed experiments, N.J. and B.M. performed experiments and analyzed data, K.K.M. and S.L.S. conducted influenza infection studies, A.P. performed LCMV infections, J.J. and C.I.T. prepared ITK inhibitors, D.L.G. provided reagents for T1D experiments, M.J.S. collaborated on fluorescence microscopy, N.J. and J.K. wrote the manuscript and J.K. and L.J.B. supervised the project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Bour-Jordan, H. et al. Intrinsic and extrinsic control of peripheral T-cell tolerance by costimulatory molecules of the CD28/ B7 family. Immunol. Rev. 241, 180–205 (2011).

2. Harding, F.A., McArthur, J.G., Gross, J.A., Raulet, D.H. & Allison, J.P. CD28 mediated signalling costimulates murine T cells and prevents the induction of anergy in T cell clones. Nature 356, 607–609 (1992).
Lenschow, D.J. et al. CD28/B7 regulation of TH1 and TH2 subsets in the development and function of autoimmune diabetes. Immunity 5, 285–293 (1996).

Salomon, B. et al. B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. Immunity 12, 431–440 (2000).

Chang, T.T., Jabs, C., Sobel, R.A., Kuchroo, V.K. & Sharpe, A.H. Studies in B7−/− deficient mice is mediated by costimulation-dependent activation of CD4+ T cells. J. Immunol. 161, 1193–1197 (1998).

Ise, W. et al. CTLA4 gene polymorphism and CD28 costimulation can promote T cell survival by enhancing CD80/CD86 expression. J. Immunol. 164, 595–599 (2000).

Collins, A.V. et al. Selective Itk inhibitors block T-cell activation and murine lung inflammation. Proc. Natl. Acad. Sci. USA 105, 6684–6689 (2008).

Peterson, J.D. & Haskins, K. Transfer of diabetes in the NOD-scid mouse by CD4 T-cell clones. Differential requirement for CD8 T-cells. Diabetes 45, 328–336 (1996).

9. Ledgerwood, L.G. et al. The sphingosine 1-phosphate receptor 1 causes tissue retention by inhibiting the entry of peripheral T lymphocytes into effenter lymphatics. Nat. Immunol. 9, 42–53 (2008).

10. Cahalan, M.D. & Parker, I. Choreography of cell motility and interaction dynamics imaged by two-photon microscopy in lymphoid organs. Annu. Rev. Immunol. 26, 585–626 (2008).

11. Sanderson, M.J. Exploring lung physiology in health and disease with lung slices. Pulm. Pharmacol. Ther. 24, 452–465 (2011).

12. Dennybny, K.M. et al. Cutting edge: monovality of CD28 maintains the antigen dependence of T cell costimulatory responses. J. Immunol. 176, 5725–5729 (2006).

13. Governan, J., Brabb, T., Paez, A., Harrington, C. & von Dassow, P. Initiation and regulation of CNS autoimmunity. Crit. Rev. Immunol. 17, 469–480 (1997).

14. Liu, L., Callahan, M.K., Huang, D. & Ransohoff, R.M. Chemokine receptor CXCR3: an unexpected enigma. Curr. Top. Dev. Biol. 68, 149–181 (2005).

15. Burkhardt, J.K., Carrizosa, E. & Shaffer, M.H. The actin cytoskeleton in T cell migration. Annu. Rev. Immunol. 19, 655–695 (2001).

16. Readinger, J.A. et al. Selective targeting of ITK blocks multiple steps of HIV replication. Proc. Natl. Acad. Sci. USA 105, 6684–6689 (2008).

17. Biencourt, J.J., Kuo, P. & Sato, K. Physiologic and aberrant regulation of memory T-cell trafficking. Annu. Rev. Immunol. 26, 267–273 (2008).

18. Kim, I.S., Zlotnik, A. & Szallasi, Z. CCR7 regulates trafficking of memory T cells to lymph nodes. J. Immunol. 176, 935–945 (2006).

19. Schaller, M.G. et al. An unexpected enigma. Curr. Top. Dev. Biol. 68, 149–181 (2005).

20. Sanderson, M.J. Exploring lung physiology in health and disease with lung slices. Pulm. Pharmacol. Ther. 24, 452–465 (2011).

21. Dennybny, K.M. et al. Cutting edge: monovality of CD28 maintains the antigen dependence of T cell costimulatory responses. J. Immunol. 176, 5725–5729 (2006).

22. Governan, J., Brabb, T., Paez, A., Harrington, C. & von Dassow, P. Initiation and regulation of CNS autoimmunity. Crit. Rev. Immunol. 17, 469–480 (1997).

23. Liu, L., Callahan, M.K., Huang, D. & Ransohoff, R.M. Chemokine receptor CXCR3: an unexpected enigma. Curr. Top. Dev. Biol. 68, 149–181 (2005).

24. Burkhardt, J.K., Carrizosa, E. & Shaffer, M.H. The actin cytoskeleton in T cell migration. Annu. Rev. Immunol. 19, 655–695 (2001).

25. Readinger, J.A. et al. Selective targeting of ITK blocks multiple steps of HIV replication. Proc. Natl. Acad. Sci. USA 105, 6684–6689 (2008).

26. Biencourt, J.J., Kuo, P. & Sato, K. Physiologic and aberrant regulation of memory T-cell trafficking. Annu. Rev. Immunol. 26, 267–273 (2008).

27. Kim, I.S., Zlotnik, A. & Szallasi, Z. CCR7 regulates trafficking of memory T cells to lymph nodes. J. Immunol. 176, 935–945 (2006).

28. Schaller, M.G. et al. An unexpected enigma. Curr. Top. Dev. Biol. 68, 149–181 (2005).
ONLINE METHODS

Mice. *Itk*Δ*+/−* mice were crossed with CIta4Δ*+/−* (DKO) mice on the C57BL6 background. Female NOD, NOD-SCID, Cxcr3*−/* and Cd80*−/*;Cd86*−/* (B7-deficient) mice were purchased from Jackson Laboratories (Bar Harbor, ME). BDC2.S/NOD mice were bred in our animal facility. Cd80*−/*;Cd86*−/*;Rag1*−/* and Sc5.C7 Itk*Δ*+/−*;CIta4Δ*+/−*;Rag1*−/* mice were generated in our colony. Rag1*−/*;H-2a-K*−/*;Cd74*−/* (lacking MHC II 1-A and 1-E); mice that lack MHC class II were provided by E. Huseby. All experiments were approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee.

Reagents, antibodies, flow cytometry and histology. Pertussis toxin was from List Biological Laboratories, Campbell CA; ITK inhibitors, BMS507944 and 10n were synthesized at the NIH’s Chemical Genomics Center and dissolved in 70% (20% wt/vol) 2-hydroxypropyl-β-cyclodextrin in water and 30% PEG300 (EMD Millipore), CD28 superagonist antibody (SACD28, clone D665; EMD Millipore) and recombinant IL-4 (2 ng ml−1, R&D Systems) were pre-incubated with neurovirulent A/PR8 strain (H1N1) of influenza virus in 50 µl of PBS. Mice were weighed every day and euthanized at day 18 after infection. Virus titers were determined at days 7 and 14 after infection by quantifying viral RNA as previously described38.

Gene expression profiling. CD4*+CD25* T cells from 3-week-old male CIta4Δ*+/−* or DKO mice were sorted in duplicates, and RNA was extracted using TRIzol reagent and microarray analysis performed with the Affymetrix MoGene 1.0 ST array based on the ImmGen protocol (http://www.immgen.org/Protocols/Total RNA Extraction with Trizol.pdf). Data were analyzed with modules of the GenePattern genomic analysis platform of the Broad Institute (http://www.broadinstitute.org/cancer/software/genepattern/). Differences in gene expression were identified by the multiplet module (coefficient of variation <0.5; P ≤ 0.05, Student’s t-test; expressed genes defined as those with mean expression value > 120 in at least one sample, 95% confidence interval, based on Immgen.org data processing of the MoGene 1.0 ST arrays), and functional categorization was performed using the Functional Annotation Tool DAVID for GO annotations. Heat maps were generated by row (gene)-based hierarchical clustering (pairwise complete linkage) of data using the hierarchical clustering module. Data were log-transformed and row-centered (subtraction from the mean), and a relative color-scale based map was generated using the HierarchicalClusteringViewer module.

Confocal microscopy and immunofluorescence. 2 × 10⁶ MACS-enriched CD4*+ T cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% saponin, and stained for actin with Alexa Fluor 488 phalloidin (5 units, Invitrogen). Images were captured using a Nikon Eclipse E 600 microscope and IPLab Spectrum software (Scanalytics, VA). At least 15 fields were photographed, and no fewer than 100 cells per sample were analyzed for each experiment. For confocal microscopy, CD4⁺ T cells were photographed using a Leica confocal microscope.

Preparation of lung slices for microscopy. The procedure was performed as described39 with some modifications. Briefly, mice were i.v. injected with 50 µM of CMTMR Orange dye (Invitrogen). After 30 min, 20 × 10⁶ to 50 × 10⁶ CFSE (Invitrogen)-labeled LN cells were i.v. injected and mice euthanized 5 min later. The trachea was cannulated, and lungs were inflated with 0.9–1.3 ml of 1.8% low-melting-point agarose in HBSSB (HBSS + 20 mM HEPES, pH 7.4). The agarose was gelled, and a lung lobe was cut into 200-µm thick serial sections. Lung slices were adhered to a glass-bottom dish (In Vitro Scientific) by serial additions of a thin film of 2% agarose and bathed in 2 ml of phenol red–free DMEM containing HEPES and 10% PBS. Microscopy was performed at 35 °C.

Fluorescence microscopy. Fluorescence imaging was performed using a custom-built two-photon or confocal microscope using a 40× or 60× objective.
lens with numerical apertures of 1.35 or 1.42, respectively. Cells were excited at 820 nm for two-photon microscopy and at 488 nm and 543 nm for confocal microscopy. Simultaneous fluorescence images were collected by separating the emitted fluorescence light with a 540-nm dichroic mirror in conjunction with a red (590-nm) and green (510-nm) barrier filter (Semrock). No substantial differences in the data were observed between the two microscopes. To follow lymphocyte motility, a time-lapse 2D image sequence was acquired by averaging 16 images (480 × 800 pixels at 15 images per second) every 5 s using Video Savant software (IO Industries). Each average image had dimensions of 240 µm × 288 µm with a pixel resolution of 0.5 × 0.36 µm using a 40× objective lens. Data was analyzed using both Video Savant and ImageJ (NIH) software. Various descriptors of lymphocyte motility (displacement, roundness) were calculated using ImageJ software.

Diabetes induction and calculation of insulitis index. 5-week-old female NOD mice were treated with ITK inhibitor as indicated, and the extent of insulitis was determined by histopathological analysis of pancreatic sections. For T cell transfer experiments, 1 × 10^6 sorted CD4+CD25− conventional T cells from 4-week-old female BDC2.5-NOD TCR transgenic mice were i.v. injected into 4-week-old female NOD-SCID mice. Mice were considered diabetic if they had three consecutive blood glucose readings of >250 mg dL−1. Insulitis index was calculated by scoring the islets as grade 0 (no infiltration), grade 1 (peri-insulitis only), grade 2 (<20% of islet mass infiltrated), grade 3 (75% of islet mass infiltrated) or grade 4 (<20% of islet mass remaining), as determined by insulin immunohistochemistry. Insulitis index was calculated using the following formula: \[I = (0 \times N0) + (1 \times N1) + (2 \times N2) + (3 \times N3) + (4 \times N4)/(N0 + N1 + N2 + N3 + N4),\] where N0, N1, N2, N3 and N4 are the number of islets showing grade 0, 1, 2, 3 and 4 pathology, respectively.

ITK inhibitor characterization and purification. For 10n: ^1^H NMR (400 MHz, DMSO-d6) δ 13.14–13.00 (br.s., 1H), 8.24–8.11 (br.s., 1H), 7.92–7.79 (br.s., 1H), 7.63 (d, J = 3.8 Hz, 1H), 7.52 (s, 1H), 7.49 (d, J = 8.4 Hz, 1H), 7.22 (d, J = 3.8 Hz, 1H), 7.20 (dd, J = 8.4, 1.0 Hz, 1H), 5.03 (s, 1H), 4.14 (s, 2H), 3.91 (d, J = 12.7 Hz, 1H); 3.66 (d, J = 12.7 Hz, 1H), 2.20 (q, J = 6.4 Hz, 1H), 1.23 (s, 6H), 0.95 (d, J = 6.4 Hz, 3H), 0.86 (s, 9H). ^13^C NMR (100 MHz, DMSO-d6) δ 168.4, 152.2, 140.8, 140.0, 136.1, 130.5, 129.2, 128.7, 125.8, 122.7, 122.3, 115.2, 111.0, 110.7, 70.8, 60.0, 52.5, 51.4, 34.0, 27.5, 26.2, and 14.0; liquid chromatography–mass spectrometry retention time 3.930 min (gradient: 4% to 100% acetonitrile (0.05% TFA) over 7 min); high-resolution mass spectrometry m/z (M + H+) = 495.2538 (calculated for C_{26}H_{35}N_{6}O_{2}S = 495.2542).

Statistical analyses. Sample size for in vivo studies including inhibitor treatment and diabetes induction were estimated by conducting pilot experiments. No samples or animals were excluded from the analyses. For animal studies, no randomization and blinding were used. Data were analyzed using Prism statistical software. Normally and non-normally distributed data were analyzed for significance by Student’s t-test and Mann-Whitney test, respectively. The variance was similar between groups being compared. Standard error and P values are shown on individual graphs.

59. McCausland, M.M. & Crotty, S. Quantitative PCR technique for detecting lymphocytic choriomeningitis virus in vivo. J. Virol. Methods 147, 167–176 (2008).
60. McKinstry, K.K. et al. IL-10 deficiency unleashes an influenza-specific T_h17 response and enhances survival against high-dose challenge. J. Immunol. 182, 7353–7363 (2009).