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Innate Immune Cell CD45 Regulates Lymphopenia-Induced T Cell Proliferation

Amy E. Saunders,1 Yaein A. Shim, and Pauline Johnson

The leukocyte-specific tyrosine phosphatase, CD45, severely impacts T cell development and activation by modulating TCR signaling. CD45-deficient (CD45KO) mice have reduced peripheral T cell numbers where CD8 T cells are underrepresented. In this article, we show that CD45KO mice are unable to support efficient homeostatic proliferation, affecting CD8 T cells more than CD4 T cells. Using CD45-RAG1 double-deficient (45RAGKO) mice, we show that lymphopenia-induced proliferation (LIP) of CD45-sufficient T cells is defective in a host environment lacking CD45 on innate immune cells. We identify two deficiencies in the 45RAGKO mice that affect LIP. One involves CD11c+ cells and the second the production of IL-7 by lymphoid stromal cells. CD45KO dendritic cells were not defective in foreign Ag–induced T cell proliferation, yet CD45KO CD11c+ cells were unable to rescue the spontaneous LIP in the 45RAGKO mice. This was in contrast with the CD45-sufficient CD11c+ cells that partially rescued this spontaneous proliferation and did so without affecting IL-7 levels. The absence of CD45 also led to reduced IL-7 production by lymphoid stromal cells, suggesting an indirect effect of CD45 on innate immune cells in influencing IL-7 production by lymphoid stromal cells. These findings demonstrate a novel role for CD45 on innate immune cells in promoting lymphopenia-induced T cell proliferation and suggest that innate immune cells may communicate with stromal cells to regulate IL-7 production. The Journal of Immunology, 2014, 193: 000–000.

T cells are maintained by survival signals such as cytokines and tonic TCR signaling stimulated by interactions with MHC molecules on dendritic cells (DCs) (1). The maintenance of both the peripheral naive and memory T cell pool is regulated by complex homeostatic mechanisms involving cytokine and, for naive T cells, TCR signaling (reviewed in Refs. 2–5). There is a drive to maintain a constant number of T cells in an organism that is demonstrated by lymphopenia-induced proliferation (LIP) that occurs when T cell numbers are reduced. This phenomenon occurs naturally in neonates and in patients on immunosuppressant drugs or infected with viruses that deplete T cells. It can also be observed experimentally when T cells are transferred into hosts lacking T cells either chronically because of genetic abnormalities or acutely because of irradiation. This mechanism of peripheral expansion is key to the homeostasis of T cells and if it becomes dysregulated, this renders the organism susceptible to infection or autoimmunity. T cell LIP has been shown to be dependent on two main mechanisms. The first leads to a population of cells that divide slowly in response to common γ-chain cytokines such as IL-7 and IL-15, which does not change their T cell phenotype. This is referred to as homeostatic or slow proliferation, because it is thought to occur as a “top-up” mechanism in normal recipients with slightly reduced T cell numbers (6). The second type of LIP is thought to be more dependent on TCR–Ag–MHC interactions and leads to the rapid division of T cells and the upregulation of activation markers such as CD44. This fast proliferation is referred to as spontaneous proliferation and is similar to cognate Ag–induced proliferation (6), except that the peptide Ags involved in this signal are thought to be either self- or commensal-derived as the absence of the microbiota in germ-free mice virtually abolished the spontaneous proliferation of transgenic CD4 T cells (7). Additional evidence for the involvement of peptide-loaded MHC is that the complexity of the TCR repertoire influences the extent of proliferation, with only polyclonal T cells being able to inhibit the proliferation of T cells in lymphopenic hosts (8, 9). There are many similarities between the signals leading to T cell activation and those leading to LIP except for the involvement of costimulation. The CD40–CD40L or 4.1BB–4.1BBL signaling pathways are not required for LIP, and the involvement of CD28–B7 interactions remains unclear (10, 11).

There has been a long-standing correlation between LIP and autoimmunity, which is now known to be due, at least in some cases, to the expansion of autoimmunity T cell subsets under lymphopenic conditions (12). CD8 T cells that undergo spontaneous LIP take on a memory-like phenotype with the upregulation of CD44, CD122, and Ly6C, gain the ability to produce IFN-γ, and have cytotoxic function in response to cognate Ag (13). This phenotype resembles that of central memory T cells in terms of cell-surface marker expression, but more closely resembles effector memory T cells with regard to their ability to produce cytokines and carry out a cytotoxic function. The differences in the signals that drive this spontaneous, Ag-driven T cell proliferation are not well understood, and a better understanding of the signals that regulate LIP would help in further identifying the link between lymphopenia and autoimmunity.

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Abbreviations used in this article: BMDC, bone marrow–derived DC; CD45KO, CD45-deficient; DC, dendritic cell; FRC, fibroelastic reticular cell; LIP, lymphopenia-induced proliferation; mLN, mesenteric lymph node; poly(I:C), polyinosinic-polycytidylic acid; QPCR, quantitative PCR; 45RAGKO, CD45-RAG1 double-deficient; WT, wild-type.

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CD45 is a leukocyte-specific transmembrane protein tyrosine phosphatase that primarily dephosphorylates specific members of the Src family of tyrosine kinases. CD45 plays a key role in regulating Ag receptor signaling in lymphocytes where it helps to maintain a pool of dephosphorylated Src family kinases (Lck in T cells, and Lyn and Fyn in B cells) primed for TCR or BCR signaling and activation. Thus, CD45 lowers the threshold required for Ag-induced proliferation. The function of CD45 to positively regulate TCR and pre-TCR signaling results in CD45-deficient (CD45KO) mice having a block in T cell development that leads to reduced numbers of mature T cells and peripheral T cell lymphopenia. In B cells, CD45 can have both positive and negative effects on B cell activation, because the Src family kinase, Lyn, associates with both positive and negative B cell regulatory molecules. CD45KO mice have increased numbers of B cells, but they have an immature phenotype. In other leukocytes such as mast cells, macrophages, and DCs, CD45 regulates adhesion, cytokine signaling, and TLR-mediated cytokine secretion (reviewed in Ref. 14).

In this report, we identified a skew in the peripheral CD4+CD8- T cell ratio in CD45KO mice. Upon further investigation, we found that CD45KO mice were defective in their ability to support LIP. Using RAG1-deficient and CD45−/−RAG1−/− double-deficient (45RAGKO) mice, we found that this was due to a deficiency of CD45 on innate immune cells, not T cells. Splenic CD11c+ cells promoted spontaneous proliferation, and this required CD45. Inactive cell CD45 was also required for optimal IL-7 production from lymphoid stromal cells, indicating an indirect effect of CD45 on IL-7 production in lymphoid organs.

Materials and Methods

Mice

C57BL/6, CD45KO Exon 9 (15), RAG1KO, OT-I, OT-II (both on the C57BL/6 background), and BoyJ (B6.SJL CD45.1) mice were obtained from The Jackson Laboratory. The CD45KO mice were backcrossed for nine generations onto the C57BL/6 background. 45RAGKO mice were generated by crossing CD45KO mice with RAG1KO mice to homozygosity. For CD45KO and wild-type (WT) mice being compared. The cells account for the larger size of spleens from 45RAGKO mice compared with CD45KO.

Bone marrow–derived DCs (BMDCs) were generated as previously described (16). In brief, the spleens were prepared as published previously (17). Intracellular staining for Bcl-2 was checked by flow cytometry and was around 95% for CD45.1 and OT-I T cells, or CD8 (53-6.7) for OT-II T cells. All of these Abs were obtained from the Biomedical Research Centre Antibody Facility. The labeled cells were separated from unlabeled cells using anti-biotin microbeads and LS MACS columns (Miltenyi Biotec). The purity of the cells was checked by flow cytometry and was around 95% for CD45.1 and OT-I T cells, and around 85% for OT-II T cells and CD45KO T cells. The T cells were labeled with 10 μM CFSE (Invitrogen Cell Probes) in RPMI 1640 at 37˚C for 12 min. For T cell LIP in RAGKO, CD45KO, or 45RAGKO mice, 1–5 × 10^6 CD45.1 T cells in PBS were injected per mouse i.v. into the tail vein and 3–12 × 10^6 T cells were injected when CD45KO and wild-type (WT) T cells were being compared. The cells were allowed to proliferate in vivo for 3–14 d.

Quantification of cytokines by ELISA

Mice were euthanized, and spleens were removed and weighed. Spleens were prepared as described previously (16). In brief, the spleens were chopped finely and incubated in 125 μL PBS containing 1000 U Collagenase VIII (Sigma) at 55˚C for 90 min. The debris was removed by centrifugation at 15,000 g for 10 min, and the supernatant was retained and used directly for the ELISAs. ELISA kits used were the R&D Systems mouse IL-7 Quantikine Immunoassay and the eBioscience Ready-SET-Go mouse IL-15/IL-15ra ELISA. The manufacturer’s instructions were followed. The cytokine levels were shown per milligram of spleen to account for the larger size of spleens from 45RAGKO mice compared with RAGKO mice.

Stromal cell isolation

Stromal cell isolation was done as described previously (17). In brief, inguinal, axillary, and brachial lymph nodes were punctured with a needle and were digested for 20 min at 37˚C with 2 ml digest mix consisting of RPMI 1640 with 0.2 mg/ml Collagenase VIII (Sigma), 0.6 mg/ml Dispase (Innitrogen), 0.1 mg/ml DNase I (Roche). The suspension was vigorously mixed and then left for large particles to settle. The supernatant was removed and washed, and the sedimented material was digested with fresh digest mix for a further 15 min. The digest was repeated a further time and the cells were pooled. Stromal cells were enriched using a biotinylated gp38 Ab (8.1.1.1; Biolegend), anti-biotin microbeads, and MS magnetic columns (Miltenyi Biotec). The cells were stained with biotinylated gp38 Ab and PE-Cy7-conjugated streptavidin, as well as a lineage mix consisting of CD11c (N418) from the Biomedical Research Centre and CD45 (GK1.5), CD8 (53-6.7), NK.1.1 (PK136), CD11b (M1/ 71), and streptavidin-conjugated PE/Cy7 from eBioscience; CD45.1 (A20) from BD Bioscience; and biotinylated gp38 (8.1.1) from Biolegend. The cells were stained with the viability dye DAPI at 25 ng/ml before running on an LSRII Flow cytometer (BD Bioscience). Data were analyzed using FlowJo (Tree Star) software.

Generation of bone marrow–derived DCs

Bone marrow–derived DCs (BMDCs) were generated as previously described (18). Cells were isolated from the femurs and tibias of mice, and plated in petri dishes at 2 × 10^6 cells/ml in medium (RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM L-glutamine, nonessential amino acids, 20 mM HEPES, sodium pyruvate, penicillin/streptomycin, and 50 μM 2-ME (all obtained from Invitrogen)) supplemented with 4% (v/v) of GM-CSF–containing J558L supernatant. On days 3 and 6, half of the culture was replaced, spun down, and the cells were replaced along with fresh medium and GM-CSF. Cells were harvested for use on day 8 and were purified using anti-CD11c microbeads and LS magnetic columns (Miltenyi Biotec).
Isolation of CD11c<sup>+</sup> cells and splenic DCs

Spleens were digested with 2 ml RPMI containing 1 mg/ml Collagenase VIII (Sigma) for 30 min at 37°C. The cells were passed through 70-μm cell strainers, and the RBCs were lysed with 0.84% ammonium chloride, 2 mM Tris-HCl pH 7.2 at room temperature for 5 min. The CD11c cells were enriched for using anti-CD11c microbeads and LS magnetic columns (Miltenyi Biotec). The enriched cells (typically ≥70% CD11c<sup>+</sup>) were used for the T cell and CD11c<sup>+</sup> cell coinjection experiments, where 0.3–3 × 10<sup>6</sup> CD11c<sup>+</sup> enriched cells were isolated from WT or CD45KO mice and injected into 45RAGKO mice with CD45<sup>+</sup> T cells. For a more purified splenic DC population, the cells were stained with Abs to PDCA-1, MHC class II (M5/114.15.2) from eBioscience and CD11c (N418) from the Biomedical Research Centre, and sorted for the PDCA-1<sup>+</sup> MHC class II<sup>high</sup> CD11c<sup>high</sup> population on an Aria cell sorter (BD Bioscience).

Quantitative PCR

RNA was extracted using TRizol (Invitrogen) in accordance with the manufacturer’s instructions. Cells were isolated as described earlier and were resuspended in 800 μl TRizol. A 160-μl aliquot of chloroform was added and the tubes were shaken vigorously. The phases were separated by centrifugation at 12,000 × g, 4°C for 15 min. The aqueous phase was transferred to a clean tube, and 400 μl isopropanol was added to precipitate the RNA. The tubes were inverted, incubated at room temperature for 10 min, and then were centrifuged at 12,000 × g, 4°C for 10 min. The RNA was washed with 75% ethanol and then air-dried. The RNA was resuspended in RNase free H<sub>2</sub>O and was treated with DNase I (Invitrogen) following the manufacturers’ instructions. The RNA was used to synthesize cDNA using the iScript cDNA synthesis kit (Bio-Rad) following the manufacturers’ instructions. For the IL-7 quantitative PCRs (QPCRs), the cDNA was purified from any unused primers using the QIAquick PCR purification kit (Qiagen).

QPCRs were set up using SSOFast EvaGreen Super mix (Bio-Rad) with 400 nM of each primer and were run on a Bio-Rad CFX96 machine. The primers used were as follows: GAPDH: Forward 5′-CACACAGTCTCAGCATCAC-3′, Reverse 5′-CACACCCCTGTGCTAGGCC-3′ (19); IL-7: Forward 5′-GGTCCATGAAATCTTCTGC-3′, Reverse 5′-TTACTACT-3′ (20); IL-15: Forward 5′-GGACATGGCTGCTGACAAGA-3′, Reverse 5′-TTCTCTCGTGTTTTCTCC-3′; IL-15Rα: Forward 5′-GCCATCTCTACTGCTTTCT-3′, Reverse 5′-TTCTTCTCTCTCTGGTCTG-3′ (designed in house using primer3).

Data were analyzed using the following formula: Fold change = 2<sup>ΔΔCt</sup>, where ΔCt is the number of cycles at which a threshold quantity of amplicon DNA is generated. ΔCt = Ct<sub>sample</sub> - Ct<sub>reference (WT1)</sub>.

In vitro T cell activation

Splenic DCs were isolated from C57BL/6 and CD45KO mice as detailed earlier using anti-CD11c microbeads and were plated in 96-well round-bottomed plates at 0.5 × 10<sup>4</sup> cells/well. The cells were loaded with OVA protein (100 μg/ml; Sigma), or peptides SIINFEKL (50 μg/ml) or OT-II peptide (1 μg/ml), and were left unstimulated or were stimulated with 200 ng/ml ultrapure LPS overnight. The supernatants were removed and the DCs were resuspended with 5 × 10<sup>5</sup> cells/well purified, CFSE-labeled OT-I or OT-II T cells. The cells were cocultured for 4 (OT-I T cells) or 5 d (OT-II T cells), then analyzed for proliferation on an LSRII flow cytometer.

In vivo T cell activation

Using BMDCs. OT-I or OT-II T cells were isolated and CFSE labeled. The T cells were injected i.p. into CD45.1 mice at 5 × 10<sup>6</sup> cells/mouse. The following day, 1 × 10<sup>5</sup> C57BL/6 or CD45KO BMDCs were injected per mouse i.p. The BMDCs had been loaded with OVA peptides at 1 μg/ml and stimulated with 1 μM CpG (for OT-II activation) or 50 μg/ml polyinosinic-polycytidylic acid (poly(C)) (for OT-I activation) overnight. On day 4 after BMDC injection, the mice were euthanized and spleens were taken and analyzed for OT-I or OT-II T cell proliferation on an LSRII flow cytometer.

Using endogenous DCs. OT-I or OT-II T cells were isolated and CFSE labeled. The T cells were injected i.p. into RAGKO and 45RAGKO mice at 5 × 10<sup>6</sup> cells/mouse. The following day, 250 μg OVA protein (endoGrade containing <1 endotoxin units/mg endotoxin from Hyglos GmbH) and 65 μg OVA ODN 1668 (IDT, for OT-I activation) or 250 μg poly(C) (for OT-II activation; Sigma) was injected per mouse i.p. On day 4 after OVA injection, the mice were euthanized, and spleens were taken and analyzed for OT-I or OT-II T cell proliferation on an LSRII flow cytometer.

Results

Innate immune cell CD45 drives the LIP of T cells

Mice deficient for CD45 were reported by three independent laboratories (15, 22, 23) between 1993 and 1999. In this study, the strain generated by Byth et al. (15) was used in which exon 9 has been disrupted but no CD45 protein of any isofrom is detectable in leukocytes. CD45KO mice have a defect in T cell development because of the essential role of CD45 in TCR signaling. This leads to decreased numbers of single-positive CD8 and CD4 T cells in the thymus and the periphery. At first sight the ratio of single-positive CD4CD8 cells was lower in the CD45KO thymus, but this was reversed when only the TCR<sup>+</sup> CD44<sup>hi</sup> cells were examined (Fig. 1A, 1B). This skewed CD4:CD8 ratio was also observed in the spleen (7.5) and lymph nodes (7.3) in CD45KO mice compared with 1.6 for spleen and 1.3 for lymph nodes in C56BL/6 mice (Fig. 1A, 1B), which had been previously noticed, but not investigated further (15, 22).

To further understand why CD45KO mice have severely reduced numbers of T cells in the periphery, we chose to examine their ability to undergo homeostatic T cell proliferation. To determine whether the CD45KO T cells had an intrinsic defect in homeostatic proliferation, we examined the ability of CD45KO T cells to proliferate by adoptively transferring CFSE-labeled CD45KO T cells into RAGKO mice (which lack both T and B cells). After 7 d, the WT T cells, the transferred CD45KO CD4 and CD8 T cells were capable of proliferating in a CD45-sufficient lymphopenic environment, as evidenced by CFSE dilution (Fig. 1C). This shows that CD45KO T cells do not have a cell-intrinsic defect that prevents their proliferation in the periphery.

To determine whether there was an extrinsic defect in the CD45KO mice in the signals that drive peripheral T cell expansion, we adoptively transferred CFSE-labeled CD45.1 congenic T cells into either CD45KO or RAGKO hosts. There was a reduced percentage of highly proliferated CD8 and CD4 T cells in the spleen, mLNs, and pooled inguinal, axillary, and brachial lymph nodes of the CD45KO hosts compared with RAGKO hosts, with the CD8 T cell proliferation reduced to a greater extent than the CD4 T cells (Fig. 1D–F and data not shown). This is consistent with innate immune cell CD45 having an extrinsic effect on T cell LIP.

Because CD45KO mice do have a small number of endogenous T and B cells that may compete for survival cytokines such as IL-7, these cells may limit the extent of LIP that can occur in the CD45KO mice. To eliminate this variable, we crossed CD45KO mice with RAGKO mice to generate 45RAGKO mice that have no T or B cells and lack CD45 in innate immune cells. After 7 d, the proliferation of adoptively transferred CFSE-labeled WT CD45.1 T cells was compared with RAGKO hosts, with the CD8 T cell proliferation reduced to a greater extent than the CD4 T cells (Fig. 1D–F and data not shown). This is consistent with innate immune cell CD45 having an extrinsic effect on T cell LIP.

Statistics

Data were analyzed using unpaired, two-tailed Student t tests or, for comparison of multiple data sets, ANOVA and Bonferroni post hoc tests were used. A p value < 0.05 was considered significant. In the figures, *p < 0.05, **p < 0.01, ***p < 0.001, and the error bars show the SD of the mean.
(categorized as spontaneous proliferation) in the 45RAGKO hosts was reduced relative to that in the RAGKO hosts (Fig. 2C). There was also a significant reduction in the number of cells that had undergone ≥5 divisions in the spleen of 45RAGKO mice compared with RAGKO mice (Fig. 2D). The same trends were seen in mLNs and pooled inguinal, axillary, and brachial lymph nodes (data not shown). The proportion of cells that had undergone zero to four divisions was significantly higher in the 45RAGKO mice; however, the number of cells in this gate was similar in 45RAGKO and RAGKO hosts, making it unclear whether there was a defect in the slow, homeostatic proliferation of T cells in the 45RAGKO mice. To determine whether there was a defect in this proliferation, the extent to which the cells in the zero to four divisions gate had proliferated was examined more closely. The amount that each cell in the zero to four divisions gate had proliferated was lower in the 45RAGKO mice compared with the RAGKO mice (Fig. 2E), and this was significant when their proliferation index was compared (Fig. 2F). These data show that the CD45KO environment significantly reduces the ability of normal T cells to undergo spontaneous proliferation and, to a lesser extent, homeostatic proliferation.

On examining the relative defect in CD4 and CD8 T cell proliferation in 45RAGKO mice, it was found that the CD45KO environment had a greater effect on the spontaneous CD8 T cell proliferation than the spontaneous CD4 T cell proliferation (Fig. 2G). This supports the conclusion that the defect in innate immune cells lacking CD45 leads to the greater impairment of CD8 LIP compared with CD4 LIP.

Lack of peripheral T cell expansion in a CD45KO environment is not due to increased cell death
The defect in T cell LIP in 45RAGKO mice could be caused by a defect in the signals leading to proliferation, or to reduced survival of the T cells after proliferation is induced. It is also a possibility that the homing signals for the T cells may be affected in the secondary lymphoid organs of 45RAGKO mice. To examine these possibilities, we examined the number of T cells recoverable on day 3 after T cell transfer, which is before much division has occurred. At this time point, the number of T cells recovered from RAGKO and 45RAGKO host spleens was equal (Fig. 3A), showing that the T cells can traffic to the spleen in both environments and...
the 45RAGKO environment does not induce cell death in the un-
divided adoptively transferred T cells, at least in the first 3 d after
adoptive transfer.

Effector T cells are known to undergo apoptosis, which is es-
sential for the contraction and homeostasis of T cells after an
immune response (24). To determine whether the defect in LIP in

FIGURE 2. Innate immune cell CD45 promotes the peripheral expansion of T cells. CFSE-labeled CD45.1 T cells were adoptively transferred via the tail
vein into C57BL/6, RAGKO, or 45RAGKO mice. The mice were euthanized on day 7 after transfer, and spleens and inguinal, brachial, axillary, and mLNs
were removed and analyzed. (A) Representative splenocyte flow-cytometry profiles showing the division of CD45.1 T cells by CFSE after gating on the
CD4 and CD8 populations. Numbers represent the percentage of cells in each population. The gates indicate cells that have divided 0–4 or ≥5 times. (B)
Numbers of T cells recovered from spleen and lymph nodes (mLN, inguinal, axillary, and brachial). (C) Percentage of cells divided ≥5 times. (D) Numbers
of cells recovered per mouse in each population. (E and F) Cells were gated on the population that had divided 0–4 times, then analyzed for proliferation by
CFSE dilution. The filled histogram indicates a C57BL/6 host, solid line indicates RAGKO host, dotted line indicates 45RAGKO host. Proliferation indexes
were calculated using the proliferation platform in FlowJo (TreeStar) software. (G) Difference in the percentage of the highly proliferated cells between
RAGKO and 45RAGKO hosts calculated by subtracting the percentage of T cells that divided ≥5 times in 45RAGKO mice from the average percentage of
T cells that divided ≥5 times in RAGKO mice. Experiments were repeated twice with a total of 9 RAGKO and 10 45RAGKO mice analyzed. For the bar
charts, n = 7–8. *p < 0.05, **p < 0.01, ***p < 0.001.
45RAGKO mice was due to enhanced T cell death after proliferation has occurred, we measured the proportion of dead cells at day 5 using the vital dye DAPI, and apoptosis was measured by Annexin V binding. As shown in Fig. 3B, there was not an increased proportion of dead or apoptotic T cells in the 45RAGKO mice; in fact, the proportion of dead and apoptotic cells T cells was equal or lower in the 45RAGKO mice.

To further examine whether T cells in the 45RAGKO mice were more susceptible to apoptosis, we isolated WT T cells from 45RAGKO and RAGKO mice after being allowed to undergo LIP for 7 d and directly assessed for caspase-3/7 activity. Fig. 3C shows an equal percentage of active caspase-3/7+ cells in the CD4 T cell population and a reduced percentage of active caspase-3/7–expressing cells in the CD8 population isolated from the 45RAGKO mice. These data support the annexin V data to show that the reduced numbers of T cells isolated from the 45RAGKO mice are not due to increased T cell apoptosis.

IL-7 is known to regulate T cell LIP and has been shown to inhibit cell death by causing the upregulation of antiapoptotic members of the Bcl-2 family (25, 26). We therefore examined Bcl-2 expression in these cells by intracellular flow cytometry. As expected, the T cells that downregulated Bcl-2, and therefore became apoptotic, were those that had undergone more than five rounds of division as shown in Fig. 3D. There was no difference in...
the proportion of the T cells that had downregulated Bcl-2 between RAGKO and 45RAGKO host mice (Fig. 3D), further supporting the premise that there is not increased death of the T cells in the 45RAGKO mice. The lack of evidence for increased T cell death in the 45RAGKO mice points to a defect in the signals stimulating T cell LIP.

A CD45KO environment does not affect the onset of T cell LIP

To determine whether there was a difference in the kinetics of T cell division, we examined the proliferation of adoptively transferred, CFSE-labeled CD45.1 T cells in RAGKO and 45RAGKO mice on days 3, 4, 5, 7, and 14. On day 3 after T cell transfer, there was very little proliferation of the T cells in either the RAGKO or 45RAGKO host mice; however, by day 5, T cells were present that had proliferated so extensively that they had lost their CFSE signal (Fig. 4A). This was true for both RAGKO and 45RAGKO hosts; however, by day 5, it was clear that the T cells had proliferated less in the 45RAGKO hosts as shown by the percentage of cells that had undergone spontaneous proliferation (Fig. 4A) and the total number of adoptively transferred T cells that were recovered on day 5 (Fig. 4B). These data show that there is a significant difference in T cell LIP by day 5 that is maintained through day 14, the last day measured. The peak number of T cells recovered from RAGKO mice was on day 7, the same day as for the 45RAGKO hosts. Thus, there is not a delay in the onset of proliferation in the 45RAGKO mice, just a reduction in the numbers of T cells undergoing spontaneous proliferation, which is apparent from day 5 onward.

Splenic CD45+ CD11c+ cells partially rescue spontaneous T cell LIP in 45RAGKO mice

The two factors known to be involved in stimulating LIP are cytokines such as IL-7 and TCR–self-Ag MHC interactions (7, 27). Whether costimulatory molecules play a role in promoting LIP is not clear because one group showed that CD4 T cell LIP is enhanced by CD28 signals (11), whereas another report showed that CD28, CD40, and 4-1BB signals are not required for LIP (10). Ag presentation and costimulation are functions normally associated with DCs, so we sought to determine whether the phenotype of the 45RAGKO mice could be rescued by the adoptive transfer of splenic CD11c+ DCs from C57BL/6 mice. The transfer of CD45+ splenic CD11c+ cells into the 45RAGKO mice significantly increased the number of T cells and the percentage that had undergone spontaneous LIP in both the CD4 and CD8 populations, whereas the adoptive transfer of CD45KO CD11c+ cells did not. The number of CD11c+ cells added varied between experiments.

FIGURE 4. The onset of spontaneous proliferation occurs simultaneously in RAGKO and 45RAGKO hosts, but occurs to a greater extent in CD45-sufficient animals. CFSE-labeled congenic CD45.1 T cells were adoptively transferred via the tail vein into RAGKO and 45RAGKO mice. Mice were euthanized and splenic CD4 and CD8 T cell proliferation analyzed on days 3, 4, 5, and 7 after T cell transfer. (A) Representative FACS plots. (B) Cumulative data. White bars indicate RAGKO hosts; black bars represent 45RAGKO hosts. The bar chart shows data from two independent experiments, n = 4–10. *p < 0.05.
(0.3–3 × 10^6) and the cumulative data are shown (Fig. 5A, 5B). There was no clear correlation between the number of CD45^+ CD11c^+ cells added and the level of T cell rescue. Although this rescue was a significant improvement, it did not match the spontaneous proliferation seen in the RAGKO mice, indicating a partial rescue of ~50%. This identifies a new role for CD45 in CD11c^+ cells, in driving spontaneous CD4 and CD8 T cell LIP in vivo.

**CD45KO DCs can activate Ag-specific T cells in vitro and in vivo**

The ability of C57BL/6, but not CD45KO, CD11c^+ cells to partially rescue the impaired T cell LIP in the 45RAGKO mice suggests a defect in CD45KO DCs. CD11c^+ DCs have been shown to promote CD8 LIP (28, 29), but the subsets of DCs involved and the signals required are not known. It was found that 45RAGKO mice have slightly increased numbers and proportions of plasmacytoid DCs (CD11c^{int} PDCA-1^+), and a decreased proportion of CD11c^{hi} MHC class II^hi conventional DCs in the spleen (data not shown). The 2-fold decrease in the proportion of conventional DCs could contribute to the reduced LIP in the 45RAGKO mice, but numbers alone cannot account for the complete lack of rescue by the CD45KO CD11c^+ cells (Fig. 5B). Thus, we began to examine the expression of MHC and costimulatory molecules on the CD45KO CD11c^{hi} MHC^{hi} DCs and determine their function in Ag presentation, to determine whether we could identify defects that may help explain the reduced LIP in these mice. We found no reduction in MHC or costimulatory molecule expression on splenic CD45KO DCs, if anything, levels were higher (data not shown), so we focused on determining whether CD45KO DCs showed any defects in cognate Ag-induced T cell activation and proliferation. Ex vivo DCs (CD11c^{hi} MHCII^{hi}) were purified from spleens, stimulated with a TLR agonist, and their ability to activate CFSE-labeled OT-I and OT-II T cells to proliferate in vitro in response to OVA peptides or OVA protein was measured. No difference was found in the ability of CD45KO DCs to activate Ag-specific T cells to proliferate (Fig. 5C).

T cell activation in vitro does not require the DCs to home to the correct location and may not accurately reflect all of the factors involved in T cell activation in vivo; therefore, we also examined T cell activation in vivo. CFSE-labeled OT-I or OT-II T cells were adoptively transferred into CD45.1 congenic mice and the following day, these mice were challenged with WT or CD45KO BMDCs that had been stimulated with a TLR agonist and loaded with OVA peptides. No difference was found in the ability of WT and CD45KO BMDCs to activate T cells to Ag (Fig. 5D). Because BMDCs are a model for DCs, but may not function in exactly the same way as endogenous DCs, and due to the fact that peptide Ags do not have to be processed to be presented, a second in vivo T cell activation experiment was also performed. In this experiment, CFSE-labeled OT-I or OT-II T cells were adoptively transferred into RAGKO and 45RAGKO mice, and the following day, these mice were challenged with 250 μg OVA protein and a TLR agonist to act as an adjuvant. No difference was found in the ability of RAGKO and 45RAGKO DCs to activate T cells to OVA Ag (Fig. 5E). Thus, we could see no defect in the ability of CD45KO DCs to induce Ag-specific T cell proliferation.

**IL-15/IL15Ra levels are not defective in CD45KO DCs**

The other component known to stimulate LIP is cytokines, in particular, IL-7 and IL-15, members of the common γ-chain family. IL-15 has been shown to enhance LIP of CD8 memory T cells (30), but as a free protein it is not particularly potent. Instead, this cytokine is presented in complex with the IL-15Ra-chain of the receptor on DCs, which greatly enhances its potency (31). Splenic lysates were prepared from untreated RAGKO and 45RAGKO mice by collagenase digestion at 55˚C for 90 min. The level of IL-15–IL-15Rα complexes in the splenic lysates was measured by ELISA and expressed per milligram of spleen weight because the spleens from 45RAGKO mice were generally larger than those from the RAGKO mice. These levels were found to be similar between the RAGKO and 45RAGKO mice (Fig. 6A).

In addition, the levels of IL-15Rα mRNA were not significantly different in WT and CD45KO ex vivo splenic DCs or BMDCs, and neither were the levels of IL-15 mRNA from C57BL/6 and CD45KO BMDCs (Fig. 6B). Thus, we have no evidence to suggest that reduced IL-15 signals are responsible for the defect in LIP in 45RAGKO mice.

**Splenic IL-7 levels are reduced in 45RAGKO mice compared with RAGKO mice**

IL-7 is known to be one of the most important cytokines for T cell survival and homeostatic proliferation (27, 32). In non-lymphopenic mice, measuring the levels of IL-7 is complicated by the fact that T cells are constantly depleting the cytokine (33). However, mice lacking T and B cells such as RAGKO mice have detectable levels of IL-7 because the cytokine is not being consumed by the lymphocytes. The level of IL-7 was measured in spleen lysates by ELISA and expressed per milligram of spleen weight, and was significantly lower (~2-fold less) in the 45RAGKO mice compared with the RAGKO mice (Fig. 6C). This is one key difference that could help account for the reduced LIP in the 45RAGKO mice.

**CD45 does not affect IL-7 transcripts in DCs, and the rescue of LIP by CD45^+ CD11c^+ DCs does not restore IL-7 levels**

IL-7 is produced mainly by stromal cells, but a couple of reports suggest that DCs can also produce IL-7 transcripts (34, 35). To check the IL-7 levels in DCs, we measured IL-7 mRNA levels by QPCR in BMDCs and ex vivo splenic DCs. We found that the IL-7 mRNA levels were very low in DCs, and there was no significant difference in the levels between WT and CD45KO mice (Fig. 6D). Thus, the lower level of IL-7 seen in the 45RAGKO mice is not due to reduced production by the CD45KO DCs.

To determine whether the rescue of the 45RAGKO LIP by C57BL/6 splenic CD11c^+ cells was due to an elevation in the IL-7 levels, we adoptively transferred ex vivo splenic CD11c^+ cells into 45RAGKO host mice, but no T cells were transferred. The levels of IL-7 were measured on day 5 after transfer, but no elevation in the splenic levels of IL-7 in 45RAGKO mice was seen (Fig. 6E). Thus, the mechanism by which WT CD11c^+ cells partially rescue T cell LIP in the 45RAGKO mice is not by restoring IL-7 levels.

**Innate immune cell CD45 regulates IL-7 production by stromal cells**

The stromal cells responsible for IL-7 production in secondary lymphoid organs are mainly fibroblastic reticular cells (FRCs) and lymphatic endothelial cells (36), which can both be identified as CD45^+ gp38^+ cells (37). Because we were unable to use CD45 as a marker for stromal cell discrimination, we developed a lineage mix (CD11b, CD11c, NK1.1, CD4, and CD8) that labeled virtually all of the CD45^+ cells in the lymph nodes of RAGKO mice. This lineage mix was used to gate out the majority of nonstromal cells, and gp38 was used as a marker to positively identify FRCs and lymphatic endothelial cells. Using this method, we determined that there were equal proportions of gp38^+ stromal cells in RAGKO and 45RAGKO lymph nodes (Fig. 6F, 6G). However, QPCR data showed that the stromal cells from 45RAGKO mice...
produced less IL-7 message than stromal cells from the RAGKO mice (Fig. 6H). Given that stromal cells do not express CD45, this defect cannot be cell intrinsic and must be due to a defect in a signal from an innate immune cell population that affects IL-7 production by stromal cells. Therefore, innate immune cell CD45 regulates IL-7 production by lymph node stromal cells.

Discussion
In this article, we have shown that a deficiency of CD45 in innate immune cells leads to the reduced ability of CD8 and CD4 T cells to undergo both spontaneous and homeostatic proliferation in a lymphopenic environment. In the CD45KO and 45RAGKO mice, the expansion of CD8 T cells was more severely affected than the CD4 T cells. Two distinct mechanisms were identified in the 45RAGKO mice to explain the reduced LIP. These mice had less IL-7 in secondary lymphoid organs, and this was attributed to an indirect effect of innate cell CD45 on the production of IL-7 by stromal cells. Second, spontaneous proliferation was enhanced by the addition of CD45+CD11c+ splenocytes. The inability of additional CD45KO CD11c+ cells to rescue the reduced LIP in 45RAGKO mice indicated that the rescue was not simply due to an increase in DC number, but is due to a defect in CD45KO CD11c+ cells that renders them unable to support spontaneous proliferation as efficiently as WT cells. It has been reported by multiple groups that DCs are required for or can enhance the LIP of T cells (28, 29, 38); however, it remains unclear exactly what signals are being provided by these cells other than the TCR signal provided by the MHC–peptide complex. We could not find a defect in the ability of CD45KO DCs to stimulate Ag-specific T cells to proliferate. CD45KO DCs were able to stimulate OT-I and OT-II T cell proliferation in response to peptide or protein Ags both in vitro and in vivo, indicating no defect in Ag loading or any other aspect of Ag processing and presentation. We also used lower-affinity peptide Ags (39) for in vitro OT-I T cell activation assays, which may mimic more closely the low affinities of self-peptide-MHC–TCR interactions involved in LIP. However, we again found no defect in the ability of the CD45KO DCs to stimulate Ag-specific T cells to proliferate (data not shown). In addition to demonstrating their T cell stimulatory function, the ability of 45RAGKO DCs to activate OT-I and OT-II T cells in vivo also shows that T cells are able to traffic to the secondary lymphoid organs in these mice and are able to support T cell proliferation and survival.

Approximately 10% of T cells in naive C56BL/6 mice have a memory phenotype, suggesting they have arisen from spontaneous proliferation. This type of LIP was abolished in germ-free mice (7), implicating a role for commensal bacteria in driving this proliferation, which has many similarities with specific Ag-induced LPS. The following day, the supernatant was removed and CFSE-labeled OT-I or OT-II T cells were added and the cells were cocultured for 3–4 d. The cells were analyzed by flow cytometry, and division indexes were calculated using the proliferation platform in FlowJo analysis software (TreeStar). n = 4–9. (D) CFSE-labeled OT-I or OT-II T cells were adoptively transferred into congenic CD45.1 mice. The following day, WT or CD45KO BMDCs that had been stimulated with CpG or poly(I:C) and loaded with OVA peptides overnight were injected i.p. On day 4 after BMDC challenge, the proliferation of T cells in the spleens was analyzed by flow cytometry. n = 5–6. (E) CFSE-labeled OT-I or OT-II T cells were adoptively transferred into RAGKO and 45RAGKO mice. The following day, OVA protein and either CpG or poly(I:C) were injected i.p. On day 4 postchallenge, the proliferation of T cells in the spleen was analyzed by flow cytometry. n = 3 or 5–7. All experiments were repeated at least twice. *p < 0.05, **p < 0.01, ***p < 0.001.
proliferation. The fact that CD45KO DCs have no apparent defect in inducing Ag-specific T cell proliferation, yet 45RAGKO mice are unable to support efficient spontaneous proliferation, suggests that there are CD45-dependent differences in how DCs induce LIP. Currently, the identity of the self-Ags or commensal Ags and how they activate T cells to spontaneously proliferate is unknown. We have examined the bacterial communities present in the feces of the C57BL/6, CD45KO and 45RAGKO, and RAGKO mice, and found that the communities in the 45RAGKO and RAGKO mice are quite closely related, more so than with CD45KO or C57BL/6 mice, indicating no major differences in their intestinal microbiota (40). However, it is an interesting possibility that specific commensals or specific bacterial communities could provide stimulating Ags or help create an environment in the secondary lymphoid organs that promotes spontaneous proliferation, but this remains to be explored.

In trying to ascertain whether there was a defect in cytokine production, we could find no evidence for a defect in IL-15 signaling because the levels of IL-15/IL-15Rα mRNA relative to GAPDH mRNA were measured in BMDCs or sorted splenic DCs (MHC class II<sup>high</sup> CD11c<sup>high</sup>) by QPCR. n = 3–6. The level of IL-7 in spleen digest supernatants was measured by ELISA. The amount of IL-7, relative to GAPDH mRNA in C57BL/6 (WT) or CD45KO BMDCs or sorted splenic DCs (MHC class II<sup>high</sup> CD11c<sup>high</sup> PDCA-1<sup>+</sup>), was measured by QPCR. n = 5–8. WT (C57BL/6) or 45KO (CD45KO or 45RAGKO) splenic CD11c<sup>+</sup> cells were adoptively transferred into 45RAGKO mice, but no T cells were transferred. Spleens were removed on day 5 after transfer and were digested with collagenase. IL-7 levels in the digest supernatant were measured by ELISA. Flow-cytometric profiles showing gating of lymph node cells that are lineage<sup>−</sup> gp38<sup>+</sup> stromal cells. Percentage of lymph node cells that are lineage<sup>−</sup> gp38<sup>+</sup> stromal cells. n = 9–10. Lineage<sup>−</sup> gp38<sup>+</sup> stromal cells were isolated from the lymph nodes of RAGKO and 45RAGKO mice by sorting. IL-7 mRNA levels were measured relative to GAPDH levels by QPCR. n = 4. *p < 0.05, **p < 0.01.
to the reduced effectiveness of the CD45KO DCs to induce T cell LIP and could help explain why the rescue with the CD45+ CD11c cells was only partial.

An interesting finding is that the IL-7 production is lower in stromal cells in 45RAGKO mice compared with RAGKO mice. Given that CD45 is not expressed by stromal cells, IL-7 production by stromal cells must be regulated by a cell type that expresses CD45. Evidence to date indicates that IL-7 production is constitutive, with IL-7 signaling being regulated at the level of receptor expression (reviewed in Refs. 43, 44). It has been suggested that IL-7 production may be upregulated in DC-like cells in lymphoproliferative animals (45), but we found that DCs made very little IL-7 message detected by QPCR, and the level of IL-7 mRNA was not altered in CD45KO DCs. An obvious candidate for the cell responsible for regulating IL-7 production by stromal cells would be DCs, which form intimate contacts with IL-7-producing FRCs in secondary lymphoid organs. However, we found no increase in IL-7 levels when C57BL/6 CD11c+ cells were adoptively transferred into 45RAGKO mice, suggesting that DCs may not be responsible. However, a role for DCs cannot be ruled out because it is possible that a longer incubation time is required to affect stromal cell IL-7 production. It is possible that a positive factor may be absent, or a negative factor may be present in CD45KO DCs. It is also possible that an additional CD45+ innate immune cell type is required in vivo to modulate or work with the DCs to regulate IL-7 production from lymph node stromal cells. Alternatively, it is possible that another innate immune cell is responsible for regulating IL-7 production in stromal cells, or that stromal cell development or function, which is dependent on cross talk from lymphoid and lymphoid inducer cells, is altered in the absence of CD45. Clearly, further work is required to understand mechanistically how innate cell CD45 regulates stromal cell production of IL-7. In these experiments, we have established a novel role for CD45 as an important regulator of T cell LIP mediated partly by CD11c+ cells, likely DCs, and partly by indirect modulation of stromal cell production of IL-7 levels.

Understanding the control of LIP is important because this process occurs in neoplasia to populate the T cell niche as well as in patients with infections that reduce the numbers of T cells, or patients undergoing cancer therapies or being treated with immunosuppressant drugs. LIP is known to be associated with some autoimmune diseases, and it has been shown to be a driving factor in some mouse models of inflammatory disease (e.g., the naive T cell transfer model of colitis). It is also a factor in susceptibility to transplant rejection, and can be advantageous, in particular in overcoming tolerance to enhance tumor rejection. An improved understanding of the factors involved in regulating LIP will allow therapies to be more targeted to either use this phenomenon to enhance tumor rejection or to suppress this phenomenon and avoid transplant rejection and autoimmune. In this study, we identified innate cell CD45 as a positive regulator of LIP.

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Disclosures
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References


