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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 indispensable 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 autoimmunity 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 (Tregs), 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 indispensable 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 Foxp3RFP+ 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+/Foxp3RFP+ and Hopx+Foxp3RFP mice. IL-2−/− mice (33) were bred with Hopx−/− and IL-2−/− heterozygotes were used to produce Hopx−/−IL-2−/− and Hopx−/−IL-2−/−/2D2 TCR-tg Foxp3RFP and non-TCR–tg Foxp3RFP mice. Hopx+/Foxp3RFP reporter mice (34) that faithfully track Hopx expression were first bred on the C57BL/6 background and then crossed with Foxp3RFP reporter mice (31), as well as with 2D2 TCR-tg mice (32). Sex- and age-matched littersmates were used for experiments. Mice were used at 6–8 wk of age, with the exception of IL-2−/− and IL-2−/− littersmate 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 University 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.
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-CD2L (MEL-14), anti-ICOS (C398.4A), anti–PD-1 (29F.1A.12), 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 (MOG35-55, 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 μg/mouse i.p.) in PBS on days 0 and 2 after MOG35-55 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% PBS, Penicillin-Streptomycin, 1-μg/ml, and 2-ME (Life Technologies) in the presence of soluble anti-CD28 (37.51; 1.5 μg/ml), IL-2 (200 U/ml), and TGFB (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 miRNAasy 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 ΔΔct cycle threshold method.

Statistical analyses

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 MOG35-55 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 but 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 Foxp3CD25 Tregs in vivo. Treatment with anti–CD25 Ab resulted in an ∼60% reduction in the numbers of Foxp3+ Tregs,
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). 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 HopxFlag-viral2A-GFP/Foxp3IRES-RFP double-reporter mouse (referred to as HopxGFP/Foxp3RFP reporter mice). We sorted GFP+ (Hopx+) RFP+ (Foxp3+) CD25+ CD4 cells from 2D2 TCR-tg HopxGFP/Foxp3RFP 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 indispensable 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. 3C). 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...
confers tolerance. As expected, in the absence of any transferred cells (PBS only), treatment with anti–DEC-MOG failed to induce tolerance in Hopx<sup>−/−</sup> mice. However, we found that mice transferred with Hopx<sup>+/+</sup> Foxp<sup>3</sup>−CD25<sup>+</sup> precursors of pTregs were protected from EAE. In contrast, mice transferred with Hopx<sup>−/−</sup> Foxp<sup>3</sup>−CD25<sup>+</sup> 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<sup>+/+</sup> or Hopx<sup>−/−</sup> 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 Foxp<sup>3</sup>−CD25<sup>−</sup>CD4<sup>+</sup> T cells isolated from Hopx<sup>+/+</sup> or Hopx<sup>−/−</sup> 2D2 TCR-tg Foxp<sup>3</sup>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 Foxp<sup>3</sup>−CD25<sup>−</sup>CD4<sup>+</sup> T cells (Supplemental Fig. 4A). We found that populations of Hopx<sup>+/+</sup> and Hopx<sup>−/−</sup> 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<sup>+/+</sup> and Hopx<sup>−/−</sup> pTregs (Supplemental Fig. 4B). To further examine the fate of such pTreg populations, we examined the percentage of Foxp<sup>3</sup>−CD25<sup>+</sup> 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.

*FIGURE 2. DCs induce Hopx<sup>+</sup> 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<sup>+</sup>/RFP<sup>+</sup>/CD25<sup>+</sup>CD4<sup>+</sup> T cells were purified from 2D2 TCR-tg Hopx<sup>Flag-vital2A-GFP/Foxp3<sup>RFP</sup></sup> double-reporter mice and then adoptively transferred into CD45.1<sup>+</sup> 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 Foxp<sup>3</sup>−CD25<sup>+</sup> 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.
consistent with a lack of downregulation of Foxp3, suggesting deletion as a possible mechanism behind the decreased numbers of Hopx<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> pTregs that express IL-2 are expected to die rapidly in vivo (Fig. 4), we examined IL-2 expression by quantitative PCR in Hopx<sup>+/+</sup> or Hopx<sup>−/−</sup> in vitro–induced Tregs after a brief restimulation (Fig. 5A). Expression of IL-2 was ∼3-fold higher in Hopx<sup>−/−</sup> than in Hopx<sup>+/+</sup> induced Tregs (Fig. 5A).

To directly examine the impact of endogenously produced IL-2 on the functions of Hopx<sup>−/−</sup> pTregs, we used T cells from IL2<sup>−/−</sup> mice that we crossed with Hopx<sup>+</sup/> and Hopx<sup>−/−</sup> mice. IL2<sup>−/−</sup> Tregs can develop in the presence of exogenous IL-2 (43). Therefore, we first confirmed induction of IL2<sup>−/−</sup> pTregs in an IL-2–sufficient environment by adoptively transferring sorted Hopx<sup>−/−</sup> or Hopx<sup>−/−</sup>/Foxp3<sup>RFP</sup> cells into IL2<sup>+/+</sup> recipient mice that were subsequently treated with anti–DEC-MOG. We found that Hopx<sup>−/−</sup>/IL2<sup>−/−</sup> and Hopx<sup>−/−</sup>/IL2<sup>−/−</sup>/Foxp3<sup>CD25−</sup> cells converted to Foxp3<sup>CD25−</sup> pTregs at a similar rate (Supplemental Fig. 4C). To examine the impact of antigenic rechallenge on Hopx<sup>−/−</sup>/IL2<sup>−/−</sup> and Hopx<sup>−/−</sup>/IL2<sup>−/−</sup>/pTregs, we immunized the recipient mice that harbored preformed pTregs with...
MOG<sub>35-55</sub> in CFA and PT. We recovered ~3-fold fewer Hopx<sup><i>-/-</i></sup> IL<sub>2</sub><sup><i>-/-</i></sup> pTregs than Hopx<sup><i>-/-</i></sup>IL<sub>2</sub><sup><i>+</i></sup> pTregs (Fig. 5B). In contrast, we recovered similar numbers of Hopx<sup><i>-/-</i></sup>IL<sub>2</sub><sup><i>+</i></sup>/ Hopx<sup><i>-/-</i></sup>IL<sub>2</sub><sup><i>++</i></sup> pTregs under the same conditions in vivo (Fig. 5C). We also determined that neither Hopx<sup><i>-/-</i></sup> nor Hopx<sup><i>-/-</i></sup> IL-2-deficient pTregs underwent apoptotic cell death following antigen rechallenge under proinflammatory conditions (Supplemental Fig. 4D). Therefore, an increased expression of IL-2 in Hopx<sup><i>-/-</i></sup> 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<sup><i>-/-</i></sup> mice with Hopx<sup><i>-/-</i></sup>IL<sub>2</sub><sup><i>++</i></sup>, Hopx<sup><i>-/-</i></sup>IL<sub>2</sub><sup><i>-/-</i></sup>, Hopx<sup><i>-/-</i></sup>IL<sub>2</sub><sup><i>++</i></sup>/ Hopx<sup><i>-/-</i></sup>IL<sub>2</sub><sup><i>-/-</i></sup>, or Hopx<sup><i>-/-</i></sup>IL<sub>2</sub><sup><i>-/-</i></sup> Foxp3<sup>+</sup>CD25<sup>+</sup> 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<sup>+</sup>CD25<sup>+</sup> cells restored tolerance, whereas Hopx<sup><i>-/-</i></sup>IL<sub>2</sub><sup><i>-/-</i></sup> Foxp3<sup>++/+</sup> cells failed to restore tolerance and prevent symptoms of EAE. In contrast, Hopx<sup><i>-/-</i></sup>IL<sub>2</sub><sup><i>-/-</i></sup> Foxp3<sup>-/-</sup>CD25<sup>-/-</sup> cells prevented EAE symptoms and T cell infiltration of spinal cords to a similar extent as observed with Hopx-sufficient Foxp3<sup>+</sup>CD25<sup>+</sup> 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 Tregs or de novo–induced pTregs to the prevention of general autoimmune 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<sup>+</sup> Tregs that then may fail to maintain their regulatory phenotype (49, 50). Our present results
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-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 ≤ 0.01, 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 Foxp3RFP 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 MOG35–55 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 Foxp3RFP 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+/− Foxp3RFP mice. All mice were treated with anti–DEC-MOG 6 wk before induction of EAE by immunization with MOG35–55 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.
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 Treg populations following their initial expansion (67).

The c-fos/c-jun AP-1 complex induces IL-2 expression in T cells (40, 63, 64). We established that, consistent with its inhibition of the expression of c-fos and c-jun, Hopx governs IL-2 expression in induced Treggs. 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<sup>−/−</sup> Treg cell numbers and functions in tolerance. Therefore, we propose that Hopx-dependent inhibition of IL-2 expression in Treggs 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 Treggs. Further, by establishing the role for Hopx as an indispensable regulator of Treg and tolerance maintenance in vivo, our results could provide the foundation for more selective and efficient immune therapies for specific immune disorders.

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Disclosures

The authors have no financial conflicts of interest.

References

Supplemental Figures for 
“Peripherally induced tolerance depends on pTreg cells that require Hopx to inhibit intrinsic IL-2 expression”

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Supplemental Figure 1. (A) Histograms show CFSE dye dilution of gated populations of CD4+ CD45.1+ 2D2 TCR tg T cells adoptively transferred into Hopx+/+ and Hopx−/− mice that were treated with either (■) αDEC-MOG, (■) IC-MOG or (■) PBS as indicated. The results represent one of two similar experiments. (B-C) Hopx+/+ or Hopx−/− CD4+ Foxp3neg CD25neg 2D2 TCR tg T cells were adoptively transferred into CD45.1+ recipient mice that were then treated with αDEC-MOG. 8 days later recipient mice were immunized with MOG/CFA and PTX. Graphs show mean number of transferred cells that stained positive for (B) RORγT or (C) Tbx21 7 days after MOG/CFA and PTX treatment +/- SD.
Results shown in (A-C) are from lymph nodes and similar results were obtained from spleens.
Supplemental Figure 2.

(A) Gating strategy starting with an initial “lymphoid gate” for spinal cords analyzed by FACS from mice with an EAE score of 0 (top panels) or an EAE score of 3 (bottom panels). (B) FACS analysis of the same spinal cord samples of as in (A) and as employed in figures 1D, 1E, 3C, 3E and 5D, based on initial “lymphoid gate”. (C) Multiple groups of mice were treated with αDEC-MOG, αDEC-OVA or PBS 5 weeks before immunization with MOG35-55 in CFA + PT. Graphs show mean disease scores (n=10 per group from 2 experiments). Results are mean +/- SEM, *** P< 0.001 and **** P< 0.0001 determined by two-way ANOVA.
Supplemental Figure 3.

(A) Foxp3\textsuperscript{RFP} mice were treated with either \(\alpha\)CD25 or the same dose of Rat IgG1. 3 weeks after treatment lymph nodes were analyzed by FACS. Graphs show the percent of Foxp3\textsuperscript{+} cells among CD4\textsuperscript{+} cells (n=3-4 per group). Results show mean +/- SEM, ** P<0.01 determined by Students T test. Similar results were obtained from spleens. (B) Two groups of C57BL/6 mice were treated with \(\alpha\)DEC-MOG. 3 weeks after treatment with \(\alpha\)DEC-MOG, individual groups of mice were injected with either \(\alpha\)CD25 or the same dose of Rat IgG1. After another 3 weeks EAE was induced. Graph shows mean disease scores (n=9-10 per group from 2 experiments). Results show mean +/- SEM, * P<0.05 determined by two-way ANOVA. (C) GFP\textsuperscript{neg}/RFP\textsuperscript{neg}/CD25\textsuperscript{neg} CD4\textsuperscript{+} T cells were purified by sorting from 2D2 TCR tg Hopx\textsuperscript{Flag-viral2A-GFP/Foxp3\textsuperscript{RFP}} double-reporter mice and then adoptively transferred into CD45.1 recipient mice. Spleens from recipient mice were
analyzed at multiple days after treatment with αDEC-MOG or IC-MOG as indicated.

Graphs show the average percentage of transferred cells that were foxp3⁺ CD25⁺ +/- SD, ** P<0.01 determined by Students T test. (D-F) CD4⁺ CD45.1⁺ Foxp3⁻/neg or Foxp3⁺ T cells were adoptively transferred into multiple CD45.2 recipient mice that were then treated with αDEC-MOG. Lymph nodes from recipient mice were analyzed by FACS 6 weeks after treatment with αDEC-MOG. (D) Graphs show the frequency of transferred cells that were Foxp3⁺CD25⁺ among total CD4⁺ cells. (E) Graphs show the frequency of transferred cells that were Foxp3⁺ CD25⁺ among total lymphocytes. D and E (n=3-7) +/- SD, ** P<0.01 determined by Students T test. (F) Representative plots show Foxp3 (RFP) expression (Y-axis) and staining intensity with anti-CD45.1 (X-axis) among CD4⁺ cells. Similar results were obtained from spleens.
Supplemental Figure 4.

(A-B) Foxp3^{neg} CD25^{neg} 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 then treated with αDEC-MOG or PBS. (A) Plots show Foxp3 (RFP) expression (Y-axis) and staining intensity with anti-CD25 (X-axis) of gated populations of adoptively transferred cells from recipient mice 10 days or 24 days after αDEC-MOG treatment or PBS treatment. The results represent one of three similar experiments. (B) Histograms show staining intensity with anti-PD-1, anti-ICOS and anti-CD73 of induced Hopx^{+/+} or Hopx^{-/-} Foxp3^{-}/CD25^{+} cells 24 days after αDEC-MOG treatment. Results represent one of two similar experiments. (C) Foxp3^{neg} CD25^{neg} 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 then treated with...
αDEC-MOG. Plots show Foxp3 (RFP) expression (Y-axis) and staining intensity with anti-CD25 (X-axis) of gated populations of adoptively transferred cells from lymph nodes of recipient mice after treatment with αDEC-MOG. Results represent one of two similar experiments. (D) Foxp3\textsuperscript{neg} CD25\textsuperscript{neg} CD4\textsuperscript{+} T cells were purified by sorting from either Hopx\textsuperscript{+/+} IL-2\textsuperscript{−/−} or Hopx\textsuperscript{−/−} IL-2\textsuperscript{−/−} I2D2 TCR tg Foxp3\textsuperscript{RFP} mice and then adoptively transferred into CD45.1\textsuperscript{+} recipient mice that were then treated with αDEC-MOG. After 9 days recipient mice were treated with MOG/CFA and PTX. Overlaid histograms show staining intensity with Annexin V in Foxp3\textsuperscript{+}CD25\textsuperscript{+} pTreg cells 3 days after MOG/CFA and PTX. Results represent one of two similar experiments. Results shown in (A-D) are from lymph nodes and similar results were obtained from spleens.