PD-L1–PD-1 interactions limit effector regulatory T cell populations at homeostasis and during infection

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Phenotypic and transcriptional proﬁling of regulatory T (Treg) cells at homeostasis reveals that T cell receptor activation promotes Treg cells with an efferent phenotype (eTreg) characterized by the production of interleukin-10 and expression of the inhibitory receptor PD-1. At homeostasis, blockade of the PD-1 pathway results in enhanced eTreg cell activity, whereas during infection with Toxoplasma gondii, early interferon-γ upregulates myeloid cell expression of PD-L1 associated with reduced Treg cell populations. In infected mice, blockade of PD-L1, complete deletion of PD-1 or lineage-speciﬁc deletion of PD-1 in Treg cells prevents loss of eTreg cells. These interventions resulted in a reduced ratio of pathogen-speciﬁc efferent T cells: eTreg cells and increased levels of interleukin-10 that mitigated the development of immunopathology, but which could compromise parasite control. Thus, eTreg cell expression of PD-1 acts as a sensor to rapidly tune the pool of eTreg cells at homeostasis and during inﬂammatory processes.

Efficacy of the PD-1 pathway in certain cancers resulted in an increase number of PD-L1+ intratumoral eTreg cells, associated with immune suppression, metastasis and increased morbidity.

The role of Treg cells during infection is complex. Enhanced Treg cell responses can promote microbial persistence, whereas inflammatory signals promote specialized subsets that limit immunopathology. Additionally, certain systemic infections result in a global collapse of Treg cell populations, which allows the emergence of efferent T cell responses that limit microbial replication.

Together, these studies have led to the concept that high efferent T:Treg cell ratios favor pathogen control while low efferent T:Treg cell ratios facilitate persistence. The basis for this Treg cell crash remains unclear but many infections lead to reduced basaIL-2 required to maintain Treg cells, whereas treatment with IL-2 complexes partially mitigate this loss. Whether IRs expressed by Treg cells inﬂuence these processes is unclear.

Here, experiments were performed to understand how PD-1 inﬂuences eTreg cells, thus at homeostasis basaIL-1 levels temper TCR signals that promote eTreg cell survival. However, during infection with the intracellular parasite Toxoplasma gondii, the early production of IFN-γ promotes myeloid cell expression of PD-L1, which leads to contraction of the eTreg cells. Antagonizing PD-1 activity during infection resulted in preservation of the eTreg cells, reduced pathogen-speciﬁc efferent T cell and subsequent co-stimulatory and TCR signals and is associated with T cell exhaustion and deletion. Although PD-1 is not essential for the development of Treg cells, there are reports that PD-1 can promote or limit peripherally induced Treg (pTreg) cells. Support for the ability of PD-1 to limit Treg cells is provided by studies in which blockade of the PD-1 pathway in certain cancers resulted in an increase number of PD-L1+ intratumoral eTreg cells, associated with immune suppression, metastasis and increased morbidity.

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immunopathology, but at the most extreme, increased parasite replication. Thus, PD-1 has a physiological role in eTreg cell homeostasis and engagement of PD-1 during infection tunes T_{reg} cell responses that balance protective and pathological responses.

**Results**

**T_{reg} heterogeneity and PD-1 expression.** To visualize the distribution of PD-1 on different CD4^+ T cell populations at homeostasis, Uniform Manifold Approximation and Projection (UMAP)
analysis of splenic CD4+ T cells from naive C57BL/6 mice was utilized (Extended Data Fig. 1a–d). Few conventional T (Tconv) cells (CD4+Foxp3−) expressed PD-1 whereas 40% of Treg cells (CD4+Foxp3+) were PD-1+ (Fig. 1a). Despite exclusion of PD-1 and Foxp3 from the UMAP analytical algorithm, Treg cells were segregated from Tconv cells due to expression of Helios, GITR and CD25 as well as PD-L1 and CTLA-4 (Extended Data Fig. 1c,e). Additional markers of activation (KLRG1, CD73 and ICOS) were associated with Treg cells, but illustrated their heterogeneity at homeostasis (Extended Data Fig. 1f). When compared to Tconv cells, Treg cells also had increased levels of activation-associated proteins (CD69, CD11a, CD44 and ICOS), but decreased expression of CD127 (Extended Data Fig. 1g,h). To compare the differences between PD-1− and PD-1+ Treg cells, UMAP analysis of Foxp3+CD4+ T cells was utilized that included markers of activation and function, but which excluded PD-1 as a calculation factor (Extended Data Fig. 2a–d). The UMAP-generated clusters were then defined via X-shift analysis and then further delineated for expression trends using the ClusterExplorer tool (Extended Data Fig. 2e,g). The comparison of Treg cells from wild-type (WT) and PD-1 knockout (KO) (Pdcd1−/−) mice allowed the identification of PD-1−, PD-1−/− and PD-1+ subsets (Extended Data Fig. 2b). Among the five Treg cell clusters identified using X-shift, four had extensive overlap in the PD-1hi regions (Fig. 1b). At homeostasis, the PD-1hi Treg cell region of the UMAP demonstrated increased expression of CD69, CD11a and Nur77 (expressed after TCR engagement) (Fig. 1c). The PD-1hi region was also associated with expression of Ki67 and cMyc, proteins connected to T cell proliferation (Fig. 1d) and overlapped with the Helios+ region of the UMAP (Fig. 1e), which is a marker of thymically derived Treg cells. The PD-1− and PD-1+ subsets did not overlap extensively with these activated, proliferative or thymic Treg cell-associated proteins, but were correlated with CD25 expression and the pro-survival Bcl-2 (Fig. 1b–e). Expression of proteins linked to Treg cell effector function (ICOS, CTLA-4, PD-1, CD27, CD43, CD73 and KLRL1) were enriched in the PD-1hi region (Fig. 1f). Notably, a KLRL1+ subset appears as a distinct cluster within the activated PD-1−CD25hi Treg cell pool, indicating heterogeneity within the PD-1+ compartment (Fig. 1c–f). Further, the expression of these Treg cells to produce IL-10 was closely associated with the PD-1− compartment (Fig. 1g and Extended Data Fig. 2h–k). Examination of the PD-1−, PD-1+ and PD-1hi Treg cell subsets demonstrates increasing proportions of Helios+ thymic Treg cells with increasing PD-1 expression (Fig. 1h). Helios+ Treg cells are associated with distinct TCR repertoires and enhanced suppressive capacity; and the levels of PD-1 correlated with increased expression of Nur77, CD11a, CD69, ICOS and CTLA-4 (Fig. 1h–j) but absence of CD25 and Bcl-2 (Fig. 1k). This combination of low CD25 with expression of IR is similar to eTreg cell populations and we will refer to PD-1− Treg cells as eTreg cells, whereas PD-1−CD25+ Treg cells will be termed central Treg (cTreg) cells.

**PD-1 restrains eTreg cell populations at homeostasis.** To assess the impact of PD-1–PD-L1 interactions on Treg cells, naive mice were dosed with an isotype control or PD-L1 antibody and splenocytes assessed after 72 h. Basal PD-1 was present on different immune populations (Extended Data Fig. 3a,c) and the in vivo PD-L1 antibody bound to these PD-L1-expressing populations (Extended Data Fig. 3b). This short-term treatment resulted in an increased number of Treg cells (Fig. 2a), most notably the proportion and total number of PD-1− Treg cells (Fig. 2b), elevated expression of Ki67 by PD-1− cells (Extended Data Fig. 4a–c) and the proportion of and number of PD-1−/− CTLA-4− eTreg cells (Extended Data Fig. 4d). In Nur77−/− GFP reporter mice, this blockade increased the proportion of eTreg cells (Nur77−/−CD11a+Ki67−, which expressed PD-1, CTLA-4 and KLRL1 but were CD25+, Extended Data Fig. 4a–c). There was also an increase in the proportion of IL-10+ Treg cells (Fig. 2c) and, consistent with the ability of IL-10 to limit co-stimulation, conventional dendritic cells (cDC2s) and macrophages expressed decreased levels of CD80 (Fig. 2d). Similarly, Pdcd1−/− mice at homeostasis had an increase in the total number of Treg cells, Ki67+ Treg cells and an increased proportion of CD25+Bcl-2+ eTreg cells (Extended Data Fig. 5) that correlated with increased production of IL-10 and reduced CD2c expression of CD80 (Extended Data Fig. 5f). Thus, at homeostasis, constitutive levels of PD-1 do not affect cTreg cells but constrain the PD-1+ eTreg cell pool.

To understand the differential impact of PD-L1 blockade on cTreg and eTreg cells, Foxp3+GFP mice were treated with an isotype control or anti-PD-L1. After 3 d, cTreg and eTreg cells were used for bulk RNA-seq (Extended Data Fig. 4f). Comparisons of transcriptomic data between cTreg and eTreg cells from both isotype and PD-L1 blockade resulted in 2,804 genes with significantly different expression regardless of treatment (Fig. 2e). There were an additional
1,969 genes with different expression between cTreg and eTreg cells in isotype-treated hosts, whereas PD-L1 blockade resulted in an additional unique set of 1,003 differently expressed genes (Fig. 2c). The use of gene set enrichment analysis (GSEA) to compare expression trends among significantly different gene signatures between cTreg and eTreg cells, revealed marked enrichment of development and migration and adhesion pathways (Fig. 2d). The expression of key cytokines and surface markers was also assessed, with a significant decrease in IL-10 expression in PD-L1-blockaded eTreg cells compared to isotype-treated cells (Fig. 2e). Furthermore, the percentage of CD80+ cells was higher in eTreg cells compared to cTreg cells, indicating a more activated phenotype (Fig. 2f). The data highlight the distinct regulatory mechanisms and functional differences between cTreg and eTreg cells, which may have implications for the development of immunotherapies.
Fig. 3 | TCR signals are necessary to maintain eT$_{reg}$ cell populations at homeostasis. Cohorts of 8-week-old male C57BL/6 mice ($n=5$ per group) were treated once daily for 4 d with subcutaneous injections of PBS or tacrolimus (FK506) and splenocytes were collected and analyzed via high-parameter flow cytometry (two experimental replicates). a, Plots depicting drop in proportion and number of T$_{reg}$ cells with FK506 treatment (two-tailed unpaired Student’s t-test, **$P=0.0012$). b, Plots depicting further changes to the T$_{reg}$ cell compartment in context of PD-1, PD-1$^+$, PD-1$^+$ subsets (two-way ANOVA with Sidak’s multiple comparisons test, $^*P=0.0162$, $^{***}P<0.0001$). c, Plots depicting proportion and numbers of activated (CD44$^+$CD11a$^+$) T$_{reg}$, Foxp3$^+$CD4$^+$ T$_{reg}$, and CD8$^+$ T cells between PBS- and FK506-treated hosts (two-tailed unpaired Student’s t-test, $^{****}P<0.0001$). d, Plots depicting changes proportion and number of Ki67$^+$ T$_{reg}$ cells among PD-1$^+$, PD-1$^+$, PD-1$^+$ subsets (two-tailed unpaired Student’s t-test, $^{***}P=0.0003$, $^{****}P<0.0001$). e, Plots demonstrating changes to the PD-1$^+$CTLA-4$^+$ T$_{reg}$ cell subset following FK506 treatment (two-tailed unpaired Student’s t-test, $^{****}P<0.0001$). f, Cytokine-stain plots of IL-10 on T$_{reg}$ cells from PBS and tacrolimus hosts (two-tailed unpaired Student’s t-test, $^{****}P<0.0001$). All data are presented as mean ± s.e.m. and show individual data points.

morphogenesis related gene sets (Sox9, Wnt3, Wnt4, Ntn4, Plag1, Tp63 and Pbx1) in cT$_{reg}$ cells, suggesting early stem-cell-like properties. Conversely, eT$_{reg}$ cells were enriched for transcripts associated with activation such as Zap70, Fyn and Pik3/akt/mTOR downstream proteins and proliferation-associated transcripts involving spindle assembly and DNA replication (Fig. 2f). Volcano-plot comparisons (Fig. 2g) of transcript differences in cT$_{reg}$ cells (values to the left of 0 on the x axis) to eT$_{reg}$ cells (values to the right of 0 on the x axis) provided transcriptional confirmation to trends previously identified in Fig. 1d–f. Thus, cT$_{reg}$ cells were enriched for IL2ra and Bcl2 transcripts, whereas eT$_{reg}$ cells were enriched for transcripts downstream of T$_{reg}$ cell activation that included Pdcd1, ICOS, Cila4, Tnsr18, NIt5e, IL10 and Klk1. In addition, eT$_{reg}$ cell subsets were enriched for TCR-signaling transcripts (Zap70, Cd3e, Lime1, Ptprc, Cd28, Mapk1, Themis2, Fyn and Ifr4) and those linked to migration and adhesion and substantial increases in metabolic (such as Pck2, Ldh, Cplt1 and Ass1), apoptotic (such as Pmaip1, Bak1 and caspases) and proliferative/growth transcripts related to the P3K/akt/mTOR pathway (such as Cdk1, Mapk1, Map2k3 and Myc). Thus, compared to cT$_{reg}$ cells, the eT$_{reg}$ cell subset is characterized by constitutive activation, metabolic function and entry into the cell cycle.

Next, the impact of anti-PD-L1 on the transcriptional profile of cT$_{reg}$ and eT$_{reg}$ cells was assessed via volcano-plot comparisons of isotype-treated hosts (values to the left of 0 on the x axis) and
These datasets highlight differences between cTreg and eTreg cells and also experiencing ongoing restriction of function and proliferation. Ki67 transcriptional activity and negatively modulates Treg cell suppression signals, a pathway that mediates disruption of Foxp3 signals and would be sensitive to mechanisms that reduce T cell impact the existing populations of activated Tconv CD4 T cells. Infection with L. monocytogenes promotes secretion of IFN-γ that promotes secretion of IFN-γ from STAT1flox mice (Extended Data Fig. 4d). Division of the T<sub>reg</sub> cell compartment into PD-1<sup>+</sup> eT<sub>reg</sub> cell pool and the considerable reduction in Ki67<sup>+</sup> T<sub>reg</sub> cells was due to reductions in the PD-1<sup>+</sup> subset (Fig. 3d). The ability of FK506 to reduce the activated T<sub>reg</sub> cells coincided with a reduction in the number of eT<sub>reg</sub> cells (PD-1<sup>+</sup>CTLA-4<sup>-</sup>) and a loss of IL-10<sup>+</sup> T<sub>reg</sub> cells (Fig. 3e,f). These results suggest that at homeostasis eT<sub>reg</sub> cells are more dependent than cT<sub>reg</sub> cells on constitutive TCR signals and would be sensitive to mechanisms that reduce T cell activation signals.

Infection-induced IFN-γ upregulates PD-L1, limiting PD-1<sup>+</sup> eT<sub>reg</sub> cells. Infection with T. gondii stimulates cDC1 production of IL-12 that promotes secretion of IFN-γ, a cytokine that signals through STAT1 and which can upregulate PD-L1 expression in other settings<sup>11,22</sup>. This infection is also accompanied by a collapse in T<sub>reg</sub> cell populations associated with CD4<sup>+</sup> T cell-mediated immunopathology<sup>11,22</sup>. To determine whether these phenomena are related, studies were performed to assess whether infection-induced IFN-γ drives expression of PD-L1 and whether disruption of this pathway impacts T<sub>reg</sub> cell populations. Therefore, mice infected with T. gondii were treated with an isotype or IFN-γ-blocking antibody. In naive hosts, basal PD-L1 was detected on multiple cell types (neutrophils, cDCs, monocytes, macrophages and T<sub>reg</sub>) cells in the spleen (Extended Data Fig. 6a) and peritoneum (Fig. 4a), with the highest levels of PD-L1 expression on cDC2s and macrophages (Fig. 4a). As early as 72 h following infection with T. gondii, there was expansion of the macrophage and monocyte compartments, which expressed high levels of PD-L1 (Fig. 4a) that dominated other cellular sources of this ligand. In infected hosts treated with anti-IFN-γ there was a failure to upregulate PD-L1 expression (Fig. 4a). When mice that lacked STAT1 in dendritic cells (DCs) or myeloid cells (STAT1<sup>fl/fl</sup>x<sup>ITGAX<sup>+</sup></sub> and STAT1<sup>fl/fl</sup>x<sup>LysM<sup>+</sup></sub>), respectively, were infected, there was a prominent reduction in the upregulation of PD-L1 on the relevant cell lineages, but PD-L1 expression was preserved on other cell types (Fig. 4b and Extended Data Fig. 6b). In these experiments, by day 7 after infection there was a marked loss of T<sub>reg</sub> cells in control and STAT1<sup>fl/fl</sup>x<sup>ITGAX<sup>+</sup></sub> and Listeria monocytogenes (Extended Data Fig. 7e). Additional analysis revealed that infection with T. gondii resulted in a preferential loss of PD-1<sup>+</sup> eT<sub>reg</sub> cells (Fig. 5b) and was abrogated by PD-L1 blockade (Extended Data Fig. 7c,d). This preferential impact on eT<sub>reg</sub> cells was verified by the ability of anti-PD-L1 to increase numbers of PD-1<sup>+</sup>CTLA-4<sup>-</sup> eT<sub>reg</sub> cells (Fig. 5c) and the proportion of IL-10<sup>+</sup> T<sub>reg</sub> cells (Fig. 5d). The T<sub>reg</sub> cell-associated expression of prosurvival BCL-2 was not enhanced by PD-L1 blockade (Fig. 5e), despite overall increased T<sub>reg</sub> cell numbers. This analysis also revealed that infection resulted in T<sub>reg</sub> cells that were characterized by deceased expression of prosurvival BCL-2 and increased levels of the pro-apoptotic BIM. While PD-L1 blockade did not affect BCL-2 it did decrease levels of BIM (Fig. 5f). Thus, PD-1 restricts...
Day 3 after infection: PEC

- Neut
- cDC1s
- cDC2s
- Mo
- Mφ
- Treg cells

Day 7 after infection: PEC

- Mo
- Mφ

Percentage Foxp3+ of CD4+ T cells

- No. of PD-1 hi Treg cells
- No. of PD-1 hi Treg cells
- Percentage Foxp3+ of CD4+ T cells

| No. of PD-1 hi Treg cells | Percentage Foxp3+ of CD4+ T cells |
|---------------------------|----------------------------------|
| 87.7                      | 12.4                             |
| 74.6                      | 12.5                             |
| 52.4                      | 9.04                             |
Fig. 5 | PD-L1 blockade ameliorates the crash of PD-1<sup>−</sup> eTreg cells during the acute phase of infection. **a**–**i.** Cohorts of 9-week-old female C57BL/6 mice were treated with an isotype antibody or PD-L1-blocking antibody 24 h before infection with T. gondii i.p. The antibody treatments were repeated every 72 h throughout the course of infection until the mice were killed on day 10 and PECs, spleen and liver were collected and analyzed via high-parameter flow cytometry. Flow plots of bulk CD4<sup>+</sup> T cells with subsequent gates on the Foxp3<sup>+</sup> T cells (T<sub>reg</sub> cells), demonstrating the drop in T<sub>reg</sub> cells comparing naïve mice (n = 4) from homeostatic levels during infection in isotype-treated hosts (n = 5) and the maintenance of T<sub>reg</sub> cells during infection with PD-L1-blocking antibody (n = 4) treatment (one-way ANOVA with Tukey’s multiple comparisons test, **P < 0.01, ***P < 0.001, four experimental replicates) (a). Flow plots of T<sub>reg</sub> cells from naïve (n = 4), infected isotype-treated (n = 5) and infected PD-L1-blockade-treated (n = 5) groups showing enrichment of the PD-1<sup>−</sup> T<sub>reg</sub> cell compartment as a consequence of PD-L1-blockade treatment during infection (one-way ANOVA with Fisher’s LSD individual comparisons test, *P = 0.0438, ***P < 0.0001, four experimental replicates) (b). Flow data for the coexpression of PD-1 and CTLA-4 on bulk T<sub>reg</sub> cells comparing the number of PD-1<sup>−</sup>CTLA-4<sup>−</sup> T<sub>reg</sub> cells (eTreg cell-associated) from animals from the infected groups, using cells from a PD-1<sup>−</sup> host as a gating control (n = 5 per group, two-tailed unpaired Student’s t-test, ****P < 0.0001, four experimental replicates) (c). Cells from spleen and liver from each group were stimulated and stained for cytokines, depicted here is IL-10 staining of T<sub>reg</sub> cells and total number of IL-10<sup>+</sup> T<sub>reg</sub> cells in the context of PD-L1 blockade (n = 5 per group, two-tailed unpaired Student’s t-test, spleen **P = 0.0009, liver ***P = 0.0006, four experimental replicates) (d). **e,f.** Flow cytometry analysis of anti-apoptotic BCL-2 (e) and BIM (f) expression on bulk T<sub>reg</sub> cells between uninfected (n = 4), infected isotype-treated (n = 5) and infected PD-L1-blockade-treated (n = 4) groups of mice (one-way ANOVA with Fisher’s LSD individual comparisons test, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, three experimental replicates). **g.** Plots depicting the proportion and number of Ki67<sup>+</sup>cMyc<sup>+</sup>T<sub>reg</sub> cells in spleen and liver from infected hosts (n = 5 per group two-tailed unpaired Student’s t-test, spleen *P = 0.0376, liver **P = 0.0025, two experimental replicates). **h.** Plots of PEC-derived cDCs (CD3<sup>+</sup>, B220<sup>−</sup>, CD19<sup>−</sup>, NK1.1<sup>−</sup>, Ly6G<sup>−</sup>, CD64<sup>−</sup>, CD11c<sup>+</sup>, MHC-II<sup>+</sup> and XCR1<sup>+</sup>) depicting changes in IL-12p40 expression (n = 5 per group, two-tailed unpaired Student’s t-test, ****P < 0.0001, two experimental replicates). **i.** Intracellular stain for IL-2 on bulk T<sub>reg</sub> cells (CD3<sup>−</sup>CD4<sup>−</sup>Foxp3<sup>−</sup>) from naïve hosts and infected hosts treated with isotype or PD-L1-blocking antibody (n = 5 per group, one-way ANOVA with Tukey’s multiple comparisons test, ***P < 0.0001, four experimental replicates). All data are presented as mean ± s.e.m. and show individual data points.
the ability of eTreg cells to proliferate, and in infected hosts treated with anti-PD-L1 there was an increase in the proportion and number of cMyc+K667+ Treg cells (Fig. 5g). Additionally, IL-10 is an important antagonist of IL-12 production during toxoplasmosis14 and PD-L1 blockade resulted in increased numbers of IL-10+ eTreg cells (Fig. 5d) and a marked reduction in the proportion of IL-12+ cDC1s (Fig. 5h). In additional experiments, the use of anti-IL-10r signals through PD-1 limit effector T cell responses, including production of IL-2 (ref. 15), it is possible that systemic blockade of PD-L1 during infection would result in enhanced parasite-specific CD4+ T cell responses and increased IL-2 that preserve the Treg cell populations; however, while infection resulted in reduced T cell production of IL-2, blockade of PD-L1 did not reverse this effect (Fig. 5i). These datasets indicate that PD-L1 blockade during infection results in enhanced eTreg cell functions and expression of IL-10 and that this is not due to a restoration of IL-2 production.
Impact of PD-L1 blockade on parasite-specific effector T cells. Parasite-specific effector CD4+ T cells produce IFN-γ required for resistance to T. gondii but the weight loss that accompanies this infection can be mediated by enhanced parasite replication or as a result of the CD4+ T cell response, which is regulated by IL-10 (ref. 34). Analysis of infected mice treated with anti-PD-L1 revealed that this intervention resulted in reduction of parasite-specific CD4+ and CD8+ T cells (Fig. 6a–d). While infection leads to acute weight loss mediated by CD4+ T cells, PD-L1 blockade antagonized the weight loss and resulted in improved physical condition (Fig. 6f) but was not associated with alterations in parasite burden (Fig. 6f). As Treg cell production of IL-10 antagonizes infection-induced immune pathology, mice were treated with anti-PD-L1 alone or in combination with anti-IL-10. In these experiments, the protective effects of anti-PD-L1 against infection-driven weight loss were antagonized by anti-IL-10 (Fig. 6g). Moreover, while anti-PD-L1 treatment alone resulted in an extensive reduction in the magnitude of the T-bet+IFN-γ+ Treg cells, treatment with anti-IL-10 antagonized this effect (Fig. 6h). The impact of these treatments on the CD4+ T cell compartment is most apparent when comparing the ratio of effector T (Teff) cells (Foxp3−CD11a−Tbet+) to Treg cells (Foxp3+CTLA-4+ICOS+) (Fig. 6i).
infection results in a high \( \text{T}_{\text{eff}}:\text{T}_{\text{reg}} \) cell ratio that is reduced following PD-L1 blockade, which in turn is negated by co-administration of anti-IL-10r (Fig. 6i). Similarly, the comparison of infected WT and \( Pdc\text{-}^{\text{Cre}} \) mice, demonstrated that absence of PD-1 resulted in the preservation of \( \text{T}_{\text{reg}} \) cells, an enrichment of the e\( \text{T}_{\text{reg}} \) cell-associated phenotypes (CD25\textsuperscript{lo}, BCL-2\textsuperscript{lo}, ICOS\textsuperscript{hi} and CTLA-4\textsuperscript{hi}), an increase in the number of IL-10\textsuperscript{+} \( \text{T}_{\text{reg}} \) cells and a reduction in the proportion of activated cDC2s, decreased number of T-bet\textsuperscript{+}KLRF1\textsuperscript{+} parasite-specific CD4\textsuperscript{+} T cells, but no substantial differences in parasite burden (Extended Data Fig. 8). Thus, PD-L1 blockade or loss of PD-1 did not result in increased CD4\textsuperscript{+} \( \text{T}_{\text{reg}} \) cell responses, but rather was associated with enhanced e\( \text{T}_{\text{reg}} \) cell activity and reduced systemic pathology.

Deletion of PD-1 in \( \text{T}_{\text{reg}} \) cells increases \( \text{T}_{\text{reg}} \) activities. While the blockade of PD-L1 or total loss of PD-1 affects e\( \text{T}_{\text{reg}} \) cell populations, these effects could be mediated through other immune cell populations. To determine whether the ability of PD-1 to limit \( \text{T}_{\text{reg}} \) cell responses was intrinsic to \( \text{T}_{\text{reg}} \) cells, \( Pdc\text{-}^{\text{fl}fl}\times\text{Foxp3}^{\text{cre}} \) mice were generated as described\textsuperscript{36}. These mice showed normal expression of PD-1 on \( \text{T}_{\text{eff}} \) cell populations, whereas the \( \text{T}_{\text{reg}} \) cell population lacked PD-1 (Fig. 7a). At homeostasis, these mice had an increased number of total \( \text{T}_{\text{reg}} \) cells (Fig. 7b) associated with preferential expansion of the e\( \text{T}_{\text{reg}} \) cell population (Fig. 7c,d). Thus, PD-1 was not required to generate e\( \text{T}_{\text{reg}} \) cells, but expression of PD-1 does limit the e\( \text{T}_{\text{reg}} \) cell pool. In response to infection with \( T.gondii, Pdc\text{-}^{\text{fl}fl}\times\text{Foxp3}^{\text{cre}} \) mice did not undergo \( \text{T}_{\text{reg}} \) cell collapse (Fig. 7e) and maintained an
enhanced proportion of eTreg cells (defined in the absence of PD-1 as ICOS+, CTLA-4+, CD25b and BCL-2+) (Fig. 7g,i). Congruent with the previous experiments, there was an increase in the number of IL-10+ Treg cells and a reduction in the number of activated cDC2s (Fig. 7h,i) and a reduced population of IL-12+ DC1s (Fig. 7j). Analysis of the CD4+ Treg cell responses revealed no evidence of increased IL-2 production (Fig. 8a). However, in context of the enhanced eTreg cell compartment, there was a reduction in the number of parasite-specific CD4+ T cells (Fig. 8b,c). Moreover, the parasite-specific CD4+ Treg compartment (Fig. 8b) was profoundly impacted with decreased T-bet expression and total number of T-bet+KLRG1+ T cells (Fig. 8d,e). In contrast to the studies with the PD-L1 blockade or Pdcd−/− mice, the lineage-specific deletion of PD-1 from Treg cells resulted in increased weight loss (Fig. 8f), but a marked reduction in pathology in the liver (Fig. 8g). Despite the reduced immunopathology, the decreased T cell responses were associated with an increase in parasite burden (Fig. 8h).

The loss of PD-1 during acute toxoplasmosis resulted in reduced immunopathology (based on gross histology) across all methods (blockade, total deletion or Pdcd−/−× Foxp3−/−), but yielded different outcomes for gross weight loss and parasite burden. To understand why the blockade of PD-L1, loss of PD-1 or Treg cell-specific PD-1 deletion produced varying outcomes, we compared Treg:eTreg cell ratios in each of these conditions. In a WT setting, infection

**Fig. 8 | Treg cell-specific deletion of PD-1 results in reduction of parasite-specific Tconv cells and a systemic increase in parasite burden.** Bulk splenocytes collected from 9- to 10-week-old infected and infected (day 10 of T. gondii infection) male C57BL/6 and Foxp3−/−× PD-1−/−× mice were stimulated with PMA/ionomycin, permeabilized and stained for IL-2. a, Intraglandular cytokine staining for IL-2 expression on splenic CD4+ Tconv cells comparing changes between naive C57BL/6 (n = 5) and Foxp3−/−× PD-1−/−× (n = 4) mice, to infected C57BL/6 (n = 5) and Foxp3−/−× PD-1−/−× (n = 5) one-way ANOVA with Tukey’s multiple comparisons test, **P < 0.0001, four experimental replicates.** b, Ex vivo flow cytometry data depicting activated parasite-specific CD4+ Tconv cells (Foxp3−, CD11a+, AS15:I-Abγ, from C57BL/6 (n = 5) and Foxp3−/−× PD-1−/−× (n = 5) mice demonstrating a reduction in parasite-specific T cells in Foxp3−/−× PD-1−/−× hosts across multiple tissues (two-way ANOVA with Sidak’s multiple comparisons test, P = 0.0335, **P < 0.0001, four experimental replicates). c, Bar graph of cytokine-stain data depicting splenic proportional changes in TH1 effector-associated IFN-γ+ T conv compartment (Fig. 8b) was profoundly impaired with decreased T-bet expression and total number of T-bet+KLRG1+ T cells (Fig. 8d,e). In contrast to the studies with
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Discussion
The initial perspective that PD-1 signaling promotes induced Treg cell formation and that PD-1 provided a mechanism for Treg cells to suppress immune activity has been altered with reports for certain cancers that PD-L1 blockade enhances function of PD-1hi Treg cells associated with hyper-progression and metastasis without further tumor mutations. Likewise, the clinical observations that PD-L1 blockade can augment Treg cell responses and that PD-1 blockade therapy is more effective when combined with strategies that also target Treg cells provides indirect evidence that PD-1 can inhibit Treg cells. Moreover, the finding that targeted deletion of PD-1 from Treg cells results in reduced severity of experimental autoimmune encephalomyelitis highlights that the inhibitory activity of PD-1 on Treg cells is not restricted to cancer. However, this seems unlikely that the ability of PD-1 to limit Treg cell activity evolved to promote cancer or autoimmunity. Treg cell function to limit a wide variety of immune-mediated conditions but pathogens can benefit from their ability to dampen Treg cell responses. Consequently, it has been proposed that the transient ‘crash’ of Treg cells during systemic infection is a compromising that allows a balanced T cell response to emerge and that infection-induced suppression of homeostatic IL-2 production contributes to this crash. The presented here reveal that at homeostasis, PD-1 regulates Treg cell populations and during infection, the production of IFN-γ drives increased PD-L1 levels, which license PD-1 to act as a rheostat that is sensitive to global levels of inflammation to contract the PD-1hi eTreg cell population.

The finding that pharmacological blockade of TCR stimulation at homeostasis leads to a reduction in the cTreg cell population (but not the cTreg cell subset) is relevant, because TCR stimulation promotes PD-1 expression. The continuous interactions of DCs with Treg cells is a process critical for Treg cell homeostasis and the presence of constitutive levels of PD-L1 on MHC-II cells and macrophages suggest that eTreg cells encounter TCR stimulation and PD-1 signaling simultaneously. Previous studies indicated that PD-1 restrains the P13K–Akt pathway and the comparison of eTreg and cTreg cells before and after PD-L1 blockade also highlighted this pathway. However, PD-1 engagement had the greatest impact on networks associated with cell division and activation that seem constitutive at homeostasis. Thus, the link of PD-1 to transcriptional alterations in P13k/AKT/mTOR pathways and changes in Bcl-2 and BIM reinforce the influence of PD-1 on proliferation and survival. While the increase in eTreg cell proliferation after short-term PD-L1 blockade is likely accompanied by alterations in cellular activity, the dominant amplification of cell cycle gene sets compared to metabolic related genes sets did not identify a clear PD-1-mediated impact on Treg cell metabolism.

In many infectious systems, Treg cells and the production of IL-10 determine the balance between protective and pathological responses and too many Treg cells or IL-10 can be associated with decreased DC function, reduced effector responses and increased microbial replication. This is exemplified during toxoplasmosis where the systemic inflammatory response promotes parasite control but can result in CD4+ T cell-mediated immunopathology. In this setting, IL-10 and Treg cells limit the magnitude of the DC and effector T cell responses and prevent immunopathology. This continuum is reflected in our datasets derived from mice or those undergoing PD-L1 blockade, which are characterized by increased eTreg cell populations, a reduction in parasite-specific CD4+ T cell responses and reduced immunopathology, but with an immune response that is sufficient for parasite control. A similar phenotype (increased Treg cells, reduced immunopathology) was observed with the Pdcdg−/− Foxp3−/− mice, but these mice had the lowest Treg cell ratio, which was accompanied by increased parasite replication. This continuum reinforces the importance of Treg cell ratios on the balance between pathological and protective responses during infection and is analogous to the hyper-progressive disease observed in certain patients with cancer treated with anti-PD-L1 that is associated with reductions in Treg cell ratios.

Many studies on the biology of PD-1 have focused on the impact of this pathway on restraining effector T cell function with the idea that blocking PD-1 signaling would invariably drive an increase in Treg cells, shifting the Treg cell ratio to enhance immune responses. However, during toxoplasmosis, the ubiquitous IFN-γ-mediated upregulation of PD-L1 did not seem to significantly restrict the development of acute Treg cell responses. Rather, PD-L1 blockade resulted in a reduction in the magnitude of the parasite-specific CD4+ T cell responses and reduced immunopathology. Because of the expectation that the ability of PD-1 to limit effector T cell responses is a dominant feature of this pathway, these findings were unanticipated but are consistent with other reports, where interfering with the PD-1 pathway does not improve effector responses. One example involves the role of PD-1 in susceptibility to Mycobacterium tuberculosis, where either genetic human deficiency or blockade of PD-1 in mice and humans results in reduced effector responses, reactivation and higher bacterial burdens.

There have been many advances in our understanding of the factors that maintain Treg cell populations and their inhibitory functions but less is known about the pathways that restrict these cells. These studies identify the constitutive expression of PD-1 on Treg cells as a homeostatic regulator of the eTreg cell population and establish that this pathway allows the development of adaptive responses to infectious challenges while balancing the development of immunopathology. Thus, PD-1 has a physiological role in Treg cell homeostasis and the ability to engage this receptor during infection provides a mechanism to rapidly tune Treg cell responses to allow the emergence of pathogen-specific T cell responses, while balancing the development of T cell-mediated immunopathology. Other IRs, such as CTLA-4, also limit Treg cell activities, even utilizing similar intracellular signaling pathways. As such, it is still unclear how signals from distinct IRs are integrated with those from PD-1 to control Treg cell homeostasis. Additionally, there are open questions about the relationship between the eTreg and cTreg cell populations that will require lineage tracing, while the long-term impact of the transient infection-induced PD-1 mediated restriction on the Treg cell repertoire has yet to be addressed.

Online content
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References
1. Josefowicz, S. Z., Lu, L.-F. & Rudensky, A. Y. Regulatory T cells: mechanisms of differentiation and function. Annu. Rev. Immunol. 30, 531–564 (2012).
2. Sakaguchi, S. et al. Regulatory T cells and human disease. Annu. Rev. Immunol. 38, 541–566 (2020).
3. Kieback, E. et al. Thymus-derived regulatory T cells are positively selected on natural self-antigen through cognate interactions of high functional avidity. *Immunity* **44**, 1114–1126 (2016).

4. Levine, A. G., Arvey, A., Jin, W. & Rudensky, A. Y. Continuous requirement for the TCR in regulatory T cell function. *Nat. Immunol.* **15**, 1070–1078 (2014).

5. Lenschow, D. J. et al. B7/CD28 costimulation is essential for the homeostasis of the CD4CD25+ immunoregulatory T cells that control autoimmune diabetes. *Immunity* **12**, 431–440 (2000).

6. Bluestone, J. A. & Tang, Q. How do CD4+CD25+ regulatory T cells control autoimmunity? *Curr. Opin. Immunol.* **17**, 638–642 (2005).

7. Chinen, T. et al. An essential role for IL-2 receptor in regulatory T cell function. *Nat. Immunol.* **17**, 1322–1335 (2016).

8. Rubtsov, Y. P. et al. Regulatory T cell-derived interleukin-10 limits homeostasis and function during type 1 inflammation. *Nature* **546**, 421–425 (2017).

9. Duhen, T., Duhen, R., Lanzavecchia, A., Sallusto, F. & Campbell, D. J. Functionally distinct subsets of human FOXP3+ Treg cells that phenotypically mirror effector Treg cells. *Blood* **119**, 4430–4440 (2012).

10. Zheng, Y. et al. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control Treg responses. *Nature* **458**, 351–356 (2009).

11. Chaudhry, A. et al. CD4+ regulatory T cells control Treg17 responses in a Stat3-dependent manner. *Science* **326**, 986–991 (2009).

12. Chinen, T. et al. The cytokines interleukin 27 and interferon-γ promote distinct Treg cell populations required to limit infection-induced pathology. *Immunity* **37**, 511–523 (2012).

13. Wyss, L. et al. Affinity for self antigen selects Treg cells with distinct functional properties. *Nat. Immunol.* **17**, 1093–1101 (2016).

14. Kamada, T. et al. PD-1 expression balance between effector and regulatory T cells predicts the clinical efficacy of PD-1 blockade therapies. *Nature* **517**, 1346–1358 (2020).

15. Oldenhove, G. et al. Decrease of Foxp3+ Treg cell number and acquisition of effector T cell phenotype during lethal infection. *Immunity* **31**, 772–786 (2009).

16. Oldenhove, G. et al. Decrease of Foxp3+ Treg cell number and acquisition of effector T cell phenotype during lethal infection. *Immunity* **31**, 772–786 (2009).

17. Chen, X. et al. PD-1 regulates extrathymic regulatory T-cell differentiation. *Eur. J. Immunol.* **44**, 3560–3572 (2014).

18. Belkaid, Y., Piccirillo, C. A., Mendez, S., Shevach, E. M. & Sacks, D. L. CD4+CD25+ regulatory T cells control *Leishmania major* persistence and immunity. *Nature* **420**, 502–507 (2002).

19. Maitzels, R. M. & Smith, K. A. Regulatory T Cells in Infection. *Advances in Immunology* Vol. 112 (Elsevier, 2011).

20. Benson, A. et al. Microbial infection-induced expansion of effector T cells overrides the suppressive effects of regulatory T cells via an IL-2 deprivation mechanism. *J. Immunol.* **188**, 800–810 (2012).

21. Oldenhove, G. et al. Decrease of Foxp3+ Treg cell number and acquisition of effector T cell phenotype during lethal infection. *Immunity* **31**, 772–786 (2009).

22. Moran, A. E. et al. T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. *J. Exp. Med.* **208**, 1279–1289 (2011).

23. Thornton, A. M. et al. Helios+ and Helios− Treg subpopulations are phenotypically and functionally distinct and express dissimilar TCR repertoires. *Eur. J. Immunol.* **49**, 398–412 (2019).

24. Kornette, M., Mason, E., Istomine, R. & Piccirillo, C. A. KLrg1 expression identifies short-lived Foxp3+ Treg effector cells with functional plasticity in islets of NOD mice. *Autoimmunity* **50**, 1–9 (2017).

25. Smigiel, K. S. et al. CCR7 provides localized access to IL-2 and defines homeostatically distinct regulatory T cell subsets. *J. Exp. Med.* **211**, 123–136 (2014).

26. van Loosdregt, J. et al. Canonical Wnt signaling negatively modulates regulatory T cell function. *Immunity* **59**, 298–310 (2013).

27. Garcia-Diaz, A. et al. Interferon receptor signaling pathways regulating PD-1+ and PD-L2 expression. *Cell Rep.* **19**, 1189–1201 (2017).

28. Gazzinelli, R. T. et al. Parasite-induced IL-12 stimulates early IFN-γ synthesis and resistance during acute infection with *Toxoplasma gondii*. *J. Immunol.* **153**, 2533–2534 (1994).

29. Chikuma, S. et al. PD-1-mediated suppression of IL-2 production induces CD8+ T cell anergy in vivo. *J. Immunol.* **182**, 6682–6689 (2009).

30. Parry, R. V. et al. PD-1 restraint of regulatory T cell suppressive activity is critical for immune tolerance. *J. Exp. Med.* https://doi.org/10.1084/jem.20182232 (2020).

31. Francisco, L. M. et al. PD-1 regulates the development, maintenance, and function of induced regulatory T cells. *J. Exp. Med.* **206**, 3015–3029 (2009).

32. Huang, A. et al. A single dose of neoadjuvant PD-1 blockade predicts clinical outcomes in resectable melanoma. *Nat. Med.* **25**, 454–461 (2019).

33. Chen, X. et al. Genetic absence of PD-1 promotes accumulation of terminally differentiated exhausted CD8+ T cells. *J. Exp. Med.* **212**, 1125–1137 (2015).

34. Hui, E. et al. T cell costimulatory receptor CD28 is a primary target for PD-1–mediated inhibition. *Science*. eaaf1297 (2019).

35. Odorizzi, P. M., Paunen, K. E., Paley, M. A., Sharpe, A. & Wherry, E. J. Genetic absence of PD-1 promotes accumulation of terminally differentiated exhausted CD8+ T cells. *J. Exp. Med.* **212**, 1125–1137 (2015).

36. Wu, E. et al. T cell regulatory T cell function. *Nat. Immunol.* **15**, 909–914 (2018).

37. Wyss, L. et al. Affinity for self antigen selects Treg cells with distinct functional properties. *Nat. Immunol.* **17**, 1093–1101 (2016).

38. Kemper, C. et al. PD-1 expression balance between effector and regulatory T cells predicts the clinical efficacy of PD-1 blockade therapies. *Nature* **517**, 1346–1358 (2020).

39. Chen, X. et al. PD-1 regulates extrathymic regulatory T-cell differentiation. *Eur. J. Immunol.* **44**, 2603–2616 (2014).

40. Elledst, K. K., Thangavelu, G., Ewen, C. L., Boon, L. & Anderson, C. C. PD-1 is not required for natural or peripherally induced regulatory T cells: severe autoimmunity despite normal production of regulatory T cells. *Eur. J. Immunol.* **44**, 3560–3572 (2014).

41. Belkaid, Y., Piccirillo, C. A., Mendez, S., Shevach, E. M. & Sacks, D. L. CD4+CD25+ regulatory T cells control *Leishmania major* persistence and immunity. *Nature* **420**, 502–507 (2002).

42. Naizels, R. M. & Smith, K. A. Regulatory T Cells in Infection. *Advances in Immunology* Vol. 112 (Elsevier, 2011).

43. Benson, A. et al. Microbial infection-induced expansion of effector T cells overcomes the suppressive effects of regulatory T cells via an IL-2 deprivation mechanism. *J. Immunol.* **188**, 800–810 (2012).

44. Oldenhove, G. et al. Decrease of Foxp3+ Treg cell number and acquisition of effector T cell phenotype during lethal infection. *Immunity* **31**, 772–786 (2009).

45. Moran, A. E. et al. T cell receptor signal strength in Treg and iNKT cell development demonstrated by a novel fluorescent reporter mouse. *J. Exp. Med.* **208**, 1279–1289 (2011).

46. Thornton, A. M. et al. Helios+ and Helios− Treg subpopulations are phenotypically and functionally distinct and express dissimilar TCR repertoires. *Eur. J. Immunol.* **49**, 398–412 (2019).
Methods

Mice. All mice used were housed in the University of Pennsylvania Department of Pathobiology vivarium with 12-h light and dark cycles, maintained at temperatures ranging between 68–77 °F and humidity ranges from 35–55% in accordance with institutional guidelines. For all strains of mice used in this study, both males and females were used. C57BL/6 mice were purchased from Taconic (Rensselaer) at 6 weeks of age and housed in the University of Pennsylvania Department of Pathobiology vivarium for 2–4 weeks until used. LysMcre × STAT1−/− mice were generated as previously described, whereas CD11ccre mice (stock no. 018967) were acquired from the Jackson Laboratory and crossed with STAT1−/− mice to generate CD11ccre × STAT1−/− mice. Foxp3EGFP reporter mice (stock no. 006772) were acquired from the Jackson Laboratory. PD-1−/− mice bred on the C57BL/6 Ntag background were created by deleting exons 2 and 3 from the Pdcd1 locus through use of CRISPR/Cas9 gene editing at Taconic Artesis. These PD-1−/− mice were then bred and maintained by Taconic Biosciences on behalf of Merck & Co. and shipped to the University of Pennsylvania. Nap77−/− mice on the C57BL/6/SJL background were acquired from the Jackson Laboratory. The mice were shipped to the University of Pennsylvania vivarium and bred in accordance with institutional guidelines. Foxp3EGFP × PD-1−/− mice bred at the University of Pennsylvania were PCR screened using digested tail tips, with a Foxp3EGFP forward primer sequence of: 5′-AGG ATG TGA GGG ACT ACC TCC TGT A-3′ and a reverse primer sequence of: 5′-GTC TCA ACA GAG GCC AGA GG-3′, which bound upstream of the loxP sequence; in addition to a third primer, which bound downstream of the loxP sequence; in addition to a third primer, which bound upstream of the loxP sequence; in addition to a third primer, which bound downstream of the loxP sequence; in addition to a third primer, which bound upstream of the loxP sequence; in addition to a third primer, which bound downstream of the loxP sequence. PCR screening was repeated three times after infection and then analyzed for cytokine concentration by ELISA. The cells were then washed and stored on ice. Lungs were collected and digested with 1 mg/ml Collagenase II (Sigma) supplemented with 0.5 mg/ml DNase I (Sigma) in complete RPMI for 45 min at 37 °C. The digested lungs were then passed through a 70-μm nylon minifilter and washed with 10 ml of complete RPMI. For liver preparations, the left renal artery was severed and the liver was perfused using 10 ml of 1× DPBS. The gallbladder was removed and the lobes of the liver were mechanically processed over a 70-μm nylon filter and washed. The single-cell preparations were then resuspended in 20 ml of 37.5% Percoll and centrifuged at 500g for 20 min at room temperature. The pellet was then resuspended in NH4Cl solution for red blood cell lysis and the cells were then washed and stored on ice.

Analysis by flow cytometry. Staining antibodies and staining reagents. Antibody viability dye, Fc block, dilutions and buffer reagent details are supplementary (Supplementary Table 1).

t cell staining. Aliquots consisting of 5×10⁶ cells were washed with ice-cold 1× DPBS in a 96-well-bottom plate, then incubated in 50 μl of vibration reconstituted in 1× DPBS for 20 min on ice and then washed in 0.2% FCS buffer. The cells were then incubated in 50 μl volume of Fc block for 30 min on ice. The cells were then washed with 0.2% FACS buffer and were stained in 50 μl volume of 0.2% FACS buffer supplemented with tetramers loaded with the parasite-specific peptides AS15 and Tgβ507 for 30 min on ice. The cells were washed in 0.2% FACS buffer and then incubated for 30 min on ice in 50 μl volume of antibody cocktail composed of surface-stain antibodies in 0.2% FACS buffer supplemented with 0.2% FACS buffer for flow cytometry analysis. The cells were washed in 0.2% FACS buffer and resuspended in 100 μl Fox3 Perm-fix cocktail (00-5232-00, Thermo Fisher Scientific) for 4 h at 4 °C. The cells were then washed twice in 1× permeabilization buffer and then resuspended in an intracellular staining cocktail composed of intracellular-stain antibodies diluted in 1× permeabilization buffer supplemented with normal goat serum of 2 h at 4 °C. The cells were then washed twice with 1× permeabilization buffer and then resuspended in 50 μl of goat anti-rabbit detection antibody diluted in 1× permeabilization buffer for 2 h at 4 °C. The cells were then washed and resuspended in 500 μl 0.2% FACS buffer for flow cytometry analysis.

Cytokine staining. To detect intracellular cytokines on T cells, cells were resuspended in a stimulation cocktail of 0.05 ng/ml IFN-γ (Sigma), 0.5 ng/ml IL-12p40 (BioXcell), 5 μg/ml IL-10 (BioLegend) and 0.75 μg/ml Golgi stop (BD Biosciences, 554724) in cRPMI for 2 h at 37 °C and 5% CO₂. Cells were then washed and surface stained and permeabilized as described above for T cell staining. The cytokine-stain-prepped cells were then intracellularly stained with a cytokine detection panel for 2 h on ice. The cells were washed and resuspended in 500 μl 0.2% FACS buffer for analysis.

Myeloid staining. Aliquots of 5×10⁶ cells were washed in ice-cold 0.2% FACS buffer in a 96-well plate and were resuspended in 50 μl stain cocktail of anti-rat IgG2b-detecting antibody in 0.2% FACS buffer for 15 min on ice. The cells were washed, viability stained and then FC-blocked as described in the T cell staining section. The cells were surface stained in 50 μl antibody cocktail consisting of dilution 1:100 with 0.2% FACS buffer supplemented with brilliant stain buffer on ice for 30 min. For stains including IL-12p40, cells were washed, permeabilized and intracellularly stained as described in the T cell staining section. For stains not requiring permeabilization, cells were washed and fixed with 2% PFA (15710-S, Electron Microscopy Sciences) and diluted in 0.2% FACS buffer for 15 min at room temperature. The cells were washed and then resuspended in 500 μl 0.2% FACS buffer for analysis.

Data acquisition. The cells were analyzed on a FACs Symphony A5 (BD Biosciences) using BD FACSDiva v.9.0 (BD Biosciences). Analysis was performed with FlowJo (v.10.5.3, BD Biosciences).

T cell sorting. To compare transcriptional profiles of cBD (CD25+ and PD-1−) to cBD (CD25− and PD-1+) cell subsets, Foxp3EGFP mice were treated with a single i.p. dose of 1 mg of isotype or PD-L1 antibody and their splenocytes were collected 72 h later. Splenocytes were CD4+ enriched using the EasySep Mouse CD4+ T cell enrichment cocktail (StemCell Technologies) following the manufacturer's instructions. Flow cytometry was performed on a BD LSR II (BD Biosciences) using BD FACSDiva v.9.0. Data were analyzed using FlowJo (v.10.5.3, BD Biosciences) and GraphPad Prism 7.0 (GraphPad Software Inc.). Statistics were performed using Kruskal-Wallis tests followed by post hoc Dunn's tests using GraphPad Prism 7.0 (GraphPad Software Inc.).
RNA-seq and analysis. Messenger RNA was isolated from sorted Treg and eTreg cells using an RNeasy Micro Plus kit (QIAGEN) and quality was assessed using a High Sensitivity RNA Screen Tape on a 4200 TapeStation (Agilent). A complementary DNA library and adaptors were prepared with Clontech SMART-Seq HT kit ( Takara). Primer cleanup was performed with AMPure XP beads (Beckman Coulter). The 75-base pair reads were sequenced on a NextSeq 500 machine (Illumina) according to the manufacturer’s protocol. Reads were pseudo-aligned to the mouse genome using with Kallisto v.0.46.1 (ref. 61). Gene counts <1 in three samples were excluded from analysis. Normalized counts per million were analyzed for differential genes in R using the limma package62 with a log, fold change cutoff of 0.3. Heat maps were generated with the heatmaply package63, GSEA was performed with GSEA software v.4.0.3 (Broad Institute) with gene sets containing fewer than ten genes being excluded. Enrichment plots were generated in Cytoscape v.3.7.1 (National Institute of General Medical Sciences) with a P value cutoff of 0.05 and false discovery rate cutoff of 0.1.

Statistics. Statistical analysis was performed using Prism 8 for Windows (v.8.4.3). For comparison of means between two groups, a two-tailed unpaired Student’s t-test was utilized with a 95% CI. Analysis for univariate statistics comparing multiple means was performed using a one-way ANOVA (family-wise significance and 95% CI), with post hoc analysis consisting of Fisher’s LSD test for direct comparison of two means within the ANOVA or Tukey’s multiple comparisons test for comparisons of all means within the test group for multiple-comparison correction. For multi-group multivariate analysis, we used a two-way ANOVA with post hoc analysis, utilizing Sidak’s multiple comparisons test for comparisons across two groups with two variables or Tukey’s multiple comparisons test for comparisons across multiple groups for multiple variables (also with a 95% CI). P values <0.05 were considered statistically significant. All error bars in the figures indicate s.e.m.

UMAP analysis. UMAP analysis was performed using the UMAP plug-in using the Euclidean distance function with a nearest neighbor score of 20 and a minimum Euclidean distance of 0.5 (v.1802.03426, 2018, 2017, Leland McInnes) for FlowJo (v.10.53). All stained parameters were included in UMAP analysis except for: Live Dead ( gated out), CD4 (pre-gated), PD-1 (avoiding grouping bias), Foxp3 (avoiding grouping bias or already pre-gated) and in instances of PD-L1 blockade treatment, PD-L1 was excluded from UMAP cluster analysis for UMAP figures depicting bulk CD4+ T cell, Treg cell or CD4+tetramer+ T cell UMAP images. The plots generated by UMAP Euclidian algorithms were analyzed via the X-shift tool (v.1.13) to identify unique clusters determined by the UMAP using a weighted k-nearest-neighbor density estimation plot. This strategy classifies and defines membership of a cell event into unique cluster groups based on a plurality of observations made from the nearest neighbor events in the UMAP. The X-shift-identified clusters were then labeled and subsequently interpreted using the ClusterExplorer (v.1.2.2) tool, which compares X-shift clusters to highlight differences in expression.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
RNA-seq datasets discussed in this publication have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus and are accessible through accession no. GSE186350. The remaining data that support the findings of this study are available on request from the corresponding author. Source data are provided with this paper.

References
53. Wang, S. et al. Infection-induced intestinal dysbiosis is mediated by macrophage activation and nitrate production. MBio 10, 1–13 (2019).
54. Klover, P., et al. Loss of STAT1 from mouse mammary epithelium results in an increased neo-induced tumor burden. Neoplasia 12, 899–905 (2010).
55. Md Sakib Hossain, D. et al. Dinaciclib induces immunogenic cell death and enhances anti-PD1-mediated tumor suppression. J. Clin. Invest. 128, 644–654 (2018).
56. Bray, N. L., Pimentel, H., Melsted, P. & Pachter, L. Near-optimal probabilistic RNA-seq quantification. Nat. Biotechnol. 34, 525–527 (2016).
57. Ritchie, M. E. et al. Limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. 43, e47 (2015).
58. Galili, T., O’Callaghan, A., Sidi, J. & Sievert, C. Heatmaply: an R package for creating interactive cluster heatmaps for online publishing. Bioinformatics 34, 1600–1602 (2018).
59. Samuisk, N., Good, Z., Spitzer, M. H., Davis, K. L. & Nolan, G. P. Automated mapping of phenotype space with single-cell data. Nat. Methods 13, 493–496 (2016).

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Author contributions
J.A.P. conceptualized the project, designed/executed all experiments and performed data analysis, produced figures and wrote the paper. L.S. performed RNA-seq, including the subsequent transcriptomic analysis and wrote the results of RNA-seq data. J.T.C., J.A.G., J.H.D., B.B.D., A.P.H., Z.L., K.O., C.K. and J.P. participated in the experiments and conceptual feedback regarding experimental design, data analysis and manuscript editing. J.R.K., R.W.M. and A.H.P. provided conceptual feedback and generated and supplied animal models (PD-1 KO and Foxp3creERT2). J.A.P. conceptualized the project, designed/executed all experiments and performed data analysis, produced figures and wrote the paper. L.S. performed RNA-seq, including the subsequent transcriptomic analysis and wrote the results of RNA-seq data. J.T.C., J.A.G., J.H.D., B.B.D., A.P.H., Z.L., K.O., C.K. and J.P. participated in the experiments and conceptual feedback regarding experimental design, data analysis and manuscript editing. J.R.K., R.W.M. and A.H.P. provided conceptual feedback and generated and supplied animal models (PD-1 KO and Foxp3creERT2). D.A.C. directly supervised experimental execution and interpretation of data and C.A.H. supervised the project in its entirety. Every author evaluated and approved this manuscript. The data presented in this manuscript were reviewed in raw form by the authors and the appropriate statistical tests were applied. The figures are accurate representations of the data and there are no manipulations of images except for general resizing for publishing. The journal policies of materials, data sharing, ethical animal use and conflicts of interest have been adhered to. We are confident that the conclusions presented here are based on accurate interpretations of the data collected for this study. Our colleagues listed as co-authors have contributed to and have earned the author designation for this manuscript.

Competing interests
At the time of the study, R.W.M. was employed by Merck & Co Inc., Palo Alto and has declared no financial interest in Merck & Co Inc. The remaining authors declare no competing interests.

Additional information
Extended data is available for this paper at https://doi.org/10.1038/s41590-022-01170-w. Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41590-022-01170-w.
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Extended Data Fig. 1 | Treg cell heterogeneity at homeostasis and Treg cell expression of PD-1. (a) Splenocytes from naïve 8 week-old male C57BL/6 mice were analyzed via high-parameter flow cytometry to identify CD4+ T cells and subset them into Foxp3+ and Foxp3− subsets, depicted is the gating strategy to identify Treg and Tconv CD4+ T cells. (b) Qualitative analysis of bulk CD3+, CD4+ T cells was conducted to produce a 2-dimensional UMAP representation using dimensional reduction algorithms (excluding CD4, Foxp3 and PD-1 expression as calculated factors). (c-d) Regions of CD4+ T cells expressing Foxp3 and or PD-1 were identified via median heatmap of expression of the generated UMAP plot. (e) The initial distribution UMAP was then qualitatively assessed using median heatmap distribution trends amongst the bulk CD4+ T cell pool of Treg cell associated proteins: Helios, GITR, CD25, PD-L1, and CTLA-4, in addition to proteins associated with effector function in Tregs (f) KLRG1, CD73, and ICOS. (g) Histogram comparisons were then made and quantified between Foxp3+ and Foxp3− subsets for the inhibitory proteins CTLA-4 and PD-L1 (n = 5/group, 2 way ANOVA with Tukey multiple comparisons test, *** = p < 0.0001, 6 experimental replicates). (h) Proteins associated with activation (CD69, CD11a, CD44, ICOS, and CD127) were also compared and quantified (n = 5/group, 2 way ANOVA with Tukey multiple comparisons test, *** = p < 0.0001, 6 experimental replicates). All data presented are means ± SEM and show individual data points.
Extended Data Fig. 2 | See next page for caption.
Extended Data Fig. 2 | Qualitative X-shift identification of Treg heterogeneity in the PD-1hi cluster of Treg cells. (a) Splenocytes from naïve 8 week-old male C57BL/6 mice were analyzed via high-parameter flow cytometry to identify CD4+ T cells and were then grouped into Foxp3+ and Foxp3− subsets. (b) CD4+ Foxp3+ T cells were then subset into PD-1−, PD-1−, and PD-1hi groups using a PD-1 KO host as a negative stain comparative control. (c) UMAP qualitative analysis was generated specifically on CD4+ Foxp3+ T cells (Treg cells), excluding CD4, PD-1, and Foxp3 as variables in the calculation. (D) Depiction of PD-1 expression as a median heatmap amongst the Treg cell UMAP. (e) The Treg cell UMAP was then reanalyzed via the X-shift algorithm (excluding CD4, PD-1, and Foxp3 from the calculation) to potentially identify Treg subsets as clusters within the UMAP, with each X-shift identified subset depicted as a separate color. (f) Within the same UMAP, the PD-1−, PD-1−, and PD-1hi groups are portrayed as black, blue, and red respectively, to compare the location of these subsets to the locations of the X-shift identified Treg subsets. (g) Graphed MFI of fluorescence of stained proteins on these cells identified in the UMAP X-shift analysis to qualitatively compare different trends amongst the Treg cell clusters at homeostasis. (h) UMAP qualitative analysis on splenocyte-derived Treg cells from naïve C57BL/6 mice following stimulation and cytokine staining. (i) Heatmaps of median expression of IL-10 and PD-1 within the UMAP generated in H. (j) Re-analysis via X-shift algorithm to identify unique clusters within the cytokine-stain UMAP, indicated by separate colors in the plot. (k) Overlay of PD-1−, PD-1−, and PD-1hi subsets within the cytokine-stain UMAP.
Extended Data Fig. 3 | Constitutive PD-L1 expression at homeostasis and anti-PD-L1 blocking antibody detection. (a) Splenocytes from 8 week-old male C57BL/6 mice were qualitatively analyzed for PD-L1 expression compared to an FMO (fluorescence minus one) via flow cytometry across multiple leukocyte populations: T reg cells (CD3⁺, CD4⁺, Foxp3⁺), B cells (CD3⁻, B220⁺, CD19⁺), cDC1s (CD3⁻, B220⁻, CD19⁻, NK1.1⁻, Ly6G⁻, CD64⁻, CD11c⁺, MHC-II⁺, XCR1⁺), cDC2s (CD3⁻, B220⁻, CD19⁻, NK1.1⁻, Ly6G⁻, CD64⁺, CD11c⁻, MHC-II⁺, SIRPa⁺), and macrophages (CD3⁻, B220⁻, CD19⁻, NK1.1⁻, Ly6G⁺, CD64⁺, CD11b⁻, MHC-II⁺, Ly6C⁻/⁻). (b) Groups of 9 week-old male C57BL/6 mice were treated with an IP injection of isotype (Rat IgG2b) (n = 4) or anti-PD-L1 blocking antibody (n = 5) for 72 hours. Splenocytes from these groups were then harvested and stained with an anti-Rat-IgG2b FITC antibody to determine if the PD-L1 blocking antibody was opsonizing the previously identified PD-L1⁺ subsets (Tregs, B cells, cDC1s, cDC2s, and Macrophages). The anti-PD-L1 blocking antibody was readily detected while subsets from the isotype treated animals had minimal anti-Rat-IgG2b staining (2-way ANOVA with Sidak’s multiple comparisons test, * = p = 0.0222, ** = p = 0.0044, *** = p = 0.0004, **** = p < 0.0001, 3 experimental replicates). (C) Example gating strategy using splenocytes from a naïve C57BL/6 host, for the populations identified in (A), starting with singlet cells, and refining down to B cells, neutrophils, monocytes, macrophages, cDC1s, and cDC2s. All data presented are means +/- SEM and show individual data points.
Extended Data Fig. 4 | Anti-PD-L1 blockade results in increased eT reg cell activation and proliferation in naïve hosts. (a–c) 9 week-old male Nur77GFP reporter mice were treated with a single dose of isotype or anti-PD-L1 blocking antibody for 72 hours. Splenocytes were then harvested and assessed via high-parameter flow cytometry. T reg cell data was then concatenated between the isotype and anti-PD-L1 treated groups, and the subsequent qualitative interpretation was conducted via UMAP analysis (excluding Foxp3, PD-1, PD-L1, and CD4 as calculation factors). (a) Side-by-side pseudo-color density plot comparison of T reg cells from isotype and anti-PD-L1 treated hosts depicting regional shifts within the same UMAP calculation. (b) Heatmap expression analysis across the total combined UMAP data from both groups, depicting median heat maps of TCR activation associated proteins Nur77, CD11a, and Ki67, with overlapping enrichment of activated T reg cells in anti-PD-L1 treated hosts. (c) Additional heatmap analysis of T reg cell associated CD25, inhibitory receptors PD-1 and CTLA-4, and KLRG1, with an enrichment of overlap between PD-1, CTLA-4, and KLRG1 expression in context of PD-L1 blockade. (d–f) 9 week-old male C57BL/6 mice were also treated with a single dose of isotype (n=4) or anti-PD-L1 blocking antibody (n=5) for 72 hours, and their splenocytes were also isolated and analyzed via high-parameter flow cytometry. (d) Flow plot data of splenic T reg cells from isotype and anti-PD-L1 treated hosts comparing changes to the PD-1+ CTLA-4hi subset following PD-L1 blockade (two-tailed unpaired student’s t-test, * = p = 0.0394, 4 experimental replicates). (e) T reg cells from isotype and PD-L1 blockade treated hosts, gated on activated (CD11a+), indicating an increase in PD-1+ T reg cells in cell cycle following treatment (2-way ANOVA with Fisher’s LSD individual comparisons test, * = p = 0.0032, ** = p = 0.0037, 4 experimental replicates). (f) Gating strategy utilized for flow cytometry sorting to isolate cT reg cells (CD25+ PD-1−) vs eT reg cells (CD25− PD-1+). (g) Flow cytometry data of Treg, CD4+ Tconv, and CD8+ T cells for the expression of Ki67 following 96 hours of tacrolimus (FK506) treatment (n = 5/group two-tailed unpaired student’s t-test, **** = p < 0.0001, 2 experimental replicates). All data presented are means +/- SEM and show individual data points.
Extended Data Fig. 5 | The development of homeostatic eTregs is not dependent on PD-1, and eTregs are limited by PD-1. (a-f) Splenocytes from naïve 8 week-old female C57BL/6 mice or total PD-1−/− mice were isolated and analyzed via high-parameter flow cytometry. (a) Pre-gated CD4+ T cells gated on Foxp3+ events (T reg cells) depicting an enrichment of T reg cells at homeostasis in PD-1−/− age matched hosts (n = 3/group two-tailed unpaired student's t-test, ** = p = 0.0037, 4 experimental replicates). (b) Comparative flow plots of T reg cells between C57BL/6 and PD-1−/− hosts with gating on activated T reg cells in cell cycle (CD11a+ Ki67+), demonstrating an increase in T reg cells undergoing proliferation at homeostasis in PD-1−/− hosts (n = 3/group two-tailed unpaired student's t-test, ** = p = 0.0044, 4 experimental replicates). (c) T reg cell staining of ICOS and CTLA-4, depicting the proportion and number of eT reg-associated (ICOS+ CTLA-4hi) T reg cells is increased in PD-1−/− mice (n = 3/group two-tailed unpaired student's t-test, ** = p = 0.0022, 4 experimental replicates), while (d) demonstrates this enhancement is specific to the eT reg compartment (BCL-2low, CD25low), as the non-eT reg compartment (BCL-2hi, CD25hi) is consistent in number when compared to C57BL/6 mice (n = 5/group, 2-way ANOVA with Sidak's multiple comparisons test, *** = p = 0.0001, 3 experimental replicates). Splenocytes from isotype and anti-PD-L1 treated groups were also stimulated and stained for IL-10 and analyzed via flow cytometry. (e) Flow plots of T reg cells from C57BL/6 and PD-1−/− hosts gated on CD11a+ IL-10+ events, depicting an increase in the proportion and number of IL-10+ T reg cells in PD-1−/− hosts (n = 3/group two-tailed unpaired student's t-test, ** = p = 0.0011, 3 experimental replicates). (f) Splenic cDC2 subsets were identified via flow cytometry (CD3−, B220−, CD19−, NK1.1−, Ly6G−, CD64+, CD11c+, MHC-II+, SIRPa+), and gated on CD80+ events based on an FMO (n = 4/group, two-tailed unpaired student's t test, * = p = 0.0122, 2 experimental replicates). All data presented are means +/- SEM and show individual data points.
Extended Data Fig. 6 IFN-γ mediated changes to myeloid PD-L1 expression. Cohorts of 8 week-old male C57BL/6 mice (n = 10/group) were treated with an isotype antibody or IFNγ blocking antibody and half of each group (n = 5) were infected with 20 cysts of ME49 intraperitoneally (IP). Splenocytes and Peritoneal exudate cells (PEC) were isolated 72 hours later and analyzed via high-parameter flow cytometry. (a) Comparative histograms evaluating 72 hour timepoint changes in the MFI of PD-L1 expression amongst splenocytes between experimental groups within leukocyte subsets: neutrophils (CD3−, B220−, CD19−, NK1.1−, Ly6G−, Ly6C+, CD11b+), cDC1s (CD3−, B220−, CD19−, NK1.1−, Ly6G−, Ly6C+, CD11c+, MHC-II+, XCR1+), cDC2s (CD3−, B220−, CD19−, NK1.1−, Ly6G−, CD64−, CD11c−, MHC-II+, SIRPα+), monocytes (CD3−, B220−, CD19−, NK1.1−, Ly6G−, CD64−, CD11b−, MHC-II+, Ly6C−), macrophages (CD3−, B220−, CD19−, NK1.1−, Ly6G−, CD64−, CD11b−, MHC-II+, Ly6C+) and T reg cells (B220−, CD19−, Ly6G−, NK1.1−, CD3+, CD4+, Foxp3+) (n = 5/group, 2-way ANOVA with Tukey’s multiple comparisons test, * = p = 0.0239, ** = p < 0.01, **** = p < 0.0001, 2 experimental replicates). (b) Cohorts of 8 week-old female STAT1flox mice without any cre expressing alleles (n = 5), or STAT1flox mice crossed onto either the CD11ccre (n = 4) or LysMcre (n = 5) background were infected with 20 cysts of ME49 IP. Splenocytes and PEC were harvested on day 7 of infection and analyzed via flow-cytometry. (b) Histogram comparisons of PD-L1 MFI changes in splenic monocytes and macrophages following conditional deletion of STAT1 (2-way ANOVA with Tukey’s multiple comparisons test, * = p = 0.0475, **** = p < 0.0001, 2 experimental replicates). All data presented are means +/− SEM and show individual data points.
Extended Data Fig. 7 | Impact of PD-L1 blockade across tissues and infection. (a–d) Cohorts of 8 week-old male C57BL/6 mice were treated with an isotype antibody or PD-L1 blocking antibody 24 hours prior to infection with 20 cysts of ME49 IP. The antibody treatments were repeated every 72 hours throughout the course of infection until the mice were killed on day 10 and PEC, spleen, and liver were harvested and analyzed via high-parameter flow cytometry. (a–b) Flow plots of bulk CD4+ T cells with subsequent gates on the Foxp3+ T cells (T reg cells) for liver (A) and PEC (B), demonstrating the drop in T reg cells from homeostatic levels during infection, and the maintenance of T reg cells during infection with PD-L1 blocking antibody treatment (n = 5/group, two-tailed unpaired student’s t-test, * = p = 0.0153, ** = p = 0.0088, 4 experimental replicates). (c–d) Flow plots of T reg cells from treatment groups showing enrichment of PD-1hi T reg compartment as a consequence of PD-L1 blockade treatment during infection in liver (C), and PEC (D) (n = 5/group, two-tailed unpaired student’s t-test, ** = p = 0.0013, **** = p < 0.0001, 4 experimental replicates). (e) Cohorts of 8 week-old male C57BL/6 mice were treated with an isotype antibody (n = 5 uninfected, n = 5 infected) or PD-L1 blocking antibody (n = 4) 24 hours prior to intravenous infection with 10⁴ pfu of L. monocytogenes cysts of ME49 IP. The antibody treatments were repeated every 72 hours until splenocytes were harvested and analyzed via high-parameter flow cytometry on day 6 of infection. Depicted are flow plots of splenocyte-derived bulk CD4+ T cells gated on Foxp3+ T cells (T reg cells), (1-way ANOVA with Tukey’s multiple comparisons test, Isotype naïve vs Isotype infected: * = p = 0.0033, Isotype infected vs anti-PD-L1 infected: ** = p = 0.0055, 1 experimental replicate). (f) Splenocyte-derived flow plots of cDC2s from cohorts of 8 week-old male C57BL/6 mice at day 10 of infection with T. gondii (20 cysts ME49 IP), that had been treated with isotype (n = 5), anti-PD-L1 (n = 5), with the inclusion of an additional cohort treated with a combination of blocking anti-IL-10r/anti-PD-L1 antibodies (n = 5) depicting ex vivo changes in the proportion of CD80+ CD86+ cells (n = 5/group, 1-way ANOVA with Tukey’s multiple comparisons test, * = p = 0.0117, ** = p = 0.0056, **** = p < 0.0001, 2 experimental replicates). All data presented are means ±/− SEM and show individual data points.
Extended Data Fig. 8 | During infection, PD-1−/− mice maintain an increased eT reg pool with diminished parasite-specific responses. (a–g) 8 week-old female C57BL/6 and PD-1−/− mice were IP infected with 20 cysts of T. gondii and splenocytes were harvested and analyzed via flow cytometry at day 10 of infection. (a) Plots of CD4+ T cells from C57BL/6 (n = 3) and PD-1−/− (n = 5) mice with gating on Foxp3+ events (T reg cells) depicting a preservation of T reg cells in PD-1−/− hosts during infection (two-tailed unpaired student’s t-test, *p = 0.0158, 3 experimental replicates). (b) T reg cell staining of BCL-2 and CD25, demonstrating an eT reg specific increase (BCL-2low, CD25low), as the non-eT reg compartment (BCL-2hi, CD25hi) is consistent in number when comparing C57BL/6 (n = 3) and PD-1−/− (n = 5) mice (2-way ANOVA with Sidak’s multiple comparisons test, **p = 0.0020, 3 experimental replicates). (c) Plots depicting an increase in the proportion and number of eT reg-associated (ICOS+ CTLA-4hi) T reg cells when comparing C57BL/6 (n = 3) to PD-1−/− (n = 5) mice (two-tailed unpaired student’s t-test, *p = 0.0177, 3 experimental replicates). (d) Splenic cDC2 subsets were identified via flow cytometry (CD3−, B220−, CD19−, NK1.1−, Ly6G−, CD64−, CD11c+, MHC-II+, SIRPα+), and gated on CD80+ events based on an FMO, comparing the proportion of CD80+ cDC2 events between C57BL/6 (n = 5) and PD-1−/− (n = 5) mice (two-tailed unpaired student’s t-test, **p = 0.0017, 2 experimental replicates). (e–g) Splenocytes from infected hosts were tetramer stained using the toxoplasma specific AS15 peptide, and the number of CD11ahi parasite-specific CD4+ T cells was compared between C57BL/6 (n = 3) and PD-1−/− (n = 4) mice (two-tailed unpaired student’s t-test, *p = 0.0108, 3 experimental replicates). (f) While the phenotype of the parasite specific CD4+ T cells (CD11a+ Tetramer+) was evaluated for the expression of KLRG1 and T-bet, resulting in a loss of observed Tbet+ KLRG1+ parasite specific T cells in PD-1−/− hosts (two-tailed unpaired student’s t-test, *p = 0.0012, 3 experimental replicates). (h) Parasite burden was assessed via qPCR from tissue samples of lungs, liver, and heart at day 10 of infection, resulting in no significant differences in parasite burden (n = 5/group, 2-way ANOVA with Sidak’s multiple comparisons test, 3 experimental replicates). All data presented are means +/− SEM and show individual data points.
Extended Data Fig. 9 | Primary T. gondii infection depletes eTreg cell populations in both C57BL/6 and hemizygous Foxp3<sup>cre</sup> x PD-1<sup>wt/flox</sup> mice, while eTreg cells in homozygous Foxp3<sup>cre</sup> x PD-1<sup>flox/flox</sup> hosts are spared. (a–e) 8-week-old male C57BL/6 (n = 5), Foxp3<sup>cre</sup> x PD-1<sup>wt</sup>/<sup>flox</sup> (n = 6), and Foxp3<sup>cre</sup> x PD-1<sup>flox/flox</sup> (n = 5) mice were IP infected with 20 cysts of T. gondii (ME49 strain), at day 10 of infection splenocytes were harvested from each group and analyzed via high parameter flow cytometry. (a) Flow plots of bulk CD4<sup>+</sup> T cells from each infected group and were gated on Foxp3<sup>+</sup> events (Treg cells), depicting similar Treg depletion in C57BL/6 and hemizygous (PD-1<sup>wt/flox</sup>) groups, with increased Treg preservation in the homozygous (PD-1<sup>flox/flox</sup>) hosts (1-way ANOVA with Tukey’s multiple comparisons test * = p = 0.0227, ** = p = 0.0050, 2 experimental replicates). (b) Flow plots of splenic Treg cells depicting an increase in eTreg-associated ICOS<sup>+</sup> CTLA-4<sup>hi</sup> cells in the Foxp3<sup>cre</sup> x PD-1<sup>flox/flox</sup> group, but not the C57BL/6 or Foxp3<sup>cre</sup> x PD-1<sup>wt/flox</sup> cohorts (1-way ANOVA with Tukey’s multiple comparisons test, ** = p < 0.01, 2 experimental replicates). (c) Flow plots depicting enhancement to the eTreg associated BCL-2<sup>lo</sup> CD25<sup>lo</sup> compartment in Foxp3<sup>cre</sup> x PD-1<sup>flox/flox</sup> mice only, while the non-eTreg compartment (BCL-2<sup>hi</sup>, CD25<sup>hi</sup>) was consistent in number across all three groups (2-way ANOVA with Tukey’s multiple comparisons test *** = p = 0.0002, **** = p < 0.0001, 2 experimental replicates). Splenocytes from all three groups were also stimulated and stained for IL-10. (d) Flow plots of Treg cells and their expression of IL-10 vs CD11a. There is no significant change between C57BL/6 and hemizygous groups, however homozygous mice have a significant increase in the number and proportion of IL-10<sup>+</sup> Treg cells (1-way ANOVA with Tukey’s multiple comparisons test *** = p < 0.001). Splenocytes were permeabilized ex vivo and stained for the downstream TCR-activation protein Nur77 and analyzed via flow cytometry. (e) Treg cell plots from the three respective groups depicting no significant differences in Nur77<sup>+</sup> Treg cells between C57BL/6 and hemizygous groups, while Treg cells from homozygous hosts (Foxp3<sup>cre</sup> x PD-1<sup>flox/flox</sup>) have an increased proportion and number of Nur77<sup>+</sup> Treg cells compared to the other two groups during infection (1-way ANOVA with Tukey’s multiple comparisons test *** = p < 0.001). All data presented are means +/- SEM and show individual data points.
Reporting Summary

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Statistics

For all statistical analyses, confirm that the following items are present in the figure legend, table legend, main text, or Methods section.

- The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection | FACSDiva v9.0 (BD Biosciences), ViiA 7TM Software

Data analysis | Prism 8 for Windows (version 8.4.3); Flowjo (10.5.3, BD biosciences); uMAP Plugin, euclidian distance function, nearest neighbor score of 20, and minimum distance rating of 0.5 (version: 1802.03426, 2018, ©2017, Leland McInness); X-Shift plugin (version 1.3) Samusik et al. Nat Methods 2016; ClusterExplorer Tool (version 1.2.2); Microsoft Excel version 2011 (Build 13426.20332); Kallisto v0.46.1 Bray et al. Nat Biotechnol 2016; Limma v3.50.0, Ritchie et al. Nucleic Acids Res 2015; Heatmaply v1.0.0, Galili et al. Bioinformatics 2018; GSEA software v4.0.3 (Broad Institute), Cytoscape v3.7.1 (National Institute of General Medical Sciences).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

RNAseq datasets discussed in this publication have been deposited in NCBI's Gene Expression Omnibus70, and are accessible through GEO Series accession number GSE186350 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE186350). The remaining data that support the findings of this study are available on request from the corresponding author C.A. Hunter.
Initial exploratory experiments for this study began with samples of 5 mice per group and were scaled up or down based on animal model availability. These studies were preliminary to evaluate if any clear phenotypes emerged. As such, consistent trends in the data occurred that were significant with basic statistical comparisons of means testing (student's t test).

All experiments have been repeated at least 2 times, with two exceptions, 1) transcriptomic RNAseq data, however this was accommodated.

The animals used in this experiment were of inbred strains, as such experiments were meticulously age and gender matched between animals.

Specific lots for each antibody used has been provided in table 1.

Due to blinding was not possible as the data was analyzed by the same individual that performed the assays and data collection.

**Reporting for specific materials, systems and methods**

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| Materials & experimental systems | Methods |
|---------------------------------|---------|
| n/a                             | n/a     |
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| - Antibodies                   | - ChIP-seq |
| - Eukaryotic cell lines         | - Flow cytometry |
| - Palaeontology and archaeology | - MRI-based neuroimaging |
| - Animals and other organisms   |         |
| - Human research participants   |         |
| - Clinical data                 |         |
| - Dual use research of concern  |         |

**Antibodies**

Specific lots for each antibody used has been provided in table 1.

- CD4 (GK1.5, 613006, BD Biosciences), α-CD8 (53-6.7, 748535, BD Biosciences), α-CD11a (2D7, 741919, BD Biosciences), α-CD25 (PC61, 102051, Biologend), α-CD16/32 (2.4G2, B9307, Bioxcell) α-CD44 (IM7, 103037, Biologend), α-CD73 (TY/11.8, 127215, Biologend), α-KLRG1 (2F1, 740279, BD Biosciences), α-Granzyme B (XMG1.2, 612769, BD Biosciences), α-CMYc (9E10, MA1-980-D488, Thermo Fisher Scientific), α-Nur77 (12.14, 25-5965-82, Thermo Fisher Scientific), Normal Goat Serum (G9023-10ML, Sigma), and α-Tbet (4B10, 644827, Biologend).

- α-CD19 (R35-38, 612978, BD Biosciences), α-CD64 (G04-77, 107609, BD Biosciences), α-ICOS (C398.4, 133536, Biologend), α-CD3 (17A2, 100249, Biologend, CA, USA), α-Foxp3 (FJK-16s, 11-5773-82, Thermo Fisher Scientific), α-Helios (22F6, 137235, Biologend), α-K67 (856, 566172, BD Biosciences), α-BCL-2 (BCL1/10C4, 633510, Biologend), α-BIM (K.912.7, MA5-14848, Thermo Fisher Scientific), α-CTLA-4 (UC10-4F10-11, 565778, BD Biosciences), α-cMyc (9E10, MA1-980-D488, Thermo Fisher Scientific), α-Nur77 (12.14, 25-5965-82, Thermo Fisher Scientific), Normal Goat Serum (G9023-10ML, Sigma), and α-Tbet (4B10, 644827, Biologend).

- α-IFNy (XMG1.2, 612769, BD Biosciences), α-IL-10 (JES5-16E3, 505021, Biologend), α-CD3 (17A2, 100249, Biologend, CA, USA), α-Foxp3 (FJK-16s, 11-5773-82, Thermo Fisher Scientific), α-Helios (22F6, 137235, Biologend), α-K67 (856, 566172, BD Biosciences), α-BCL-2 (BCL1/10C4, 633510, Biologend), α-BIM (K.912.7, MA5-14848, Thermo Fisher Scientific), α-CTLA-4 (UC10-4F10-11, 565778, BD Biosciences), α-cMyc (9E10, MA1-980-D488, Thermo Fisher Scientific), α-Nur77 (12.14, 25-5965-82, Thermo Fisher Scientific), Normal Goat Serum (G9023-10ML, Sigma), and α-Tbet (4B10, 644827, Biologend).

- α-IL-2 (JES6-5H4, 25-7021-80, Thermo Fisher Scientific), α-µMHC (M5/114.15.2, 106763, Biologend), α-CD154 (P84, 144016, Biologend), and α-CD64 (X54-5/7.1, 139314, Biologend). For blockade, α-l-10r antibody (clone: 181.3A, BEO050, Bioxcell), and α-PD-L1 (clone: 10F.9G2,
Validation
Thermo-fisher verified the Foxp3 clone FJK-16s via relative expression to ensure the antibody is specific to the antigen Foxp3. This was done by comparing staining between CD4+ and CD8+ T cells, or by pre-gating on CD4+ CD25+ T cells, and demonstrating an enrichment of FJK-16s+ stained cells. The FJK-16s clone was further validated by staining Foxp3creFP reporter mice with a PE-conjugated clone of FJK-16s, resulting in a clear and concise double-positive event of YFP and PE when utilized in tandem as verification of the antibody. The clone for PD-1 (29F.1A12) was validated in the lab by staining a PD-1KO host (Presented in Figure 2D, and in Supplemental Figure 2B). The remaining clones utilized in this study from Thermo-Fisher/Invitrogen/ebiosciences, Biolegend, or BD Biosciences are established and well cited, there are no novel clones here that have not undergone validation, and the validation methods can be obtained by looking up the catalog #s provided above.

Animals and other organisms
Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals
Mice were exclusively used as the animal model in these studies. Mice were all utilized for study within the age ranges of 8-12 weeks old. All mice used were housed in the University of Pennsylvania Department of Pathobiology vivarium with 12 hour light and dark cycles, maintained at temperature ranges of 68° - 77°F and humidity ranges from 35% - 55% humidity in accordance with institutional guidelines. Within experiments, control mice and experimental mice were carefully age matched within 7 days of birth. The strains used were the C57BL/6 mouse from Taconic Laboratories, the PD-1KO on the C57BL/6 background from Merck and generated by Taconic Laboratories, and The Foxp3cre YFP x PD-1 flox mouse on the C57BL/6 background as generated in the Sharpe lab at Harvard and bred at the University of Pennsylvania. CD11cCre, LysMcre, and STAT1flox mice were acquired from Jackson laboratories and crossbred at the University of Pennsylvania. Male and female mice from all strains were used in this study based on litters available with gender-matched controls within individual experiments. Ethical oversight of all animal use in this study was approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Wild animals
No wild animals were used.

Field-collected samples
No field-collected samples were used.

Ethics oversight
Animal protocol approved by the University of Pennsylvania Institutional Animal Care and Use Committee

Note that full information on the approval of the study protocol must also be provided in the manuscript.

Flow Cytometry

Plots
Confirm that:
- The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).
- The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a ‘group’ is an analysis of identical markers).
- All plots are contour plots with outliers or pseudocolor plots.
- A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation
Tissue Preparation: Single cell suspensions were prepared from spleen, lung, liver, bone marrow, and peritoneal exudate cells (PEC) for flow cytometry analysis. Spleens were mechanically processed and passed through a 70µm nylon filter and then lysed in 1ml of 0.846% solution of NH4Cl for red blood cell lysis. The cells were then washed and stored on ice. Lungs were harvested and digested with 1mg/ml Collagenase I (Sigma, MO, USA) supplemented with 0.5mg/ml DNAse I (Sigma, MO, USA) in complete RPMI for 45 minutes at 37°C. The digested lungs were then passed through a 70µm nylon filter and washed with 10ml of complete RPMI. For liver preparations, the left renal artery was severed, and the mice were perfused using 10ml of 1xDPBS. The gallbladder was removed, and the lobes of the liver were mechanically processed over a 70µm nylon filter and washed. The single cell preparations were then re-suspended in 20ml of 37.5% percoll and centrifuged at 2000rpm for 20mins at RT. The pellet was then re-suspended in NH4Cl solution for red blood cell lysis, and the cells were then washed and stored on ice. The bone marrow from the femur and tibia of mice was harvested and pooled RBC lysed, and these single cell preparations used for analysis.

Instrument
FACS Symphony A5 (BD Biosciences)

Software
BD FACSDiva v9.0 (BD Biosciences)

Cell population abundance
Treg cells are between 1-5% of lymphocytes in the spleen.

Gating strategy
The gating strategy for Treg and non Treg CD4+ T cells is depicted in supplemental figure 1A, and supplemental figure 2A. Singlets were gated using FSC-A vs FSC-W, and then SSC-A vs SSC-W. Live cells were then identified by gating on Live/Dead aqua negative cells. CD3+ cells were gated, and then subgated on CD4 and CD8. From CD4+ T cells, Foxp3 was then subgated on to identify Treg cells.
Gating for B cells, and myeloid subsets (neutrophils, monocytes, macrophages, cDC1s and cDC2s) are demonstrated in supplemental figure 3C. The subsets are also described in the images where myeloid cells are displayed.

☑ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.