The Journal of Immunology

Peripherally Induced Tolerance Depends on Peripheral Regulatory T Cells That Require Hopx To Inhibit Intrinsic IL-2 Expression

Andrew Jones, Adeleye Opejin, Jacob G. Henderson, Cindy Gross, Rajan Jain, Jonathan A. Epstein, Richard A. Flavell and Daniel Hawiger

J Immunol 2015; 195:1489-1497; Prepublished online 13 July 2015;
doi: 10.4049/jimmunol.1500174
http://www.jimmunol.org/content/195/4/1489

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Peripherally Induced Tolerance Depends on Peripheral Regulatory T Cells That Require Hopx To Inhibit Intrinsic IL-2 Expression

Andrew Jones,* Adeleye Opejin,* Jacob G. Henderson,* Cindy Gross,* Rajan Jain,† Jonathan A. Epstein,† Richard A. Flavell,‡ and Daniel Hawiger*

Dendritic cells (DCs) can induce peripheral immune tolerance that prevents autoimmune responses. Ag presentation by peripheral DCs under steady-state conditions leads to a conversion of some peripheral CD4$^+$ T cells into regulatory T cells (Tregs) that require homeodomain-only protein (Hopx) to mediate T cell unresponsiveness. However, the roles of these peripheral Tregs (pTregs) in averting autoimmune responses, as well as immunological mechanisms of Hopx, remain unknown. We report that Hopx$^+$ pTregs converted by DCs from Hopx$^-$ T cells are indispensible to sustain tolerance that prevents autoimmune responses directed at self-Ags during experimental acute encephalomyelitis. Our studies further reveal that Hopx inhibits intrinsic IL-2 expression in pTregs after antigenic rechallenge. In the absence of Hopx, increased levels of IL-2 lead to death and decreased numbers of pTregs. Therefore, formation of Hopx$^+$ pTregs represents a crucial pathway of sustained tolerance induced by peripheral DCs, and the maintenance of such pTregs and tolerance requires functions of Hopx to block intrinsic IL-2 production in pTregs. ***The Journal of Immunology*****, 2015, 195: 1489–1497.**

The task of silencing autoimmune responses mediated by autoreactive T cells is a complex process referred to as immune tolerance; it begins in the thymus and continues in the peripheral lymphoid system (1–8). Mechanisms of peripheral tolerance can inactivate Ag-specific T cell responses after exposure to noninflammatory forms of Ags introduced as soluble peptides/proteins or as cell-bound material. Therefore, peripheral tolerance induced by defined tolerizing neural Ags, including myelin oligodendrocyte glycoprotein (MOG), can prevent a specific autoimmune process, such as experimental acute encephalomyelitis (EAE), a model of multiple sclerosis (MS) (9–17). Dendritic cells (DCs) play a central role in peripheral tolerance to prevent autoimmune EAE because DCs present specific Ags and induce tolerogenic responses in T cells. Mechanisms of T cell tolerance mediated by DCs include T cell anergy, deletion, skewing of effector T cell responses, expansion of thymically derived regulatory T cells (tTregs), and de novo induction of peripheral Tregs (pTregs) (18–27). However, the relative roles of these mechanisms, as well as their specific molecular pathways in tolerance, remain unclear (20–23, 28). We recently discovered that the transcription cofactor homeodomain-only protein (Hopx) is required for regulatory T cell (Treg)-mediated immune unresponsiveness induced by DCs, but the specific roles for Hopx in the regulation of autoimmune responses remain unknown (29). We report that maintenance of Ag-specific peripheral tolerance requires de novo–induced Hopx$^+$ pTregs that develop from Hopx$^-$Foxp3$^-$CD25$^-$ precursors in response to tolerizing Ags presented by DCs. Our findings show indispensible functions of pTregs in Ag-specific peripheral tolerance induced by DCs, and they also reveal that by inhibiting intrinsic IL-2 expression in induced pTregs, Hopx promotes the maintenance of pTregs and peripheral tolerance.

Materials and Methods

Mice

Hopx$^{-/-}$ mice (30), bred on the C57BL/6 background and also crossed with Foxp3^{RFP} mice (31), were described previously (29). They were also bred with MOG-specific TCR-transgenic (tg) (2D2 TCR-tg) mice (32) to produce 2D2 TCR-tg and non-TCR–tg/*Hopx*$^{-/-}$Foxp3^{RFP} and *Hopx*$^{+/+}$Foxp3^{RFP} mice. *IL-2*$^{-/-}$ mice (33) were bred with *Hopx*$^{-/-}$ mice, and *IL-2*$^{+/-}$ heterozygotes were used to produce *Hopx*$^{-/-}$*IL-2*$^{-/-}$ and *Hopx*$^{-/-}$*IL-2*$^{+/+}$/2D2 TCR-tg Foxp3^{RFP} and non-TCR–tg Foxp3^{RFP} mice. *Hopx*$^{Flag\text{-}viral2A\text{-}GFP}$ reporter mice (34) that faithfully track Hopx expression were first bred on the C57BL/6 background and then crossed with Foxp3^{RFP} reporter mice (31), as well as with 2D2 TCR-tg mice (32). Sex- and age-matched littermates were used for experiments. Mice were used at 6–8 wk of age, with the exception of *IL-2*$^{-/-}$ and *IL-2*$^{+/+}$ littermate control mice, which were used at 4–5 wk of age, as donors for adoptive transfers. All mice were maintained in the Department of Comparative Medicine at Saint Louis Univeristy School of Medicine under specific pathogen–free conditions and used in accordance with the guidelines of Saint Louis University's Institutional Animal Care and Use Committee.

Production of chimeric Abs

Chimeric Abs were produced as previously described (28, 35). Briefly, Abs were expressed in A293 cells by transient transfection using the calcium/phosphate method. Cells were grown in serum-free DMEM supplemented with Nutridoma-SP (Roche), and Abs were purified on protein G columns. Chimeric Abs in PBS were injected i.p.

*Department of Molecular Microbiology and Immunology, Saint Louis University School of Medicine, St. Louis, MO 63104; †Department of Cell and Developmental Biology and Institute for Regenerative Medicine and Cardiovascular Institute, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104; and ‡Department of Immunobiology and Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT 06519

Received for publication January 26, 2015. Accepted for publication June 15, 2015.

This work was supported by grants from the National Multiple Sclerosis Society (RG5019A) and the National Institute of Allergy and Infectious Diseases of the National Institutes of Health (R01AI113903) (both to D.H.). Generation of the original *Hopx*$^{Flag\text{-}viral2A\text{-}GFP}$ reporter mice was supported by R01 HL071546 from the National Heart, Lung, and Blood Institute of the National Institutes of Health (to J.A.E.).

This publication is solely the responsibility of the authors and does not necessarily represent the official view of the National Institutes of Health.

Address correspondence and reprint requests to Dr. Daniel Hawiger, Department of Molecular Microbiology and Immunology, Saint Louis University School of Medicine, Edward A. Doisy Research Center, 1100 South Grand Boulevard, Room 715, St. Louis, MO 63104. E-mail address: dhawiger@slu.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: DC, dendritic cell; EAE, experimental autoimmune encephalomyelitis; Hopx, homeodomain-only protein; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; PT, pertussis toxin; pTreg, peripheral Treg; tg, transgenic; Treg, regulatory T cell; tTreg, thymically derived Treg.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1500174

Flow cytometry and Abs used for staining

Anti-CD4 (GK1.5), anti-CD25 (PC-61), anti-Vα3.2 (RR3-16), anti-CD45.2 (104), anti-CD44 (IM7), anti-CD62L (MEL-14), anti-ICOS (C398.4A), anti–PD-1 (29F.1A12), and anti-CD73 (TY/11.8) were from BioLegend. Anti-Foxp3 (FJK-16a) was from eBioscience. Cell sorting and analyses were performed using a FACSAria III, FACSCalibur, LSR II, or FACSCanto (BD). An FITC Annexin V Apoptosis Detection Kit and Zombie Violet Fixable Viability Dye were used to detect apoptosis (BioLegend). For intracellular staining, cells were fixed and permeabilized using Fixation/Permeabilization buffers from eBioscience and BD, according to the manufacturers' manual.

Adoptive transfers

Lymph nodes and spleen cells from multiple mice were pooled, and $CD4^+$ T cells were enriched by depletion of $CD8^+$, $B220^+$, $CD11c^+$, $CD11b^+$, and $NK1.1^+$ cells with magnetic MicroBeads (Miltenyi Biotec), and Foxp3 $(RFP)^-/CD25^-$ or Foxp3$(RFP)^+$ cells were purified by subsequent automated cell sorting performed on a FACSAria III (BD). Cells were washed three times with PBS, and 1×10^6 $Foxp3^+$ cells, 10×10^6 $RFP^-/CD25^-$ cells, or 5×10^6 2D2 TCR-tg $RFP^-/CD25^-$ cells were transferred into mice by i.v. injection into a tail vein. In some experiments, cells were labeled with 3 μM CFSE (Sigma) in 5% FCS RPMI 1640 at 37°C for 20 min and washed three times with PBS, and 5×10^6 cells were injected i.v. into each mouse.

EAE model

To induce EAE, mice were injected s.c. in each flank with 100 μg synthetic MOG peptide ($MOG_{35–55}$; Yale Keck Protein Synthesis Facility) in CFA (Difco). CFA was enriched with *Mycobacterium tuberculosis* (10 ml CFA + 40 mg *M. tuberculosis*; Difco). Pertussis toxin (PT; List Biological Laboratories) was injected (200 ng/mouse i.p.) in PBS on days 0 and 2 after $MOG_{35–55}$ injections. EAE was scored daily on a scale of 1 to 4: 0, no clinical signs; 1, flaccid tail; 2, hind limb weakness, abnormal gait; 3, complete hind limb paralysis; and 4, complete hind limb paralysis and forelimb weakness or paralysis. Each experimental group was scored in a blinded fashion. Spinal cords were extracted from the spinal columns of experimental mice, mashed through 70-μm filters using a 5-ml syringe plunger, and prepared for FACS analysis.

Chimeric Ab and anti-CD25 Ab injections

Each mouse received anti–DEC-MOG or IC-MOG chimeric Abs in PBS (15 μg i.p.). A total of 250 μg anti–CD25 (PC-61.5.3) or rat IgG1 (Bio X Cell) Abs in PBS was injected (i.p.) per mouse.

Cell cultures

$CD4^+$ cells were enriched using magnetic MicroBeads (Miltenyi Biotec), and Foxp3$(RFP)^-/CD25^-$ cells were purified by subsequent automated cell sorting performed on a FACSAria III (BD). Tregs were differentiated for 5 d in 96-well plates (Thermo Fisher) coated with anti-CD3 (145-2C11; 1 μg/ml) in Click's media containing 10% FBS, Penicillin-Streptomycin, L-glutamine, and 2-ME (Life Technologies) in the presence of soluble anti-CD28 (37.51; 1.5 μg/ml), rIL-2 (200 U /ml), and TGF-β (4 ng/ml; all from BioLegend). Foxp3 $(RFP)^+$ cells were sorted and restimulated with PMA (100 ng/ml) for 90 min.

Real-time RT-PCR analysis

RNA was isolated from in vitro–cultured induced Tregs using TRIzol reagent (Invitrogen) and an miRNeasy Kit (QIAGEN). Total RNA was reverse transcribed, and cDNA was subsequently used for real-time PCR on an ABI Prism instrument using commercial primer-probe sets (Applied Biosystems) for HPRT and IL-2. The results of quantitative PCR were standardized to HPRT expression levels and analyzed by the ΔΔ cycle threshold method.

Statistical analysis

Mice of particular genotypes were randomly assigned to individual experimental groups. The numbers of groups and mice in each group were determined to achieve statistical significance based on commonly used statistical techniques: two-way ANOVA, one-way ANOVA, and the Student *t* test. All experimental groups and individual mice were included in the statistical analyses. Individual *p* values were calculated using the Student *t* test with the Welch correction, one-way ANOVA, or two-way ANOVA.

Results

Peripherally induced tolerance requires Hopx

To study peripherally induced tolerance, we used a well-established method to target DCs in vivo with a recombinant chimeric Ab specific for DEC205 (28, 35). MOG is delivered as a fusion protein linked to the C terminus of the H chain of a chimeric anti–DEC-MOG Ab that targets DCs in a process resembling in vivo uptake of self-Ags (21, 28, 35–37). C57BL/6 mice administered a single treatment with anti–DEC-MOG acquired T cell tolerance that protects from autoimmune EAE induced by subsequent immunization with $MOG_{35–55}$ peptide in CFA and PT (21, 28). DCs mediate several distinct pathways of T cell tolerance, including a de novo induction of pTregs (18–27). Hopx is expressed in Tregs induced by DCs, but the roles of these pTregs in averting autoimmune responses, as well as the immunological mechanisms of Hopx, remain unknown (29). To directly examine the role of Hopx in tolerance, we used $Hopx^{-/-}$ mice (30) bred on a C57BL/6 background (29). The induction of T cell tolerance by DCs requires initial T cell proliferation, and such T cell activation is independent of Hopx expression in T cells (29, 35, 36). To exclude that the absence of Hopx might affect presentation of tolerogenic Ags by DCs, we transferred CFSE-labeled congenic $CD4^+$ T cells from MOG-specific TCR-tg (2D2) mice into $Hopx^{+/+}$ and $Hopx^{-/-}$ mice and measured their proliferation in response to anti–DEC-MOG–targeted DCs (Supplemental Fig. 1A). We found that the proliferation of the transferred 2D2 T cells in response to MOG-targeted DCs was similar in $Hopx^{+/+}$ and $Hopx^{-/-}$ mice (Supplemental Fig. 1A). To directly test a requirement for Hopx in tolerance, we induced EAE in $Hopx^{+/+}$ and $Hopx^{-/-}$ mice 1, 3, or 6 wk after the initial treatment with either anti–DEC-MOG or IC-MOG (Fig. 1A–C). We found that treatment with anti–DEC-MOG protected both $Hopx^{+/+}$ and $Hopx^{-/-}$ mice from EAE that was induced 1 wk later (Fig. 1A). Further, we found that pretreatment with anti–DEC-MOG limited skewing toward Th1 and Th17 effector phenotypes similarly in $Hopx^{+/+}$ and $Hopx^{-/-}$ T cells (Supplemental Fig. 1B, 1C). We also examined the spinal cords of anti–DEC-MOG–treated and control mice by flow cytometry to examine $CD4^+$ T cells within the "lymphoid gate" that consists of ∼90% live $CD45^+$ cells (Fig. 1D, Supplemental Fig. 2A, 2B). In contrast to the similar increased numbers of $CD4^+$ T cells in the spinal cords of $Hopx^{+/+}$ or $Hopx^{-/-}$ mice that were not treated with anti–DEC-MOG and developed EAE, we observed that treatment with anti–DEC-MOG prevented T cell spinal cord infiltration, consistent with the absence of effector T cells (Fig. 1D). However, when we delayed induction of EAE after the initial treatment with anti–DEC-MOG, we observed that the infiltration of T cells in the spinal cords of $Hopx^{-/-}$ mice was comparable to that observed in the spinal cords of $Hopx^{+/+}$ mice or $Hopx^{-/-}$ mice that were not treated with anti–DEC-MOG (Fig. 1E). Consistent with the presence of encephalitogenic T cells in their spinal cords, such $Hopx^{-/-}$ mice developed symptoms of EAE comparable to $Hopx^{+/+}$ mice or $Hopx^{-/-}$ mice that were not treated with anti–DEC-MOG (Fig. 1B, 1C). In contrast, $Hopx^{+/+}$ mice remained protected from EAE 3 wk (Fig. 1B) and 6 wk (Fig. 1C) after their initial treatment with anti–DEC-MOG. Therefore, DCs induced sustained tolerance that requires Hopx to prevent a subsequently triggered autoimmune attack. To confirm that such tolerance induced by DCs is Ag specific, we used anti–DEC-OVA that targets unrelated OVA Ag to DCs and found that treatment with anti–DEC-OVA did not protect from EAE that was induced by immunization with MOG (Supplemental Fig. 2C).

DCs induce $Hopx^+$ pTregs and Treg-dependent tolerance

A sustained long-lasting immunological tolerance may require the presence and suppressor functions of Tregs (6, 7). Therefore, to test the general requirement for Tregs in such DC-induced tolerance, we used a well-established treatment with anti–CD25 Ab (PC-61.5.3) to remove $Foxp3^+CD25^+$ Tregs in vivo. Treatment with anti–CD25 Ab resulted in an ∼60% reduction in the numbers of $Foxp3^+$ Tregs,

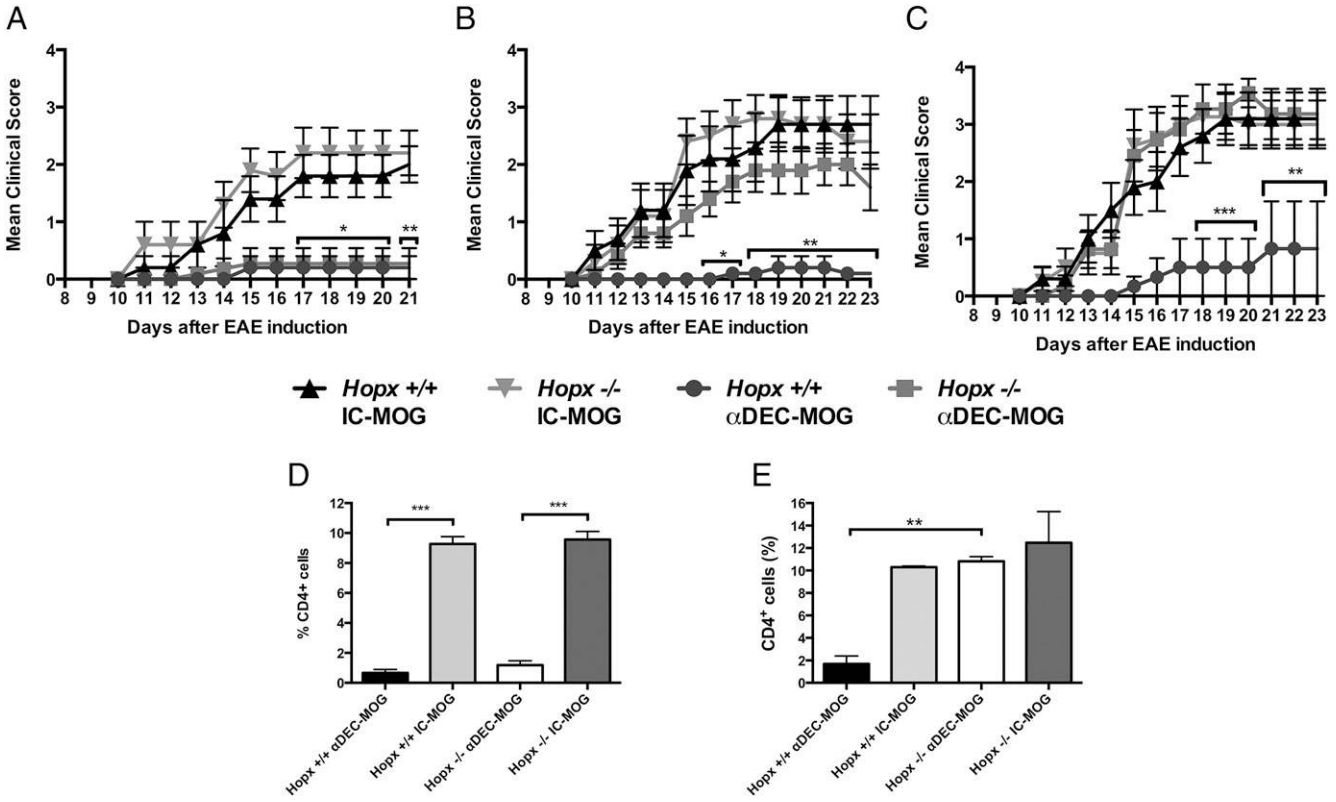

FIGURE 1. Hopx is required to sustain peripherally induced tolerance. Multiple groups of $Hopx^{+/+}$ and $Hopx^{-/-}$ mice were treated with anti–DEC-MOG or IC-MOG 1 wk (**A**), 3 wk (**B**), or 6 wk (**C**) before immunization with $MOG_{35–55}$ in CFA + PT to induce EAE. Graphs show mean disease scores (n = 10–15/group from two experiments). (**D**) Multiple groups of $Hopx^{+/+}$ and $Hopx^{-/-}$ mice were treated as in (A). Results show mean percentages of $CD4^+$ T cells in the "lymphoid" gate of spinal cord cell suspensions 21 d after EAE induction (n = 3–4/group from two experiments). (**E**) Multiple groups of $Hopx^{+/+}$ and $Hopx^{-/-}$ mice were treated as in (C). Results show mean percentages of $CD4^+$ T cells in the "lymphoid" gate of spinal cord cell suspensions 17 d after EAE induction (n = 3–4/group from two experiments). All data are mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, two-way ANOVA.

and this reduction lasted for ≥3 wk (Supplemental Fig. 3A). We treated C57BL/6 mice with anti–DEC-MOG or IC-MOG and 5 d later injected anti–CD25 Ab or an IgG1 isotype-control Ab. EAE was induced in these mice after an additional 2 d (or 1 wk after chimeric Ab treatment) (Fig. 2A). Alternatively, we treated C57BL/6 mice with anti–DEC-MOG or IC-MOG and 7 d later injected anti-CD25 Ab or an IgG1 isotype-control Ab. After another 2 wk (3 wk after chimeric Ab treatment), EAE was induced in these mice (Fig. 2B). Although Treg depletion did not affect tolerance to EAE induced 1 wk after treatment with anti–DEC-MOG (Fig. 2A), depletion of Tregs completely abolished tolerance when EAE was induced 3 wk after anti–DEC-MOG treatment (Fig. 2B). To further substantiate these results, we depleted Tregs 3 wk after treatment with anti–DEC-MOG and also found an abolished protection from EAE induced after an additional 3 wk (6 wk after the initial tolerance induction by treatment with anti–DEC-MOG) (Supplemental Fig. 3B). We conclude that, after the initial exposure to MOG, tolerance induced within 1 wk by DCs does not rely on Hopx or Tregs. However, Tregs are required for tolerance lasting >3 wk after exposure of DCs to MOG. Therefore, DCs induce a sustained, long-lasting tolerance that requires both Tregs and Hopx.

It remains unknown whether Hopx-expressing pTregs induced by DCs can develop from $Hopx^-$ precursor T cells. To study the expression of Hopx and Foxp3 in T cells upon induction of tolerance by DCs, we produced a $Hopx^{Flag\text{-}viral2A\text{-}GFP}$/$Foxp3^{IRES\text{-}RFP}$ double-reporter mouse (referred to as $Hopx^{GFP}Foxp3^{RFP}$ reporter mice). We sorted $GFP^-(Hopx^-)RFP^-(Foxp3^-)CD25^-$ CD4 cells from 2D2 TCR-tg $Hopx^{GFP}Foxp3^{RFP}$ mice and transferred the isolated T cells into new recipient mice (Fig. 2C–E, Supplemental Fig. 3C). Within 21 d after treatment of the recipients with anti–DEC-MOG, >70% of T cells responding to MOG in lymph nodes and spleens became $Foxp3^+CD25^+$ double-positive pTregs, and these pTregs also induced the expression of Hopx. In contrast, no induction of CD25, Foxp3, or Hopx expression was observed in MOG-specific T cells 8 or 21 d after treatment with IC-MOG (Fig. 2C–E, Supplemental Fig. 3C). Therefore, presentation of MOG by DCs to T cells leads to an induction of $Hopx^+$ pTregs from precursor T cells.

$Hopx^+$ pTregs are indispensible for tolerance

In addition to converting pTregs, tolerogenic stimulation by DCs can increase the numbers and enhance the functions of pre-existing $Foxp3^+CD25^+$ tTregs; therefore, it remains unclear whether the newly induced pTregs or the expanded tTregs are required to ameliorate the symptoms of EAE and MS (17, 19–21, 23, 38, 39). To distinguish between the roles of these different types of Tregs in tolerance, we attempted to restore DC-induced and Hopx-dependent tolerance by transferring either pre-existing $Foxp3^+$ Tregs that include mostly tTregs or the $Foxp3^-CD25^-$ precursors of pTregs (6, 7) into $Hopx^{-/-}$ mice that were treated with anti–DEC-MOG and immunized 6 wk later to induce EAE (Fig. 3). In separate experiments, we determined the numbers of transferred pre-existing tTregs and pTregs that were induced from the transferred $Foxp3^-CD25^-$ cells 6 wk after the initial presentation by DCs of MOG delivered by anti–DEC-MOG. We found ∼6-fold more remaining pre-existing tTregs than induced pTregs (Supplemental Fig. 3D–F). Further, by using $Hopx^{+/+}$ or $Hopx^{-/-}$ transferred cells, we directly tested the role of Hopx in Tregs to

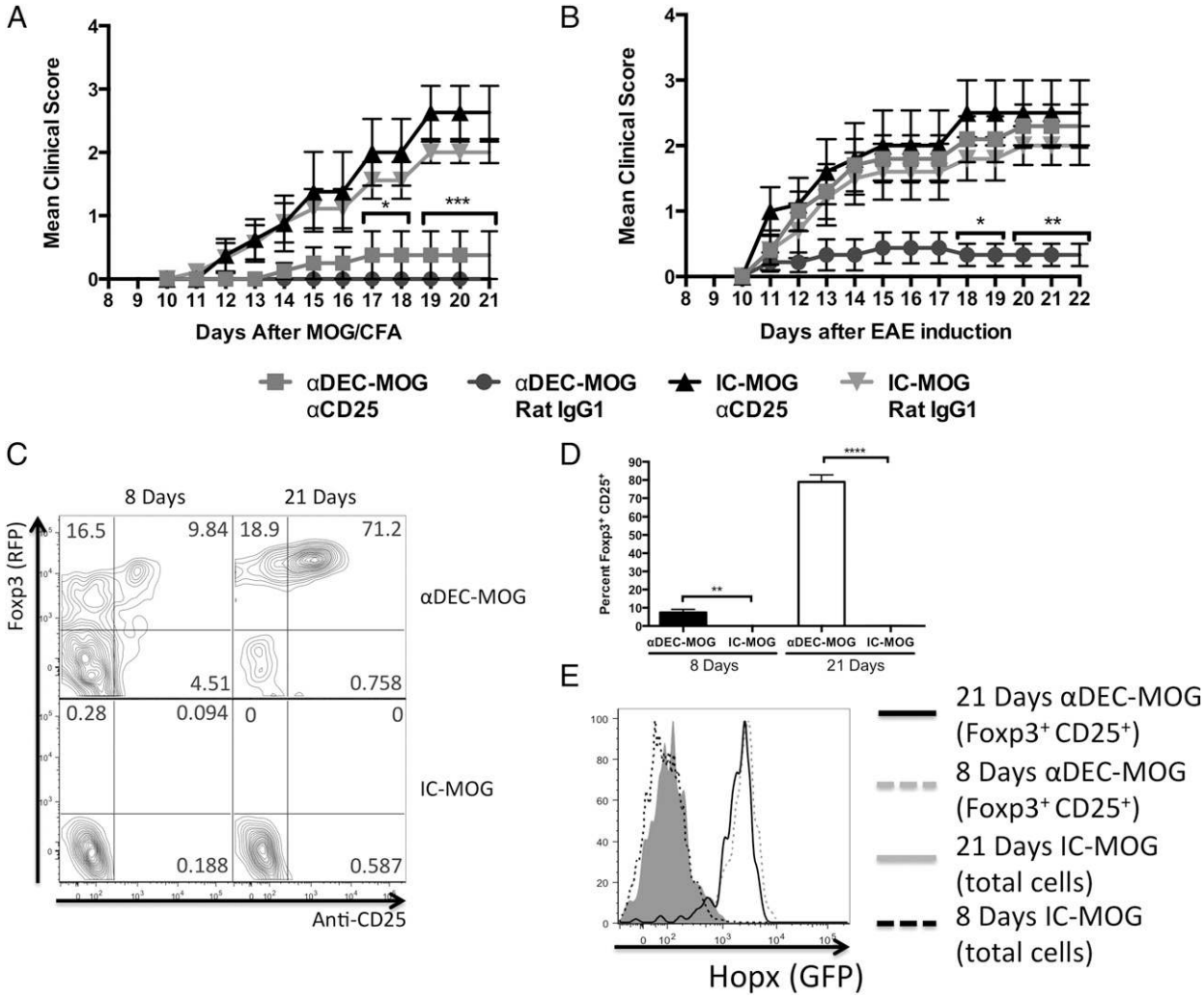

FIGURE 2. DCs induce Hopx$^+$ pTregs and Treg-dependent long-lasting tolerance. (**A** and **B**) Multiple groups of C57BL/6 mice were treated with anti–DEC-MOG or IC-MOG. Five days (A) or 1 wk (B) after treatment with chimeric Abs, individual groups of mice were injected with anti-CD25 or the same dose of rat IgG1. EAE was induced after an additional 2 d (A) or 2 wk (B). Graphs show mean disease scores (n = 10/group from two experiments). Data are mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, two-way ANOVA. (**C**–**E**) GFP$^-$/RFP$^-$/CD25$^-$ CD4$^+$ T cells were purified by sorting from 2D2 TCR-tg Hopx$^{\text{Flag-viral2A-GFP}}$/Foxp3$^{\text{RFP}}$ double-reporter mice and then adoptively transferred into CD45.1$^+$ recipient mice. (C) Plots show Foxp3 (RFP) expression (y-axis) and staining intensity with anti-CD25 (x-axis) of gated populations of adoptively transferred cells at multiple days after treatment with anti–DEC-MOG. (D) Graphs show mean percentages of Foxp3$^+$CD25$^+$ pTregs among transferred cells as in (C) (n = 3–5/group). **$p < 0.01$, ****$p < 0.0001$, one-way ANOVA with the Turkey multiple-comparison test. (E) Induction of Hopx (GFP) expression from (C). The results in (C) and (E) represent one of two similar experiments. Data in (C)–(E) are from lymph nodes; similar results were obtained from spleens.

confer tolerance. As expected, in the absence of any transferred cells (PBS only), treatment with anti–DEC-MOG failed to induce tolerance in *Hopx*$^{-/-}$ mice. However, we found that mice transferred with *Hopx*$^{+/+}$ Foxp3$^-$CD25$^-$ precursors of pTregs were protected from EAE. In contrast, mice transferred with *Hopx*$^{-/-}$ Foxp3$^-$CD25$^-$ cells developed symptoms of EAE and infiltration of spinal cords that were similar to those of mice that did not receive precursors of pTregs (Fig. 3B, 3C). However, transfer of *Hopx*$^{+/+}$ or *Hopx*$^{-/-}$ tTregs did not prevent symptoms of EAE or block T cell infiltration of spinal cords in recipient mice (Fig. 3D, 3E). Thus, we conclude that tolerance induced by DCs depends on Hopx expression in de novo–induced pTregs.

Hopx is required for maintenance of DC-induced pTregs after antigenic rechallenge under inflammatory conditions

To examine Hopx-dependent responses in pTregs, we transferred Foxp3$^-$CD25$^-$ CD4 T cells isolated from *Hopx*$^{+/+}$ or *Hopx*$^{-/-}$ 2D2 TCR-tg Foxp3$^{\text{RFP}}$ mice into individual groups of recipient mice that were subsequently treated with anti–DEC-MOG. We then analyzed expression of Foxp3 and CD25 in the transferred cells (Supplemental Fig. 4A). We found that populations of *Hopx*$^{+/+}$ and *Hopx*$^{-/-}$ pTregs developed similarly after treatment with anti–DEC-MOG (Supplemental Fig. 4A). Also in agreement with our previous studies (29), we found similar expression of PD-1, ICOS, and CD73 in *Hopx*$^{+/+}$ and *Hopx*$^{-/-}$ pTregs (Supplemental Fig. 4B). To further examine the fate of such pTreg populations, we examined the percentage of Foxp3$^+$CD25$^+$ cells converted from the transferred Foxp3$^-$CD25$^-$ cells among the total CD4 cells in recipient mice before and after immunization with $MOG_{35–55}$ in CFA and PT and also determined the absolute numbers of such pTregs (Fig. 4A, 4B). We found that, after a similar initial formation of *Hopx*$^{+/+}$ and *Hopx*$^{-/-}$ pTregs, we recovered more than twice as many *Hopx*$^{+/+}$ pTregs as *Hopx*$^{-/-}$ pTregs after we immunized the recipient mice with MOG (Fig. 4A, 4B). To examine the specific downregulation of Foxp3 expression, we analyzed Foxp3 expression in the remaining *Hopx*$^{-/-}$ and *Hopx*$^{+/+}$ cells (Fig. 4C). We observed only negligible numbers of Foxp3$^-$ T cells among remaining *Hopx*$^{-/-}$ and *Hopx*$^{+/+}$ cells,

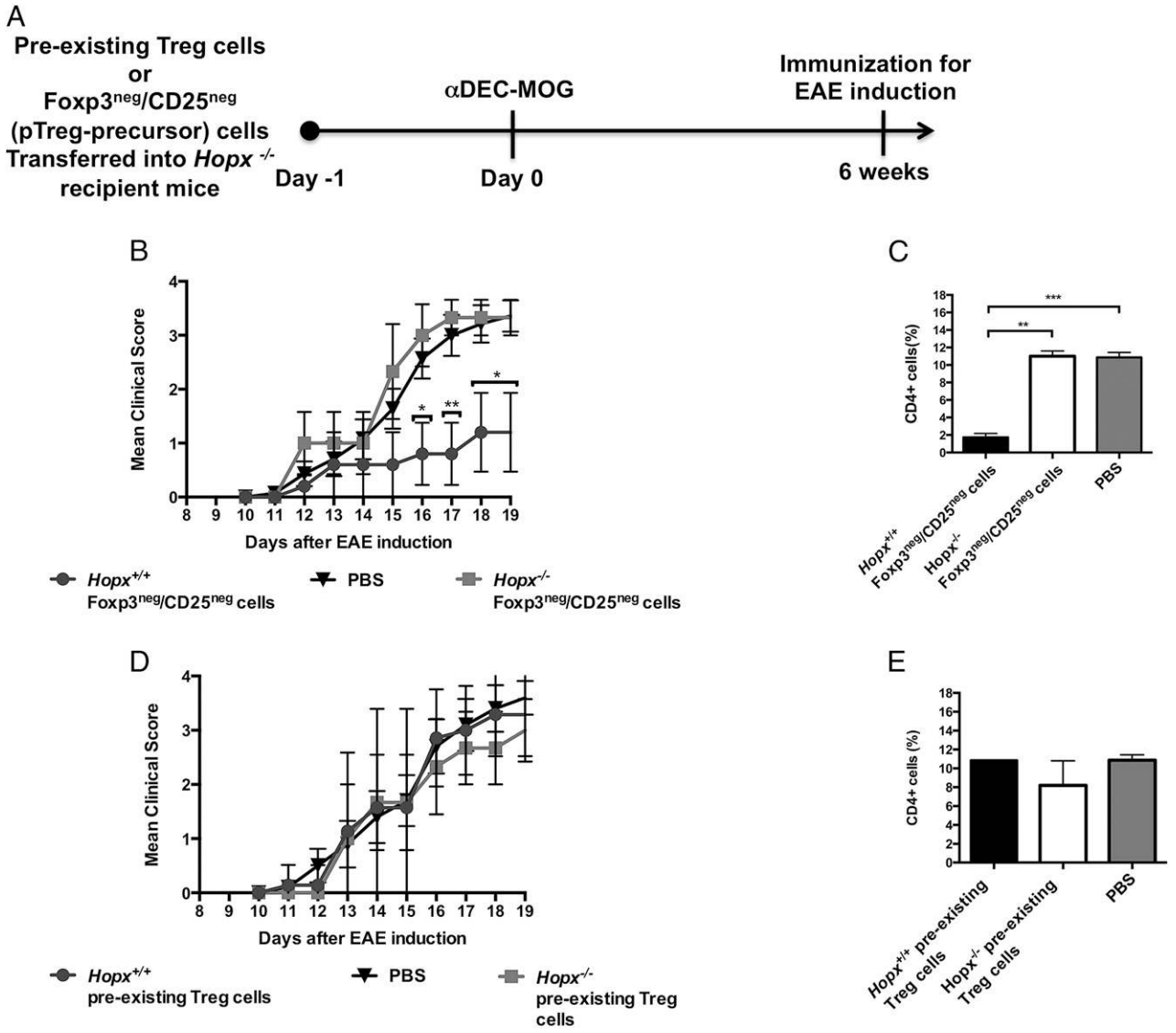

FIGURE 3. Hopx$^+$ pTregs are indispensible for peripherally induced tolerance. (**A**) Schematic outline of the experimental design to restore induced tolerance in *Hopx*$^{-/-}$ mice. (**B**) Multiple groups of *Hopx*$^{-/-}$ mice were transferred with PBS or Foxp3$^-$CD25$^-$ CD4$^+$ cells purified by sorting from *Hopx*$^{+/+}$ or *Hopx*$^{-/-}$ Foxp3^{RFP} mice. All mice were treated with anti–DEC-MOG 6 wk before EAE induction by immunization with $MOG_{35–55}$ in CFA + PT. Line graph shows mean disease scores (n = 6–8/group from two experiments). (**C**) Multiple groups of *Hopx*$^{-/-}$ mice were treated as in (B). Results show mean percentages of CD4$^+$ T cells in the "lymphoid" gate of spinal cord cell suspensions 19 d after EAE induction (n = 3–4/group from two experiments). (**D**) Multiple groups of *Hopx*$^{-/-}$ mice were transferred with PBS or Foxp3$^+$ CD4$^+$ cells purified by sorting from either *Hopx*$^{+/+}$ or *Hopx*$^{-/-}$ Foxp3^{RFP} mice. All mice were treated with anti–DEC-MOG 6 wk before EAE induction. Line graph shows mean disease scores (n = 6–7/group from two experiments). (**E**) Multiple groups of *Hopx*$^{-/-}$ mice were treated as in (D). Results show mean percentages of CD4$^+$ T cells in the "lymphoid" gate of spinal cord cell suspensions 19 d after EAE induction (n = 2–3/group from two experiments). Data in (B)–(E) are mean ± SEM. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, one-way or two-way ANOVA.

consistent with a lack of downregulation of Foxp3, suggesting deletion as a possible mechanism behind the decreased numbers of *Hopx*$^{-/-}$ pTregs (Fig. 4C). To confirm apoptotic death of pTregs in the absence of Hopx, we examined binding of Annexin V and found increased staining of *Hopx*$^{-/-}$ pTregs with Annexin V (Fig. 4D). We conclude that maintenance of pTregs after antigenic rechallenge depends on Hopx.

Hopx-mediated inhibition of IL-2 expression is required for maintenance of induced pTregs

The c-fos/c-jun AP-1 complex induces IL-2 expression in T cells (6, 40, 41). Because Hopx inhibits the expression of c-fos and c-jun in various types of cells, including T cells (29, 30, 42), we hypothesized that Hopx could govern IL-2 expression in induced pTregs. Because Hopx$^{-/-}$ pTregs that express IL-2 are expected to die rapidly in vivo (Fig. 4), we examined IL-2 expression by quantitative PCR in Hopx$^{+/+}$ or Hopx$^{-/-}$ in vitro–induced Tregs after a brief restimulation (Fig. 5A). Expression of IL-2 was ∼3-fold higher in Hopx$^{-/-}$ than in Hopx$^{+/+}$ induced Tregs (Fig. 5A). To directly examine the impact of endogenously produced IL-2 on the functions of *Hopx*$^{-/-}$ pTregs, we used T cells from *IL2*$^{-/-}$ mice that we crossed with *Hopx*$^{+/+}$ and *Hopx*$^{-/-}$ mice. *IL2*$^{-/-}$ Tregs can develop in the presence of exogenous IL-2 (43). Therefore, we first confirmed induction of *IL2*$^{-/-}$ pTregs in an IL-2–sufficient environment by adoptively transferring sorted *Hopx*$^{-/-}$ *IL2*$^{+/+}$ and *Hopx*$^{-/-}$*IL2*$^{-/-}$ Foxp3$^-$CD25$^-$ 2D2 cells into *IL2*$^{+/+}$ recipient mice that were subsequently treated with anti–DEC-MOG. We found that *Hopx*$^{-/-}$*IL2*$^{+/+}$ and *Hopx*$^{-/-}$*IL2*$^{-/-}$ Foxp3$^-$CD25$^-$ cells converted to Foxp3$^+$CD25$^+$ pTregs at a similar rate (Supplemental Fig. 4C). To examine the impact of antigenic rechallenge on *Hopx*$^{-/-}$*IL2*$^{-/-}$ and *Hopx*$^{-/-}$*IL2*$^{+/+}$ pTregs, we immunized the recipient mice that harbored preformed pTregs with

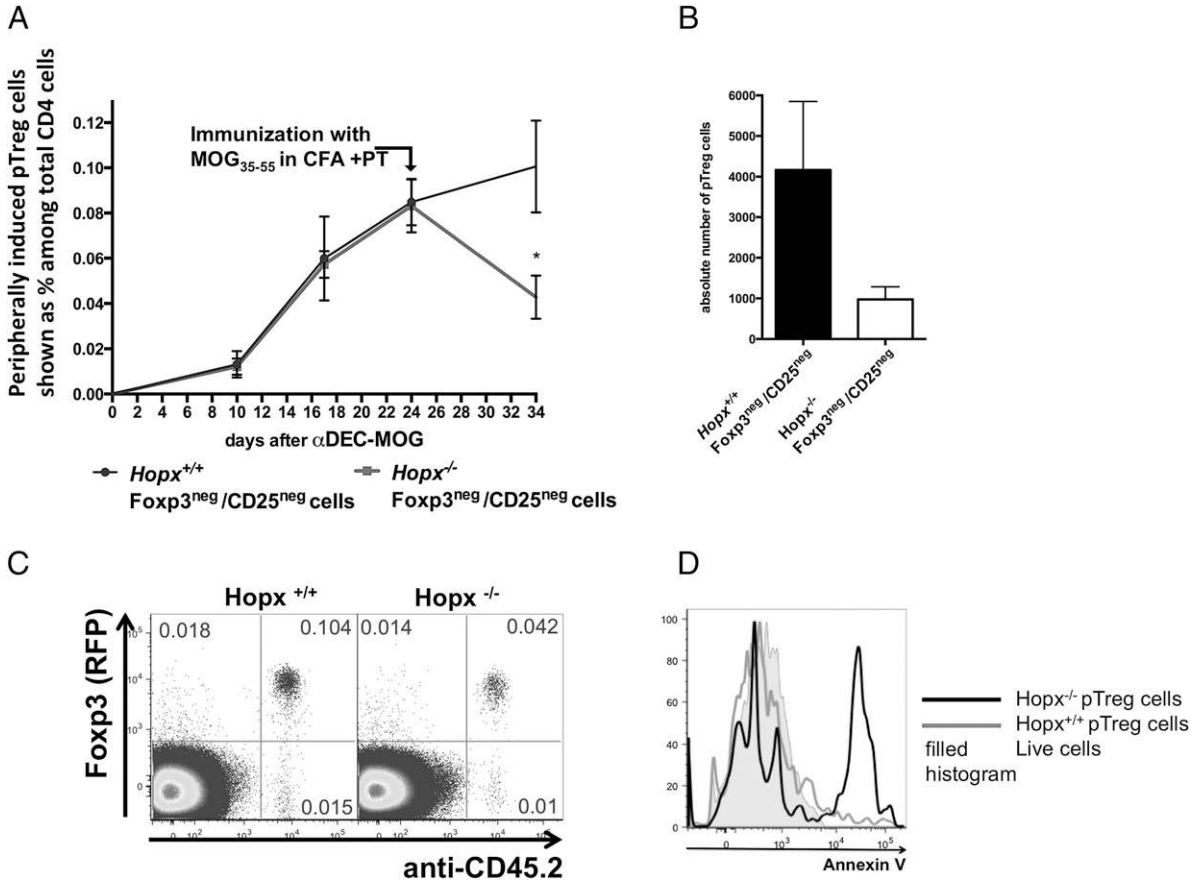

FIGURE 4. Hopx is required for maintenance of pTregs after antigenic rechallenge under inflammatory conditions. (**A**–**D**) Foxp3$^-$CD25$^-$ CD4$^+$ T cells were purified by sorting from either *Hopx*$^{+/+}$ or *Hopx*$^{-/-}$ 2D2 TCR-tg Foxp3^{RFP} mice and then adoptively transferred into CD45.1$^+$ recipient mice that were treated with anti–DEC-MOG. (A) Results show mean percentages of induced *Hopx*$^{+/+}$ or *Hopx*$^{-/-}$ Foxp3$^+$CD25$^+$ pTregs among the total CD4$^+$ cells in the recipients before and after immunization with $MOG_{35–55}$ in CFA + PT (n = 5/group for each time point and type of cells transferred). (B) Absolute numbers of induced *Hopx*$^{+/+}$ or *Hopx*$^{-/-}$ Foxp3$^+$CD25$^+$ pTregs after immunization with MOG in CFA + PT as in (A) (n = 5/group). Data in (A) and (B) are mean ± SEM. (C) Plots show Foxp3 (RFP) expression (y-axis) and staining intensity with anti-CD45.2 (x-axis) among CD4$^+$ cells in mice transferred with either *Hopx*$^{+/+}$ or *Hopx*$^{-/-}$ cells after immunization with MOG in CFA + PT, as in (A). The results represent one of three similar experiments. (D) Staining intensity with Annexin V in *Hopx*$^{+/+}$ or *Hopx*$^{-/-}$ Foxp3$^+$CD25$^+$ pTregs 5 d after immunization with MOG in CFA + PT. The results shown represent one of two similar experiments. All data are from lymph nodes; similar results were obtained from spleens. *$p < 0.05$, two-way ANOVA or Student t test with Welch correction.

$MOG_{35–55}$ in CFA and PT. We recovered ~3-fold fewer *Hopx*$^{-/-}$*IL2*$^{+/+}$ pTregs than *Hopx*$^{-/-}$*IL2*$^{-/-}$ pTregs (Fig. 5B). In contrast, we recovered similar numbers of *Hopx*$^{+/+}$*IL2*$^{+/+}$ and *Hopx*$^{+/+}$*IL2*$^{-/-}$ pTregs under the same conditions in vivo (Fig. 5C). We also determined that neither *Hopx* $^{-/-}$ nor *Hopx*$^{+/+}$ *IL-2*–deficient pTregs underwent apoptotic cell death following antigenic rechallenge under proinflammatory conditions (Supplemental Fig. 4D). Therefore, an increased expression of IL-2 in *Hopx*$^{-/-}$ pTregs adversely affects their maintenance after antigenic rechallenge. To directly examine the impact of such increased expression of IL-2 on tolerance, we followed the experimental design in Fig. 3. We transferred multiple groups of *Hopx*$^{-/-}$ mice with *Hopx*$^{+/+}$*IL2*$^{+/+}$, *Hopx*$^{+/+}$*IL2*$^{-/-}$, *Hopx*$^{-/-}$*IL2*$^{+/+}$, or *Hopx*$^{-/-}$*IL2*$^{-/-}$ Foxp3$^-$CD25$^-$ cells or PBS only. We treated all of the recipients with anti–DEC-MOG and immunized them 6 wk later to induce EAE (Fig. 5D, 5E). As expected, all Hopx-sufficient Foxp3$^-$CD25$^-$ cells restored tolerance, whereas *Hopx*$^{-/-}$*IL2*$^{+/+}$ Foxp3$^-$CD25$^-$ cells failed to restore tolerance and prevent symptoms of EAE. In contrast, *Hopx*$^{-/-}$*IL2*$^{-/-}$ Foxp3$^-$CD25$^-$ cells prevented EAE symptoms and T cell infiltration of spinal cords to a similar extent as observed with Hopx-sufficient Foxp3$^-$CD25$^-$ cells (Fig. 5D, 5E). Thus, a genetic deletion of IL-2 restores defective tolerance in the absence of Hopx. We conclude that Hopx blocks intrinsically expressed IL-2 from disrupting pTreg-dependent maintenance of long-term tolerance.

Discussion

We propose that, under steady-state tolerogenic conditions, DCs confer long-lasting tolerance that is sustained by pTregs whose maintenance depends on the functions of Hopx to inhibit intrinsically produced IL-2. Tolerogenic presentation by peripheral DCs of Ags, such as peptides from neural proteins, either expressed in DCs or targeted to these APCs in vivo, can inactivate T cell responses by several mechanisms, including T cell anergy, skewing of effector T cell responses, and induction of Treg functions preventing a subsequent induction of EAE (1, 3–8, 20–24, 28, 36). However, it has remained unknown how these DC-dependent mechanisms of tolerance are orchestrated and maintained.

A division of labor between various types of Tregs results in different contributions of expanded pre-existing tTregs or de novo–induced pTregs to the prevention of general autoinflammatory responses, maternal–fetal conflict, and mucosal tolerance in the airways and gut (44–47). However, Tregs that accumulate during proinflammatory processes, such as EAE, a model of MS, are inefficient in controlling autoimmunity (48, 49). Such Tregs appear to originate from expanded pre-existing Foxp3$^+$ Tregs that then may fail to maintain their regulatory phenotype (49, 50). Our present results

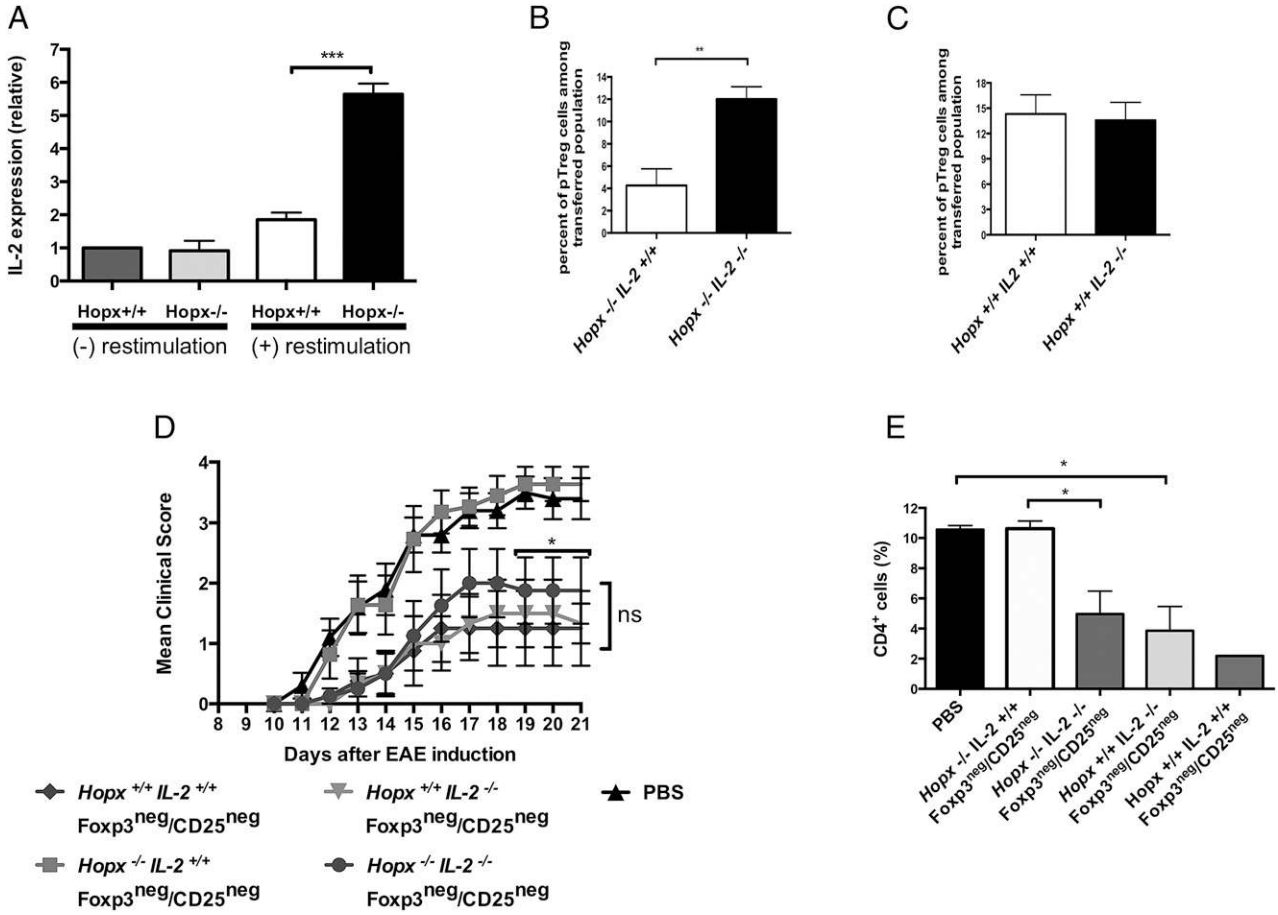

FIGURE 5. Hopx-mediated inhibition of IL-2 expression is required for maintenance of pTregs and tolerance. (**A**) IL-2 transcripts from Hopx$^{+/+}$ or Hopx$^{-/-}$ Tregs induced in vitro and then restimulated or not were analyzed by real-time PCR. The results were normalized for expression of *Hprt* and standardized by the ΔΔ cycle threshold method to the expression of IL-2 in nonrestimulated Hopx$^{+/+}$ induced Tregs. Error bars represent SD. ***$p \leq 0.001$, one-way ANOVA. Results represent one of two similar experiments. (**B**) Foxp3^{-}CD25^{-} CD4^{+} T cells were purified by sorting from either *Hopx*$^{-/-}$ IL-2$^{+/+}$ or *Hopx*$^{-/-}$ IL-2$^{-/-}$/2D2 TCR-tg Foxp3^{RFP} mice and then adoptively transferred into CD45.1^{+} recipient mice. Bar graph shows percentages of induced Foxp3^{+}CD25^{+} pTregs among adoptively transferred cells in the indicated groups of mice after treatment with anti–DEC-MOG and immunization with $MOG_{35–55}$ in CFA + PT (n = 2–4/group from two experiments). (**C**) Foxp3^{-}CD25^{-} CD4^{+} T cells were purified by sorting from either *Hopx*$^{+/+}$ IL-2$^{+/+}$ or *Hopx*$^{+/+}$ IL-2$^{-/-}$/2D2 TCR-tg Foxp3^{RFP} mice and then adoptively transferred into CD45.1^{+} recipient mice that were treated as in (B) (n = 3–4/group from two experiments). Results in (B) and (C) are from lymph nodes; similar results were obtained from spleens. (**D**) Multiple groups of *Hopx*$^{-/-}$ mice were transferred with PBS or Foxp3^{-}CD25^{-} cells purified by sorting from *Hopx*$^{+/+}$ IL-2$^{+/+}$, *Hopx*$^{-/-}$ IL-2$^{+/+}$, *Hopx*$^{-/-}$ IL-2$^{-/-}$, or *Hopx*$^{+/+}$ IL-2$^{-/-}$ Foxp3^{RFP} mice. All mice were treated with anti–DEC-MOG 6 wk before induction of EAE by immunization with $MOG_{35–55}$ in CFA + PT. Line graph shows mean disease scores (n = 6–11/group from two experiments). (**E**) Multiple groups of *Hopx*$^{-/-}$ mice were treated as in (D). Results show mean percentages of CD4^{+} T cells in the "lymphoid" gate of spinal cord cell suspensions 21 d after EAE induction (n = 2–3/group from two experiments). All data are mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, one-way or two-way ANOVA. ns, not significant.

establish that sustained Ag-specific tolerance induced by DCs relies on the functions of de novo–induced, Hopx-expressing pTregs.

Hopx is an evolutionarily conserved, homeodomain-containing, small transcription cofactor expressed in stem cells, tumor cells, myocytes, and various lymphocytes (29, 30, 42, 51–60). Expression of Hopx in effector memory T cells has been linked with their increased survival, consistent with versatile and context-dependent functions of Hopx (60). However, Hopx is not required for the functions of encephalitogenic effector T cells, and *Hopx*$^{-/-}$ mice succumb to EAE at a similar rate and severity as Hopx-sufficient mice. Further, Hopx is dispensable for the initially developing tolerance shortly after an exposure of DCs to MOG. Instead, the Hopx- and Treg-independent tolerance appears to rely on anergic cell-intrinsic mechanisms. Within 3 wk, initially tolerized T cells develop into pTregs that sustain tolerance long-term. However, only *Hopx*$^{+/+}$ pTregs can survive the antigenic rechallenge and then prevent subsequent infiltration of CNS by the newly activated encephalitogenic T cells. Therefore, *Hopx*$^{-/-}$ mice fail to sustain Ag-induced tolerance long-term. Consistent with the specific Hopx-dependent mechanisms in pTregs, only Hopx$^{+/+}$ pTregs can restore the defective tolerance in *Hopx*$^{-/-}$ mice. Hopx is also expressed in tTregs, but the functions of Hopx in these T cells remain unknown. Because tTregs are crucial for the maintenance of general immune homeostasis, future studies would elucidate any role of these tTregs in the Hopx-dependent functions of pTregs. Overall, our results establish a crucial role for Hopx-expressing pTregs in the maintenance of Ag-specific peripheral tolerance induced by DCs.

Although IL-2–mediated signals are essential for Treg development, Tregs block their own expression of IL-2 through multiple mechanisms, including Foxp3 and Helios-dependent pathways, and the intrinsic inhibition of IL-2 production in Tregs may be crucial for some suppressor functions of these cells (6, 61, 62). For example, capture of effector cell IL-2 by Tregs may enhance immune regulation by depriving the effector T cells of IL-2, in addition to other mechanisms, including blocking of IL-2

production in effector T cells (41, 63, 64). Because Tregs rely on extracellular sources of IL-2 for their proliferation and survival, treatment with rIL-2 promotes the proliferation and functions of Tregs (6, 41, 49, 65–71). However, increased IL-2 concentrations in vivo also lead to a disappearance of tTreg populations following their initial expansion (67).

The c-fos/c-jun AP-1 complex induces IL-2 expression in T cells (6, 40, 41). We established that, consistent with its inhibition of the expression of c-fos and c-jun, Hopx governs IL-2 expression in induced pTregs. We further established that increased production of IL-2 caused by the absence of Hopx is deleterious for pTreg maintenance and functions, and genetic deletion of IL-2 restores the maintenance of *Hopx*$^{-/-}$ pTreg cell numbers and functions in tolerance. Therefore, we propose that Hopx-dependent inhibition of IL-2 expression in pTregs is necessary for their maintenance and tolerance.

In conclusion, our results define how DCs orchestrate sustained tolerance to transiently available neural Ags by inducing Ag-specific pTregs. Further, by establishing the role for Hopx as an indispensible regulator of pTreg and tolerance maintenance in vivo, our results could provide the foundation for more selective and efficient immune therapies for specific immune disorders.

Acknowledgments

We thank Dr. Eric Olson for sharing *Hopx*$^{-/-}$ mice, as well as Joy Eslick and Sherri L. Koehm for expert help with flow cytometry.

Disclosures

The authors have no financial conflicts of interest.

References

1. Mathis, D., and C. Benoist. 2009. Aire. *Annu. Rev. Immunol.* 27: 287–312.
2. Curotto de Lafaille, M. A., and J. J. Lafaille. 2009. Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? *Immunity* 30: 626–635.
3. Belkaid, Y., and K. Tarbell. 2009. Regulatory T cells in the control of host-microorganism interactions (*). *Annu. Rev. Immunol.* 27: 551–589.
4. von Boehmer, H., and F. Melchers. 2010. Checkpoints in lymphocyte development and autoimmune disease. *Nat. Immunol.* 11: 14–20.
5. Bilate, A. M., and J. J. Lafaille. 2012. Induced CD4+Foxp3+ regulatory T cells in immune tolerance. *Annu. Rev. Immunol.* 30: 733–758.
6. Josefowicz, S. Z., L. F. Lu, and A. Y. Rudensky. 2012. Regulatory T cells: mechanisms of differentiation and function. *Annu. Rev. Immunol.* 30: 531–564.
7. Sakaguchi, S., D. A. Vignali, A. Y. Rudensky, R. E. Niec, and H. Waldmann. 2013. The plasticity and stability of regulatory T cells. *Nat. Rev. Immunol.* 13: 461–467.
8. Bollrath, J., and F. M. Powrie. 2013. Controlling the frontier: regulatory T-cells and intestinal homeostasis. *Semin. Immunol.* 25: 352–357.
9. Miller, S. D., R. P. Wetzig, and H. N. Claman. 1979. The induction of cell-mediated immunity and tolerance with protein antigens coupled to syngeneic lymphoid cells. *J. Exp. Med.* 149: 758–773.
10. Critchfield, J. M., M. K. Racke, J. C. Zúñiga-Pflücker, B. Cannella, C. S. Raine, J. Goverman, and M. J. Lenardo. 1994. T cell deletion in high antigen dose therapy of autoimmune encephalomyelitis. *Science* 263: 1139–1143.
11. Smilek, D. E., A. M. Gautam, C. Pearson, L. Steinman, and H. O. McDevitt. 1992. EAE: a model for immune intervention with synthetic peptides. *Int. Rev. Immunol.* 9: 223–230.
12. Whitham, R. H., B. L. Kotzin, A. C. Buenafe, A. D. Weinberg, R. E. Jones, G. A. Hashim, C. M. Hoy, A. A. Vandenbark, and H. Offner. 1993. Treatment of relapsing experimental autoimmune encephalomyelitis with T cell receptor peptides. *J. Neurosci. Res.* 35: 115–128.
13. Turley, D. M., and S. D. Miller. 2007. Peripheral tolerance induction using ethylenecarbodiimide-fixed APCs uses both direct and indirect mechanisms of antigen presentation for prevention of experimental autoimmune encephalomyelitis. *J. Immunol.* 178: 2212–2220.
14. Wasserman, H. A., and B. D. Evavold. 2008. Induction of anergy by antibody blockade of TCR in myelin oligodendrocyte glycoprotein-specific cells. *J. Immunol.* 180: 7259–7264.
15. Getts, D. R., D. M. Turley, C. E. Smith, C. T. Harp, D. McCarthy, E. M. Feeney, M. T. Getts, A. J. Martin, X. Luo, R. L. Terry, et al. 2011. Tolerance induced by apoptotic antigen-coupled leukocytes is induced by PD-L1+ and IL-10-producing splenic macrophages and maintained by T regulatory cells. *J. Immunol.* 187: 2405–2417.
16. Zhang, H., J. R. Podojil, J. Chang, X. Luo, and S. D. Miller. 2010. TGF-beta-induced myelin peptide-specific regulatory T cells mediate antigen-specific suppression of induction of experimental autoimmune encephalomyelitis. *J. Immunol.* 184: 6629–6636.
17. Getts, D. R., A. J. Martin, D. P. McCarthy, R. L. Terry, Z. N. Hunter, W. T. Yap, M. T. Getts, M. Pleiss, X. Luo, N. J. King, et al. 2012. Microparticles bearing encephalitogenic peptides induce T-cell tolerance and ameliorate experimental autoimmune encephalomyelitis. *Nat. Biotechnol.* 30: 1217–1224.
18. Darrasse-Jèze, G., S. Deroubaix, H. Mouquet, G. D. Victora, T. Eisenreich, K. H. Yao, R. F. Masilamani, M. L. Dustin, A. Rudensky, K. Liu, and M. C. Nussenzweig. 2009. Feedback control of regulatory T cell homeostasis by dendritic cells in vivo. *J. Exp. Med.* 206: 1853–1862.
19. Kretschmer, K., I. Apostolou, D. Hawiger, K. Khazaie, M. C. Nussenzweig, and H. von Boehmer. 2005. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat. Immunol.* 6: 1219–1227.
20. Yogev, N., F. Frommer, D. Lukas, K. Kautz-Neu, K. Karram, D. Ielo, E. von Stebut, H. C. Probst, M. van den Broek, D. Riethmacher, et al. 2012. Dendritic cells ameliorate autoimmunity in the CNS by controlling the homeostasis of PD-1 receptor(+) regulatory T cells. *Immunity* 37: 264–275.
21. Idoyaga, J., C. Fiorese, L. Zbytnuik, A. Lubkin, J. Miller, B. Malissen, D. Mucida, M. Merad, and R. M. Steinman. 2013. Specialized role of migratory dendritic cells in peripheral tolerance induction. *J. Clin. Invest.* 123: 844–854.
22. Stern, J. N., D. B. Keskin, Z. Kato, H. Waldner, S. Schallenberg, A. Anderson, H. von Boehmer, K. Kretschmer, and J. L. Strominger. 2010. Promoting tolerance to proteolipid protein-induced experimental autoimmune encephalomyelitis through targeting dendritic cells. *Proc. Natl. Acad. Sci. USA* 107: 17280–17285.
23. Ring, S., M. Maas, D. M. Nettelbeck, A. H. Enk, and K. Mahnke. 2013. Targeting of autoantigens to DEC205^{+} dendritic cells in vivo suppresses experimental allergic encephalomyelitis in mice. *J. Immunol.* 191: 2938–2947.
24. Mascanfroni, I. D., A. Yeste, S. M. Vieira, E. J. Burns, B. Patel, I. Sloma, Y. Wu, L. Mayo, R. Ben-Hamo, S. Efroni, et al. 2013. IL-27 acts on DCs to suppress the T cell response and autoimmunity by inducing expression of the immunoregulatory molecule CD39. *Nat. Immunol.* 14: 1054–1063.
25. Manicassamy, S., and B. Pulendran. 2011. Dendritic cell control of tolerogenic responses. *Immunol. Rev.* 241: 206–227.
26. Satpathy, A. T., X. Wu, J. C. Albring, and K. M. Murphy. 2012. Re(de)fining the dendritic cell lineage. *Nat. Immunol.* 13: 1145–1154.
27. Merad, M., P. Sathe, J. Helft, J. Miller, and A. Mortha. 2013. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu. Rev. Immunol.* 31: 563–604.
28. Hawiger, D., R. F. Masilamani, E. Bettelli, V. K. Kuchroo, and M. C. Nussenzweig. 2004. Immunological unresponsiveness characterized by increased expression of CD5 on peripheral T cells induced by dendritic cells in vivo. *Immunity* 20: 695–705.
29. Hawiger, D., Y. Y. Wan, E. E. Eynon, and R. A. Flavell. 2010. The transcription cofactor Hopx is required for regulatory T cell function in dendritic cell-mediated peripheral T cell unresponsiveness. *Nat. Immunol.* 11: 962–968.
30. Shin, C. H., Z. P. Liu, R. Passier, C. L. Zhang, D. Z. Wang, T. M. Harris, H. Yamagishi, J. A. Richardson, G. Childs, and E. N. Olson. 2002. Modulation of cardiac growth and development by HOP, an unusual homeodomain protein. *Cell* 110: 725–735.
31. Wan, Y. Y., and R. A. Flavell. 2005. Identifying Foxp3-expressing suppressor T cells with a bicistronic reporter. *Proc. Natl. Acad. Sci. USA* 102: 5126–5131.
32. Bettelli, E., M. Pagany, H. L. Weiner, C. Linington, R. A. Sobel, and V. K. Kuchroo. 2003. Myelin oligodendrocyte glycoprotein-specific T cell receptor transgenic mice develop spontaneous autoimmune optic neuritis. *J. Exp. Med.* 197: 1073–1081.
33. Schorle, H., T. Holtschke, T. Hünig, A. Schimpl, and I. Horak. 1991. Development and function of T cells in mice rendered interleukin-2 deficient by gene targeting. *Nature* 352: 621–624.
34. Takeda, N., R. Jain, M. R. Leboeuf, A. Padmanabhan, Q. Wang, L. Li, M. M. Lu, S. E. Millar, and J. A. Epstein. 2013. Hopx expression defines a subset of multipotent hair follicle stem cells and a progenitor population primed to give rise to K6+ niche cells. *Development* 140: 1655–1664.
35. Hawiger, D., K. Inaba, Y. Dorsett, M. Guo, K. Mahnke, M. Rivera, J. V. Ravetch, R. M. Steinman, and M. C. Nussenzweig. 2001. Dendritic cells induce peripheral T cell unresponsiveness under steady state conditions in vivo. *J. Exp. Med.* 194: 769–779.
36. Steinman, R. M., D. Hawiger, and M. C. Nussenzweig. 2003. Tolerogenic dendritic cells. *Annu. Rev. Immunol.* 21: 685–711.
37. Bonifaz, L., D. Bonnyay, K. Mahnke, M. Rivera, M. C. Nussenzweig, and R. M. Steinman. 2002. Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. *J. Exp. Med.* 196: 1627–1638.
38. Yamazaki, S., D. Dudziak, G. F. Heidkamp, C. Fiorese, A. J. Bonito, K. Inaba, M. C. Nussenzweig, and R. M. Steinman. 2008. CD8+ CD205+ splenic dendritic cells are specialized to induce Foxp3+ regulatory T cells. *J. Immunol.* 181: 6923–6933.
39. Viglietta, V., C. Baecher-Allan, H. L. Weiner, and D. A. Hafler. 2004. Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. *J. Exp. Med.* 199: 971–979.
40. Wu, Y., M. Borde, V. Heissmeyer, M. Feuerer, A. D. Lapan, J. C. Stroud, D. L. Bates, L. Guo, A. Han, S. F. Ziegler, et al. 2006. FOXP3 controls regulatory T cell function through cooperation with NFAT. *Cell* 126: 375–387.
41. Malek, T. R., and I. Castro. 2010. Interleukin-2 receptor signaling: at the interface between tolerance and immunity. *Immunity* 33: 153–165.

42. Chen, F., H. Kook, R. Milewski, A. D. Gitler, M. M. Lu, J. Li, R. Nazarian, R. Schnepp, K. Jen, C. Biben, et al. 2002. Hop is an unusual homeobox gene that modulates cardiac development. *Cell* 110: 713–723.
43. Davidson, T. S., R. J. DiPaolo, J. Andersson, and E. M. Shevach. 2007. Cutting Edge: IL-2 is essential for TGF-beta-mediated induction of Foxp3+ T regulatory cells. *J. Immunol.* 178: 4022–4026.
44. Curotto de Lafaille, M. A., N. Kutchukhidze, S. Shen, Y. Ding, H. Yee, and J. J. Lafaille. 2008. Adaptive Foxp3+ regulatory T cell-dependent and -independent control of allergic inflammation. *Immunity* 29: 114–126.
45. Josefowicz, S. Z., R. E. Niec, H. Y. Kim, P. Treuting, T. Chinen, Y. Zheng, D. T. Umetsu, and A. Y. Rudensky. 2012. Extrathymically generated regulatory T cells control mucosal TH2 inflammation. *Nature* 482: 395–399.
46. Samstein, R. M., S. Z. Josefowicz, A. Arvey, P. M. Treuting, and A. Y. Rudensky. 2012. Extrathymic generation of regulatory T cells in placental mammals mitigates maternal-fetal conflict. *Cell* 150: 29–38.
47. Lathrop, S. K., S. M. Bloom, S. M. Rao, K. Nutsch, C. W. Lio, N. Santacruz, D. A. Peterson, T. S. Stappenbeck, and C. S. Hsieh. 2011. Peripheral education of the immune system by colonic commensal microbiota. *Nature* 478: 250–254.
48. Korn, T., J. Reddy, W. Gao, E. Bettelli, A. Awasthi, T. R. Petersen, B. T. Bäckström, R. A. Sobel, K. W. Wucherpfennig, T. B. Strom, et al. 2007. Myelin-specific regulatory T cells accumulate in the CNS but fail to control autoimmune inflammation. *Nat. Med.* 13: 423–431.
49. Bailey-Bucktrout, S. L., M. Martinez-Llordella, X. Zhou, B. Anthony, W. Rosenthal, H. Luche, H. J. Fehling, and J. A. Bluestone. 2013. Self-antigen-driven activation induces instability of regulatory T cells during an inflammatory autoimmune response. *Immunity* 39: 949–962.
50. Zhou, X., S. L. Bailey-Bucktrout, L. T. Jeker, C. Penaranda, M. Martínez-Llordella, M. Ashby, M. Nakayama, W. Rosenthal, and J. A. Bluestone. 2009. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat. Immunol.* 10: 1000–1007.
51. Kee, H. J., J. R. Kim, K. I. Nam, H. Y. Park, S. Shin, J. C. Kim, Y. Shimono, M. Takahashi, M. H. Jeong, N. Kim, et al. 2007. Enhancer of polycomb1, a novel homeodomain only protein-binding partner, induces skeletal muscle differentiation. *J. Biol. Chem.* 282: 7700–7709.
52. Hamamori, Y., and M. D. Schneider. 2003. HATs off to Hop: recruitment of a class I histone deacetylase incriminates a novel transcriptional pathway that opposes cardiac hypertrophy. *J. Clin. Invest.* 112: 824–826.
53. Kook, H., J. J. Lepore, A. D. Gitler, M. M. Lu, W. Wing-Man Yung, J. Mackay, R. Zhou, V. Ferrari, P. Gruber, and J. A. Epstein. 2003. Cardiac hypertrophy and histone deacetylase-dependent transcriptional repression mediated by the atypical homeodomain protein Hop. *J. Clin. Invest.* 112: 863–871.
54. Kook, H., W. W. Yung, R. J. Simpson, H. J. Kee, S. Shin, J. A. Lowry, F. E. Loughlin, Z. Yin, J. A. Epstein, and J. P. Mackay. 2006. Analysis of the structure and function of the transcriptional coregulator HOP. *Biochemistry* 45: 10584–10590.
55. Yin, Z., L. Gonzales, V. Kolla, N. Rath, Y. Zhang, M. M. Lu, S. Kimura, P. L. Ballard, M. F. Beers, J. A. Epstein, and E. E. Morrisey. 2006. Hop functions downstream of Nkx2.1 and GATA6 to mediate HDAC-dependent negative regulation of pulmonary gene expression. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 291: L191–L199.
56. Takeda, N., R. Jain, M. R. LeBoeuf, Q. Wang, M. M. Lu, and J. A. Epstein. 2011. Interconversion between intestinal stem cell populations in distinct niches. *Science* 334: 1420–1424.
57. Katoh, H., K. Yamashita, M. Waraya, O. Margalit, A. Ooki, H. Tamaki, H. Sakagami, K. Kokubo, D. Sidransky, and M. Watanabe. 2012. Epigenetic silencing of HOPX promotes cancer progression in colorectal cancer. *Neoplasia* 14: 559–571.
58. Cheung, W. K., M. Zhao, Z. Liu, L. E. Stevens, P. D. Cao, J. E. Fang, T. F. Westbrook, and D. X. Nguyen. 2013. Control of alveolar differentiation by the lineage transcription factors GATA6 and HOPX inhibits lung adenocarcinoma metastasis. *Cancer Cell* 23: 725–738.
59. Watanabe, H., and M. Meyerson. 2013. Hopping between differentiation states in lung adenocarcinoma. *Cancer Cell* 23: 707–709.
60. Albrecht, I., U. Niesner, M. Janke, A. Menning, C. Loddenkemper, A. A. Kühl, I. Lepenies, M. H. Lexberg, K. Westendorf, K. Hradilkova, et al. 2010. Persistence of effector memory Th1 cells is regulated by Hopx. *Eur. J. Immunol.* 40: 2993–3006.
61. Yamaguchi, T., A. Kishi, M. Osaki, H. Morikawa, P. Prieto-Martin, K. Wing, T. Saito, and S. Sakaguchi. 2013. Construction of self-recognizing regulatory T cells from conventional T cells by controlling CTLA-4 and IL-2 expression. *Proc. Natl. Acad. Sci. USA* 110: E2116–E2125.
62. Baine, I., S. Basu, R. Ames, R. S. Sellers, and F. Macian. 2013. Helios induces epigenetic silencing of IL2 gene expression in regulatory T cells. *J. Immunol.* 190: 1008–1016.
63. Thornton, A. M., and E. M. Shevach. 1998. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J. Exp. Med.* 188: 287–296.
64. Liao, W., J. X. Lin, and W. J. Leonard. 2013. Interleukin-2 at the crossroads of effector responses, tolerance, and immunotherapy. *Immunity* 38: 13–25.
65. Boyman, O., M. Kovar, M. P. Rubinstein, C. D. Surh, and J. Sprent. 2006. Selective stimulation of T cell subsets with antibody-cytokine immune complexes. *Science* 311: 1924–1927.
66. Tang, Q., J. Y. Adams, C. Penaranda, K. Melli, E. Piaggio, E. Sgouroudis, C. A. Piccirillo, B. L. Salomon, and J. A. Bluestone. 2008. Central role of defective interleukin-2 production in the triggering of islet autoimmune destruction. *Immunity* 28: 687–697.
67. Webster, K. E., S. Walters, R. E. Kohler, T. Mrkvan, O. Boyman, C. D. Surh, S. T. Grey, and J. Sprent. 2009. In vivo expansion of T reg cells with IL-2-mAb complexes: induction of resistance to EAE and long-term acceptance of islet allografts without immunosuppression. *J. Exp. Med.* 206: 751–760.
68. Grigorian, A., H. Mkhikian, and M. Demetriou. 2012. Interleukin-2, Interleukin-7, T cell-mediated autoimmunity, and N-glycosylation. *Ann. N. Y. Acad. Sci.* 1253: 49–57.
69. Kim, M. G., T. Y. Koo, J. J. Yan, E. Lee, K. H. Han, J. C. Jeong, H. Ro, B. S. Kim, S. K. Jo, K. H. Oh, et al. 2013. IL-2/anti-IL-2 complex attenuates renal ischemia-reperfusion injury through expansion of regulatory T cells. *J. Am. Soc. Nephrol.* 24: 1529–1536.
70. Pierson, W., B. Cauwe, A. Policheni, S. M. Schlenner, D. Franckaert, J. Berges, S. Humblet-Baron, S. Schönefeldt, M. J. Herold, D. Hildeman, et al. 2013. Antiapoptotic Mcl-1 is critical for the survival and niche-filling capacity of Foxp3$^+$ regulatory T cells. *Nat. Immunol.* 14: 959–965.
71. Carbone, F., V. De Rosa, P. B. Carrieri, S. Montella, D. Bruzzese, A. Porcellini, C. Procaccini, A. La Cava, and G. Matarese. 2014. Regulatory T cell proliferative potential is impaired in human autoimmune disease. *Nat. Med.* 20: 69–74.