Natural regulatory T cells are resistant to calcium release-activated calcium (CRAC/ORAI) channel inhibition

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Received 12 December 2012, accepted 7 March 2013

Abstract

Organ transplant patients are often treated with immunosuppressants, such as the calcineurin phosphatase inhibitor, cyclosporin A, to block T cell-mediated graft rejection. The calcium release-activated calcium (CRAC/ORAI) channels, which act upstream of calcineurin, are essential for calcium entry and CD4+ T-cell activation. Although cyclosporine A has also been shown to inhibit FoxP3+ Tregs both in vitro and in vivo, the role of ORAI channel inhibition in natural Tregs (nTregs) or inducible Tregs (iTregs) has not been investigated. We found that, despite inhibition of calcium influx through the ORAI channels, ORAI channel inhibitors were unable to repress FoxP3 expression in mouse and human nTregs, whereas FoxP3 expression was inhibited in iTregs. In contrast, cyclosporin A inhibited FoxP3 expression in both nTregs and iTregs. We also generated mice with a T cell-specific, conditional knockout of ORAI1 and found that the mice have normal nTreg development and suppressive activity. Moreover, iTregs derived from ORAI1 conditional knockout mice develop normally and are still susceptible to ORAI channel inhibition. Our data indicate that unlike CD4+ T cells and iTregs, nTregs are resistant to ORAI-mediated inhibition. Targeting ORAI channels potentially offers a novel way to inhibit pathologic T cells, while sparing nTreg-mediated tolerance.

Keywords: cyclosporin A, FoxP3, ORAI channels, Tregs

Introduction

Immune suppressants, including calcineurin inhibitors (CNIs) such as cyclosporin A (CsA) and FK506, have long been used in clinical transplant patients and in autoimmune disease settings, to control rampant anti-graft or anti-self-immune responses (1). CNIs inhibit IL-2 production from T cells, thereby blocking T-cell expansion and activation. In addition to its negative effects on T lymphocytes, CsA inhibits Treg expansion and homeostasis in vitro and in vivo (1–4). FoxP3+ Tregs can be derived from the thymus [natural Tregs (nTregs)] or induced in the periphery via conversion of naive CD4+ T cells into FoxP3+ Tregs [inducible Tregs (iTregs)] in the presence of TGF-β and IL-2 (5). FoxP3 is the forkhead family transcription factor responsible for driving and coordinating Treg development and function (6–8). Recently, the Helios transcription factor was identified as being selectively expressed in nTregs but generally not in iTregs (9, 10). Under homeostatic conditions, nTregs are responsible for maintaining peripheral tolerance via suppression of self-reactive T cells and prevention of autoimmunity; during pathologic conditions, both nTregs and iTreg conversion may be critical for controlling disease (11, 12). Because maintaining Treg populations in transplant and autoimmune patients is essential for establishing tolerance, identification of novel therapies that inhibit T-cell activation, but preserve Treg function and stability would provide long-term efficacy and benefits.

As their name implies, the CNIs inhibit calcium-dependent calcineurin phosphatase activity, downstream of calcium release-activated calcium (CRAC/ORAI) channels. This mechanism of calcium entry, known as store-operated calcium entry (SOCE), is required for proper T-cell activation, but preserve Treg function and stability would provide long-term efficacy and benefits.
ER-resident calcium sensor molecule, STIM1 and STIM2, to the plasma membrane. There, STIM molecules bind to and open the ORAI channels (comprising ORAI1, ORAI2 and ORAI3 subunits) allowing calcium to enter the cell and activate calcineurin phosphatase activity (14, 15). Calcineurin dephosphorylates the transcription factor, NFAT, which then translocates into the nucleus and regulates various target genes, such as IL-2 that are responsible for driving T-cell proliferation and activation (16, 17).

The ORAI signaling pathway is essential for T-lymphocyte function and clonal expansion, as patients with ORAI1 loss of function mutations develop a SCID phenotype. However, less is known about the role of the ORAI channels in nTreg development and function, as the nTregs from the ORAI1 mutant patient were not analyzed (18). In comparison, the role of calcineurin and nuclear factor of activated T cells (NFAT) both downstream of ORAI channels, in nTreg function has been well established (19). First, NFAT induces FoxP3 expression by binding directly to the FoxP3 promoter and enhancer regions (20). Second, once FoxP3 is expressed in nTregs, NFAT binds to FoxP3 and inhibits IL-2 production and reduces CTLA4 expression (21, 22). Moreover, CsA and FK506 inhibit NFAT-FoxP3-mediated gene responses (1, 23). Thus, although the downstream pathway has been well documented, the mechanism(s) of calcium entry into nTregs has not been investigated.

Two approaches have been taken to generate mice globally deficient in ORAI1, resulting in two starkly different T-cell phenotypes. One model did not have significant defects in T-cell development or functions, whereas the second model had a severe T-cell phenotype (24, 25). Although there is significant discrepancy between the two models regarding the T-cell phenotype, the effect of ORAI channel loss or inhibition in mouse nTregs has not been adequately evaluated.

In this study, we analyzed the effects of ORAI-specific channel inhibitors (ORAIci) on human and mouse nTregs and iTregs. Even though ORAIci potently inhibit rapid calcium influx in nTregs, ORAIci do not inhibit calcineurin activity, NFAT activity or FoxP3 expression, whereas CsA, which inhibits downstream of ORAI, does inhibit FoxP3 expression. In contrast, iTregs are susceptible to FoxP3 inhibition by both CsA and ORAIci. Thus, our data indicate that unlike iTregs and CD4+ T cells, nTregs are resistant to ORAI channel inhibition.

Methods

nTreg and T-cell experiments

Human nTregs and iTregs were generated as previously published (26–28). In brief, human nTregs were isolated from whole blood (n = 4 donors) via FACS sorting on CD4+ CD25hi and then expanded with high-dose coated anti-CD3 and IL-2 for 14 days. This protocol selectively protects Tregs and induces cell death in any contaminating CD4+ T cells. Following expansion, >95% of the cells were FoxP3+ and were then frozen down in aliquots. Each nTreg donor was tested for FoxP3 expression and proliferation, and each donor was tested 3–4 times. Tregs were thawed and briefly expanded with anti-CD3/CD28 Treg expander beads (Invitrogen, Carlsbad, CA, USA) plus 50–100 ng/ml IL-2 (R&D Systems, Minneapolis, MN, USA) prior to use in functional assays. Intracellular mouse or human FoxP3 expression was analyzed via FoxP3 staining kit (e-Bioscience, San Diego, CA, USA). For the calcium-free media experiment, Tregs were cultured in MEM with or without CaCl2 (Sigma, St Louis, MO, USA) with 10% fetal bovine serum for 72 h. Cell proliferation and viability were measured using Cell Titer Glo kit or Cyto Tox-One Homogenous Membrane Integrity Assay, respectively (Promega, Madison, WI, USA). The ORAIci compound, RO6712, originally generated by Yamanouchi Pharmaceutical corporation and designated as YM-58483, was synthesized by Hoffmann-La Roche on the basis of previously published reports and reconstituted in DMSO (29, 30). Naive human CD4+ T cells were purified from peripheral blood of healthy donors using Miltenyi Naive CD4+ T Isolation Kit II, which depletes CD4+CD45RO+ memory T cells and non-CD4+ T cells (Auburn, CA, USA). In some cases, 50 ng/ml of recombinant hIL-2 was added. To obtain peripheral blood from healthy donors, Roche established an internal blood donation program, which was reviewed and received approval from an outside IRB (Copernicus Group IRB, Research Triangle Park, NC, USA). All volunteers gave proper consent before becoming an active donor.

NFAT and calcineurin assays

CD4+ T cells were treated with αCD3/αCD28 beads for 20 h, washed, pre-treated with 1 µM compound or DMSO control, and then treated with activator beads for another 20 h. Nuclear extraction and NFATc1 activity were assayed using the TransAM NFATc1 kit, according to the manufacturer’s protocol (Active Motif, Carlsbad, CA, USA). The calcineurin cellular activity assay kit (Enzo Farmingdale, NY, USA) was used with several modifications. Briefly, cell pellets were re-suspended in 200 µl of lysis buffer [50 mM Tris-HCL (pH 7.7), 1.0 mM dithiothreitol, 5.0 mM ascorbic acid, 0.02% (v/v) tween 20] with EDTA-free protease inhibitor and were lysed by three freeze-thaw cycles (liquid N2/30°C). Lysate was used to analyze the calcineurin assay. Functional assays were performed three times each.

Fluorescent Imaging Plate Reader (FLIPR) assays

CD4+ T cells were activated using αCD3/αCD28 beads for 24–48 h. Activated CD4+ T cells were washed with HBSS buffer with 20 mM HEPES, 2.5 mM probenecid and 5 mM glucose. One hundred thousand cells per well plus an equal volume of Fluo-4 NW dye in assay buffer (Invitrogen) were incubated at 37°C for 1–2 h. Cells were stimulated with 10 µM thapsigargin or 20 µg/ml PHA plus increasing compound concentrations for 10 min. An equal volume of 4 mM calcium assay buffer was added and fluorescence intensity was recorded for 10 min at Ex/Em (494/516 nm) using FLIPR TERA (Molecular Devices, Silicon Valley, CA, USA). FLIPR assays were performed with three different healthy donors and Treg donors.

Quantitative real-time PCR studies

RNA was isolated from CD4+ T cell samples of mouse and human using PerfectPure RNA 96 Cell Kit (5 Prime, Gaithersburg, MD, USA). For CDNA synthesis, 4 µl of the RNA template was reverse transcribed (Transcriptor First Strand
cDNA Synthesis Kit, Roche Custom Biotech) using random hexamers. Quantitative real-time PCR (qPCR) was performed using Applied Biosystems 7900 HT under normal settings. Raw data were analyzed using tools developed internally. Normalized relative expression values were generated, respective to species-specific housekeeping genes.

Wild-type and ORAI conditional knockout experiments

Generation of ORAI1 conditional knockout (CKO) mice is described in Supplementary Material, available at International Immunology Online. Mouse CD4+ T cells were purified from spleens using CD4+CD62L+ T Cell Isolation Kit II, which depletes non-CD4+ T cells and then isolates CD4+CD62L+ via positive selection or CD4 (L3T4) MicroBeads, which depletes non-CD4+ cells and then isolates CD4+CD62L+ cells with positive selection. Mouse nTregs were purified using the CD4+CD25+ regulatory T cell isolation kit, which depletes non-CD4+ cells and then isolates CD4+CD25+ T cells via positive selection (Miltenyi). More than 90% of the cells were consistently FoxP3+. Splenocytes were analyzed using anti-CD3, anti-CD4, anti-CD8 and anti-CD19 (BD Pharmingen, Franklin Lakes, NJ, USA). For suppressor assays, target cells were labeled with 5 μM CFSE and then cultured with increasing concentrations of Tregs, for 5 days. Proliferation was measured using flow cytometry.

Results

Because ORAI1 is believed to be the dominant and best-characterized ORAI channel subunit in humans (31), we first determined the levels of human ORAI1 by qPCR in αCD3/αCD28 activated T cells versus human nTregs, defined as CD4+CD25+FoxP3+ T cells with potent suppressive activity (26, and data not shown). Activated CD4+ T cells and nTregs express comparable levels of ORAI1 (P = 0.11), whereas naive T cells express significantly more ORAI1 than nTregs (P = 0.001) (Supplementary Figure 1A, available at International Immunology Online). Immunofluorescence confirmed comparable levels of ORAI1 (~70% ORAI1+ cells) on the cell surface and comparable levels of ORAI2, but undetectable levels of ORAI3 on both activated human CD4+ T cells and Tregs (Supplementary Figure 1B, available at International Immunology Online). Both STIM1 and STIM2 were comparably expressed in activated CD4+ T cells and nTregs (data not shown).

Several ORAI1c have been developed to (i) better understand ORAI activity, (ii) differentiate from the effects of CsA and (iii) provide a better safety profile than CsA (29). We synthesized the previously characterized Yamamoto compound, YM-58483, which we designated here as ORAI1c RO6712 (29, 30, 32). ORAI1c RO6712 can inhibit ORAI1, ORAI2 and ORAI3 with similar potency (data not shown). Although ORAI1c RO6712 has previously been reported to inhibit human and mouse T cells, neither it, nor any other ORAI1c has been tested on Tregs.

Human nTregs or activated human CD4+ T cells were stimulated with either (i) thapsigargin, to release intracellular ER-Ca2+, stores or (ii) PHA, to cross-link TCR and trigger cellular calcium influx (26). Using FLIPR assays, ORAI1c RO6712 was capable of rapidly and completely inhibiting calcium influx back to baseline levels in both nTregs and CD4+ T cells (IC50 = 0.2–0.5 μM for both cells). As expected, the immunosuppressant, CsA which inhibits calcineurin activity downstream of ORAI, did not inhibit calcium entry (Fig. 1A and B).

We next measured effects of CsA and ORAI1c on αCD3-αCD28-IL-2- and IL-2-induced proliferation. Both CD4+ T cells and nTregs expanded comparably in response to TCR cross-linking plus IL-2 (Fig. 1C, left panel). However, only CD4+ T cell expansion, but not nTregs, could be inhibited by both ORAI1c and CsA (percent inhibition, Fig. 1C right panel and raw values, Supplementary Figure 1C, available at International Immunology Online). In addition, neither compound could inhibit IL-2-driven proliferation in nTregs or pre-activated T cells, even though both cell populations expanded comparably (Fig. 1D). As a positive control for inhibition of nTreg expansion, we also tested a Jak1/3 inhibitor that blocks downstream of the IL-2 pathway because Tregs rely on IL-2 signaling for expansion, viability and maintenance of suppressive activity (33, 34). Unlike CsA and ORAI1c, the Jak inhibitor did block Treg expansion in response to PMA-ionomycin plus IL-2 or IL-2 alone (Supplementary Figure 1D, available at International Immunology Online). To ensure that the cells were not dying in response to CsA or ORAI1c treatment, loss of membrane integrity and cell death were measured. Although CD4+ T cells were growth inhibited, both CD4+ T cells and Tregs were viable following CsA and ORAI1c treatment, indicating that the compounds were not toxic and were not causing death. Previous studies with ORAI1c on Jurkat T cells and CD4+ T cells also support the conclusion that there is growth inhibition, as opposed to cell death (29, 35).

Moreover, up-regulation of CD25, which is responsible for binding to IL-2, leading to cell cycle progression, was inhibited by both CsA and ORAI1c in CD4+ T cells, as was IL-2 production (Supplementary Figure 2A and B, available at International Immunology Online). In activated CD4+ T cells, CsA and ORAI1c did not repress CD25 expression but did inhibit IL-2 expression (Supplementary Figure 2C and D, available at International Immunology Online). Thus, the lack of growth inhibition in activated CD4+ T cells is likely due, in part, to the fact that IL-2 signaling is calcium independent.

To determine whether ORAI1c had a similar effect on mouse T cells, calcium influx was analyzed in ORAI1c RO6712-treated mouse CD4+ T cells. Consistently, ORAI1c RO6712 could also inhibit calcium influx in mouse PHA-treated T cells, in a dose-dependent manner (Fig. 1E). Consistent with human cells, both CsA and ORAI1c RO6712 inhibited mouse T-cell proliferation, but they did not inhibit freshly purified murine CD4+ CD25+FoxP3+ nTreg proliferation (Fig. 1F and G, respectively).

Both nTregs and iTregs express the transcription factor, FoxP3, whereas the transcription factor, Helios, is selectively expressed in nTregs, but not in iTregs and can be used to discriminate between the two Treg populations (9, 36). We next investigated whether calcium entry was required to maintain both FoxP3 and Helios expression. When nTregs were cultured in calcium-free media, FoxP3 expression levels plummeted, whereas Helios expression remained stable (Fig. 2A). Even in the absence of calcium, there was no effect on Treg viability, indicating that the loss of FoxP3 in Tregs was not due to cell death. Moreover, the residual calcium released from ER stores was not sufficient to maintain FoxP3 expression long term, indicating that calcium influx into Tregs is required for FoxP3, but
not Helios, expression. As expected, IL-2-dependent FoxP3 expression was inhibited by the Jak inhibitor as measured by both qPCR (data not shown) and flow cytometry (Supplementary Figure 1F, available at International Immunology Online).

Expression of FoxP3, along with other transcription factors, coordinates the full suppressive activity of Tregs (37). The FoxP3 promoter and enhancer both contain calcium-dependent NFAT-binding sites essential for FoxP3 expression (20). Fixing the concentration of ORAIci RO6712 and CsA at 1 µM, ORAIci still did not inhibit FoxP3 expression, whereas CsA could partially, but significantly, block expression at 96 h (Fig. 2B). In addition, expression of several downstream FoxP3 target genes, CD25, CTLA4, and GITR was again partially, but significantly inhibited by CsA, but not by ORAIci RO6712 (Fig. 2D). At 1 µM, CD25 protein expression was also partially inhibited by CsA (CsA versus DMSO, P = 0.0007), but not by ORAIci compared with DMSO-treated cells (ORAIci versus DMSO, P = 0.8) (Fig. 2D). In addition, we found that CsA begins to inhibit FoxP3 expression at 72 h (Fig. 2E).

Following calcium entry into T cells, the phosphatase, calcineurin, is activated via binding to calcium and calmodulin, which in turn induces NFAT translocation and transcriptional activity (17). To further probe the downstream pathway, we analyzed whether cellular calcineurin phosphatase activity and nuclear NFAT transcriptional activity were inhibited in CsA- or ORAIci-treated nTregs. Consistent with the FoxP3 data, both CsA and ORAIci RO6712 inhibited cellular calcineurin activity in CD4+ T cells (90 versus 60%, respectively), whereas only CsA inhibited calcineurin activity in nTregs (100 versus 25%, respectively, Fig. 2F). Similarly, CsA and ORAIci demonstrated comparable inhibition of

Fig. 1. ORAIcis block calcium influx in both CD4+ T cells and nTregs, but only suppress CD4+ T-cell proliferation. (A and B) Human nTregs or activated CD4+ T cells were labeled with Fluo-4 NW dye and then incubated with increasing concentrations of CsA or ORAIci RO6712. Cells were then treated with either thapsigargin (A) or PHA (B) to trigger ORAI channel opening, and calcium influx was measured using FLIPR assays. (C) CD4+ T cells and nTregs were activated with αCD3–αCD28 beads plus IL-2 in the presence of ORAIci RO6712 and CsA for 96 h. Cell proliferation was measured by Cell Titer Glo. The amount of T-cell and Treg proliferation over baseline is shown in the bar graph (left panel). (D) CD4+ T cells or nTregs were activated with αCD3-αCD28 beads for 48 h and then cultured with IL-2 alone. Proliferation was measured as above. (E) Mouse CD4+ T cells were activated for 24 h, treated with increasing concentrations of CsA or ORAIci RO612, and then PHA-induced calcium influx was measured by FLIPR, as in (A). (F and G) Mouse CD4+ T cells (F) or mouse FoxP3– nTregs (G) were activated in the presence of DMSO or increasing concentrations of CsA or ORAIci RO6712 and proliferation was measured as in (C).
nTregs are resistant to ORAI channel inhibition

501

nuclear NFAT activity in T cells, but virtually no inhibition by ORAIci in nTregs (Fig. 2G).

In contrast to nTregs, which are derived from the thymus, iTregs are generated in the periphery, converted from naive CD4+ T cells into FoxP3-expressing Tregs with suppressive activity (27). We next investigated whether CsA or ORAIci have the same effects on FoxP3 expression in iTregs, as measured by the amount and the percentage of cells expressing FoxP3 (Fig. 3A and B) or CD25 (Fig. 3C). ORAIci selectively inhibit FoxP3 in developing and established iTregs, but not nTregs.

However, these results do not address the role of ORAI channels in Treg development. In addition because T cells themselves are susceptible to ORAIci, we were unable to test the effects of ORAIci on Treg suppressive function. Although two different ORAI1-deficient mouse models have previously been generated, resulting in two disparate T-cell phenotypes, neither study fully investigated the effect of ORAI1 deletion on Tregs (24, 25). Thus, ORAI1 CKO mice were generated with ORAI1 deleted specifically in T cells, using Lck-Cre recombinase (Supplementary Figure 3A and B, available at International Immunology Online). ORAI1 CKO mice were born at the normal frequency and were healthy and viable. Mice developed normally, with no overt signs of immunodeficiency or autoimmunity. When expression of ORAI1 was analyzed in naive and activated ORAI1 CKO CD4+ T cells and CD4+CD25+ Tregs, we found 70–80% reduction, but not complete loss of ORAI1 expression (Figs 4A and 5B, respectively). This residual ORAI1 expression

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was likely due to contaminating non-T cells and/or inefficient Cre recombinase tissue activity (Supplementary Figure 3C, available at International Immunology Online). Comparable expression of ORAI2 and ORAI3 was observed in wild-type (WT) and ORAI1 CKO CD4+ T cells, with higher ORAI2 levels compared with ORAI1 levels (Supplementary Figure 3D, available at International Immunology Online).

The splenic percentages of CD4+ and CD8+ T cells and B cells were comparable between WT and ORAI1 CKO mice, indicating normal lymphocyte development (Fig. 4B). When activated with αCD3/αCD28 WT and ORAI1 CKO CD4+ T cells demonstrated comparable proliferation, CD25 expression and IL-2 or IFN-γ cytokine production (Fig. 4C-E). We next analyzed whether ORAI1 CKO T cells were still susceptible to inhibition by ORAIci. Using FLIPR assays, we found that ORAIci could still block rapid calcium influx in PHA-stimulated ORAI1 CKO CD4+ T cells (Fig. 4F). Correspondingly, ORAI1 CKO T cells were susceptible to growth inhibition by both ORAIci and CsA (Fig. 4G). Finally, similar to WT mice, CD25 expression as well as IL-2 and IFN–γ cytokine production were inhibited by ORAIci in ORAI1 CKO T cells (Fig. 4H and I, respectively).

When we analyzed the effects of ORAIci on freshly purified mouse WT nTregs, only CsA, but not ORAIci, could partially inhibit FoxP3 expression, similar to human Tregs (Fig. 5A). The Jak inhibitor also inhibited FoxP3 expression in mouse Tregs (data not shown). When the percentage of CD4+CD25+FoxP3+ Tregs was measured, no apparent defect in nTreg development was observed in ORAI1 CKO spleens (Fig. 5C). Similar to T cells, both WT and ORAI1 CKO nTregs also grow comparably in response to αCD3 and IL-2 (Fig. 5D). A recent study demonstrated that nTregs mediate T-cell suppression via inhibiting release of intracellular calcium stores, and subsequently blocking calcium entry through ORAI channels (38). However, whether ORAI channels on nTregs play a role in Treg suppressor function was not discussed. To address this question, we performed a suppressor assay where WT and ORAI1 CKO nTregs were incubated with labeled target T cells, and proliferation was analyzed after 5 days. Both WT and ORAI1 CKO nTregs were
nTregs are resistant to ORAI channel inhibition

capable of comparably inhibiting WT and ORAI1 CKO CD4+ T-cell proliferation (Fig. 5E and F).

Finally, we generated iTregs from WT and ORAI1 CKO CD4+ T cells and analyzed the effects of CsA and ORAIci on their development. After 72 h, both ORAIci and CsA comparably inhibited iTreg expansion (Fig. 5G). In addition, FoxP3 and CD25 expression levels were inhibited in WT and ORAI1 CKO iTregs by CsA and ORAIci (Fig. 5H and I), consistent with the human data. Thus, the remaining ORAI2 and 3 channel subunits appear functional in vitro and may compensate for the loss of ORAI1 in mice. Future studies should address whether iTreg conversion or maintenance is affected by ORAIci in vivo.

Discussion

The main conduit for calcium entry into T cells is via the ORAI channels (14). However, little is known about the role of ORAIci in Tregs. In this study, we investigated the role of calcium entry via ORAI channels in both human and mouse nTregs and iTregs in vitro. We found that, in the presence of ORAIci, both human and mouse nTregs maintain FoxP3 expression, despite blockade of calcium influx through ORAI channels. This is in direct contrast to CD4+ T cells, where inhibition of ORAI channels leads to impaired T-cell activation, cytokine production and clonal expansion (29, 35). Because the CNI, CsA, inhibits FoxP3+ Tregs in vitro and in vivo, it is surprising that ORAIci, which act upstream of calcineurin, do not inhibit nTregs. Interestingly, ORAIci block FoxP3 expression in iTregs, which are converted from naive CD4+ T cells. It appears from our data that unlike nTregs, iTregs are unable to compensate for ORAIci. Therefore, only nTregs that develop in the thymus appear resistant to ORAIci. In both mice and humans, ORAIci appear to selectively inhibit iTreg development and full T-cell activation, while sparing nTregs (summarized in Fig. 6).
Fig. 5. ORAI1 CKO nTregs have comparable suppressive activity against WT and ORAI1 CKO T cells, whereas ORAI1 CKO iTregs are still susceptible to ORAIci. (A) Freshly purified mouse WT FoxP3<sup>+</sup> Tregs were treated with increasing concentrations of CsA or ORAIci and FoxP3 was measured after 96 h. (B) RNA was harvested from WT and ORAI1 CKO mouse nTregs and ORAI expression was analyzed by qPCR. (C) WT or ORAI1 CKO spleen cells were gated on CD4<sup>+</sup> T cells and then analyzed for the percentages of CD25<sup>+</sup> FoxP3<sup>+</sup> Tregs. (D) WT and ORAI1 CKO Tregs were purified and treated with IL-2 alone or with αCD3-αCD28 beads plus IL-2, and proliferation was measured as in Fig. 1. WT (E) or ORAI1 CKO (F) CD4<sup>+</sup> T cells were labeled with CFSE and cultured with αCD3-αCD28 beads and IL-2, plus increasing concentrations of WT or ORAI1 CKO Tregs for 96 h. Proliferation was measured by flow cytometry. (G–I) WT and ORAI1 CKO iTregs were generated in the presence of DMSO, or 1 µM CsA or ORAIci for 72 h. Cell proliferation (G), percent FoxP3<sup>+</sup> cells (H) or CD25<sup>+</sup> cells (I) were measured by flow cytometry.

Fig. 6. Mechanism of CsA and ORAI channel inhibition in CD4<sup>+</sup> T cells and iTregs compared with nTregs. CsA potently inhibits IL-2 production and T-cell proliferation and activation while also inhibiting FoxP3 expression in nTregs and iTregs. In contrast, ORAIci blocks IL-2 production and T-cell proliferation, but only inhibits FoxP3 expression in iTregs but not in nTregs. Our data suggest that an independent calcium-permissive channel exists in nTregs that compensates for the loss or inhibition of ORAI channels, to maintain FoxP3 expression and suppressive function.
Although ORAI channels are upstream of calcineurin and CsA blocks FoxP3 expression, nTregs remain resistant to ORAI channel loss or inhibition. Our data suggest that there may be a compensatory calcium-permissive channel in nTregs that acts as fail-safe mechanism to maintain FoxP3 expression, in the face of ORAI channel inhibition, to preserve immune tolerance. Several pieces of data support this conclusion. (i) If calcium is removed from the surrounding media, FoxP3 expression in human nTregs is dramatically reduced, with no corresponding loss in viability. (ii) ORAIci completely block rapid calcium influx through ORAI channels, but nTregs still maintain FoxP3 expression. (iii) CsA, which abrogates calcium-dependent calcineurin activity downstream of the ORAI channels, inhibits FoxP3 expression in nTregs and FoxP3 target genes. (iv) Both calcium-dependent calcineurin activity and NFAT activity are maintained in nTregs treated with ORAIci, whereas their activity is inhibited in T cells. Taken together, our data suggest that nTREG channels are not the only mode of calcium entry in nTregs, thereby sustaining FoxP3 expression in the presence of ORAIci.

Using FLIPR assays, we were only able to measure calcium influx through the ORAI channels within 10–20 min following T-cell stimulation, thereby making it difficult to observe later responses, when we presume the back-up calcium channel opens. In an attempt to identify the elusive calcium channel, commercially available broad spectrum calcium inhibitors and STIM inhibitors were tested in both T-cell and Treg assays, with no effect on FoxP3 expression (data not shown). In addition, qPCR comparison of an array of known calcium channels did not identify a calcium channel expressed exclusively in nTregs, thereby sustaining FoxP3 expression in the presence of ORAIci.

Because ORAI channels not only regulates FoxP3 expression but also affects Treg expansion. In response to TCR stimulation plus IL-2, both CsA and ORAIci were able to inhibit proliferation in T cells, but not in nTregs. In addition, neither CD4+ T cells nor Treg expansion could be rescued with IL-2 because CD25 induction was inhibited by ORAIci and CsA. However, CsA and ORAIci were unable to inhibit IL-2-induced T-cell proliferation, in part because levels of CD25 were already stable (Supplementary Figure 2C, available at International Immunology Online). Moreover, the IL-2 pathway is calcium independent and would be resistant to the effects of ORAIci and CsA.

In transplant patients, there is keen interest in immunosuppressants that inhibit T cell-mediated graft destruction while maintaining Treg-induced tolerance. In contrast to CsA, another immunosuppressive drug, rapamycin, which inhibits the serine-threonine kinase, mTOR, has been shown to preserve Treg function and even induce Tregs in vitro (23, 39). However, even though they have different mechanisms of action, both rapamycin and CsA have tissue toxicity issues associated with long-term use. Thus, new therapies are still warranted that bring added safety as well as protection of Treg suppressive function (40).

Most clinical and mouse in vivo data have focused on FoxP3+ Tregs but have not differentiated the effects of immunosuppressant therapies on nTregs versus iTregs (41). Our data indicate that nTregs and in vitro-generated iTregs respond similarly to CsA, whereas nTregs remain selectively resistant to ORAIci. However, it is likely that both nTregs and iTregs influence the development and progression of autoimmunity and transplant rejection, with CNIs likely inhibiting both Tregs in vivo. Because there is some controversy about how comparable TGF-β-generated iTregs are to disease-generated iTregs in vivo, futures studies should address the effects of CsA versus ORAIci on iTreg development and stability compared with nTreg expansion in vivo (11, 12). Using markers such as FoxP3 and Helios to discriminate between nTregs and iTregs, the respective contributions of each population could be monitored, leading to a better understanding of the role of each Treg in disease progression.

Despite loss of ORAI1 in mouse T lymphocytes, there was no defect in lymphocyte development or function and no corresponding autoimmune disease. It is likely that ORAI2 and/or ORAI3 is compensating for the reduced levels of ORAI1, thereby maintaining ORAI channel function in both T cells and Tregs. ORAI1 KO mice resemble the global ORAI1 KO mice generated by Vig et al. (24), which also have a rather modest T-cell phenotype; those authors also support a compensatory role for the remaining ORAI subunits. Along these lines, it is possible that the remaining channel subunits can compensate for the loss of ORAI1 in T cells because (i) ORAI2 and ORAI3 levels are higher than ORAI1 in activated CD4+ T cells and (ii) ORAIci can still block T-cell expansion and cytokine production in ORAI1 KO T cells, suggesting that the other channel subunits can maintain channel function. Because ORAIci can comparably block all three individual ORAI subunits (data not shown), it is unclear whether ORAI2 or ORAI3 or both can compensate for the loss of ORAI1. Future studies should address this issue using either more selective ORAIci or ORAI2/ORAI3 knockout mice.

In summary, we have shown that inhibition of ORAI channels in human and mouse nTregs does not diminish FoxP3 expression, due to sustained calcineurin and NFAT activity. Our studies suggest that there is a novel calcium-permissive channel that allows nTregs to preserve FoxP3 expression, as a fail-safe mechanism to maintain systemic tolerance in the presence of ORAIci or loss. Identification of this nTreg-specific channel could potentially provide the means to selectively target the nTreg population, while sparing the effector T-cell population, in cancer and other immunosuppressive diseases.

Supplementary data

Supplementary data are available at International Immunology Online.

Funding

Immunity Department at Hoffmann-La Roche.

Acknowledgements

We thank Dr Nagendra Singh of the Medical College of Georgia for generating human Tregs for our studies, the Gene Targeting and Transgenic Facility at the University of Connecticut Health Center for generation of the ORAI1 CKO mice and Charles River Laboratories for housing and breeding the ORAI1 CKO mice.

nTregs are resistant to ORAI channel inhibition 505
nTregs are resistant to ORAI channel inhibition

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