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Connexin 43 Signaling Enhances the Generation of Foxp3+ Regulatory T Cells

Michal Kuczma,* Jeffrey R. Lee,†‡§¶ and Piotr Kraj*

Despite their importance for the functioning of the immune system, thymic development and peripheral maintenance of Foxp3+ regulatory T (T<sub>R</sub>) cells are poorly understood. We have found that connexin 43 (Cx43), expressed by thymic T<sub>R</sub> cells progenitors, supports T<sub>R</sub> development. Mice with deletion of the Cx43 gene induced in T cells produce only few T<sub>R</sub> cells and had increased proportion of activated T cells in the lymph nodes, suggesting impaired peripheral tolerance. Reduction of the T<sub>R</sub> cell numbers was accompanied by increased presence of CD4<sup+</sup>CD25<sup+</sup>GITR<sup+</sup>Foxp3<sup+</sup> T cells, which did not produce inflammatory cytokines and lost suppressor function. These results strongly argue that we have discovered a novel signaling pathway, controlled by Cx43, that enhances the generation of T<sub>R</sub> cells. We propose that a possible mechanism of Cx43 activity is by regulating Foxp3 expression in T<sub>R</sub> lineage cells. The Journal of Immunology, 2011, 187: 000–000.

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Abbreviations used in this article: Cx43, connexin 43; Cx43NT, Cx43 consisting of amino acids 1–228 (N-terminal portion); SEB, staphylococcal enterotoxin B; T<sub>R</sub> cell, regulatory T cell.

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peripheral T cells (35). In Treg cells, Cx43 contributes to the formation of gap junctions with target cells, which is necessary for Treg-mediated suppressor function (36).

In this article, we report that Cx43 supports lineage commitment of double- and single-positive Treg precursors. We found that Cx43 is expressed in double- and single-positive thymocytes at the developmental stages preceding Foxp3 expression and T cell-specific deletion of Cx43 gene resulted in a profound deficiency of Treg cells in the thymus and in the periphery. Aging Cx43 mutant mice had progressively increasing numbers of activated CD4+ T cells in the lymph nodes and spleen. Surprisingly, mice deficient in Cx43 in T cells had an expanded population of nonsuppressive CD4+CD25+GITR+Foxp3GFP- cells, whose phenotype suggests that they are related to Treg cells deficient in Foxp3 expression. Cx43-deficient thymocytes were less sensitive to signaling by IL-2, which likely contributed to impaired generation of Treg cells in the thymus. In conclusion, our data show that Cx43 supports Treg lineage development, and we postulate that it occurs by a mechanism involving regulation of Foxp3 expression.

Materials and Methods

Mice

Mice with conditional knockout of the Cx43 gene (Cx43loxP mouse; Jackson Labs) were crossed to Cdc4-cre (Taconic) and Foxp3GFP reporter mice (23, 29, 37, 38). All mouse strains used for crossing were on the C57BL6 background. Scurfy, TCRα37, 38) were purchased from Jackson Labs. Mice were housed under specific pathogen-free conditions and used according to the guidelines of the Institutional Animal Care and Use Committee of the Medical College of Georgia.

T cell activation, proliferation, and Treg cell suppression assay

T cells were activated in vivo by injecting Foxp3GFP mice with 50 μg staphylococcal enterotoxin B (SEB) in a footpad, and T cells were isolated after 4 d from popliteal lymph nodes. Naïve CD4+ T cells were activated with plate-bound anti-CD3e (10 μg/ml) and anti-CD28 (1 μg/ml) Abs. After culturing cells for 3 d, proliferation was measured by adding 1 μCi/well 3H-thymidine. IL-2 (50 U/ml) was added to indicated wells in some experiments. Activated Treg cells were produced by activating naive CD4+ T cells in the presence of TGF-β (3 ng/ml) for 4 d and by sorting Foxp3GFP+ cells. Activated Treg cells were produced by incubating Foxp3GFP+ cells with plate-bound anti-CD3e and anti-CD28 Abs in the presence of 100 U/ml IL-2. For Th1 differentiation, cells were stimulated in the presence of anti-IL-4 Ab (10 μg/ml) and IL-12 (10 ng/ml). For Th2 differentiation, cells were stimulated in the presence of IL-4 (1000 U/ml), anti-IFN-γ (10 μg/ml), and anti-IL-12 (10 μg/ml). Abs. Finally, for Th17 priming, cells were stimulated in the presence of TGF-β (3 ng/ml) and IL-6 (20 ng/ml). Cells were cultured for 4 d. Lymph node proliferation and Treg cell suppression assays were performed as previously described (39).

Cell purification, flow cytometry, and cell sorting

Single-cell suspensions were stained with Abs available commercially (BD Biosciences, eBioscience, and BioLegend). Cells were analyzed using FACSComp flow cytometer (Becton Dickinson) and sorted on a MoFlo cell sorter (Cytomation). Purity of sorted populations exceeded 98.5%.

Production of mixed bone marrow chimeras

Bone marrow from Cx43Tw1 and Cx43T-/- mice (expressing congenic markers Ly5.1- and Ly5.2+) was mixed 1:1, and a total of 6 × 105 cells were injected i.v. into sublethally irradiated (600 rad) RAG-/- mice. Mice were analyzed 8 wk after transfer.

In vitro cultures of T cells and cytokine detection

Cytokine production was measured in supernatants of sorted T cell subsets activated and cultured in vitro. CD4+CD25+ GFP-, CD4+CD25+GFP+ , and CD4+CD25-GFP cells (2 × 105 cells/well of 24-well plate) from Cx43Tw1 and Cx43T-/- mice were sorted and stimulated with plate-bound anti-CD3 (10 μg/ml) and anti-CD28 (1 μg/ml) Abs for 40 h. Supernatants were then collected and used for cytokine detection using commercially available kits (eBioscience). All samples were done in triplicate using pooled cells from two mice. Experiments were repeated three times.

Adaptive transfer

Total CD4+ cells from Cx43Tw1, Cx43T-/-, and 3-wk-old scurfy male mice were sorted on AutoMACS (Miltenyi). Purify of sorted cells exceeded 97%. A total of 5 × 105 cells from Cx43Tw1 or Cx43T-/- were mixed with 5 × 105 scurfy cells and transferred (i.v.) into TCRα-/- recipient mice. Recipient mice were weighed every day with weight at the day of transfer marked as 100%. Five mice of each kind were analyzed.

CD4+Foxp3GFP- effectors from Cx43Tw1 and Cx43T-/- mice, CD4+CD25+Foxp3GFP- T cells from Cx43Tw1 or Cx43T-/- mice, and CD4+Foxp3GFP- Treg cells from Cx43Tw1 mice were isolated by flow cytometry. A total of 5 × 105 effector cells were transferred (i.v.) into RAG knockout mice alone or mixed with 1.5 × 106 CD4+CD25+Foxp3GFP- T cells from Cx43T-/- mice or CD4+Foxp3GFP+ cells from Cx43Tw1 mice. Recipient mice were sacrificed after 10 wk, and colon sections were examined for inflammatory bowel disease as described previously (40).

RT-PCR

RNA was isolated from sorted cells with RNeasy Mini Kit (Qiagen) and reverse transcribed using SuperScript kit (Invitrogen). β-actin was used to normalize cDNA quantities. PCR products were resolved on agarose gels, with the gel being scanned. PCR primers used to detect Cx43, Foxp3, and β-actin transcripts were as follows: Cx43 sense: 5'-CAGACAGTGTGAGAGGGCGGA-3', antisense: 5'-AAGGAGCCAGAGAGCACGTGAGAG-3'; Foxp3 sense: 5'-ATCCAGCCTGGCTTCAGAAGAACCC-3', antisense: 5'-GGGTGTGTCGACGTGACACTTGAGG-3'; β-actin sense: 5'-CCCTCTCAATGAGCCTGCTGTGGC-3', antisense: 5'-CATGAGGTAGTCTGCTAGOTCC-3'. Foxp3-specific primers distinguish between amplification product of the endogenous Foxp3 gene (401 bp) and the transgenic transcript (1357 bp). PCR conditions were 94°C for 2 min, 30 cycles of 94°C for 30 s, 56°C for 20 s, and 72°C for 50 s; and 72°C for 2 min.

Western blotting

Foxp3 protein was detected in sorted (105 cells/sample) CD4+CD25+Foxp3GFP-, CD4+CD25+Foxp3GFP-, and CD4+CD25+Foxp3GFP+ cells. Cells were lysed in the gel-loading buffer and resolved on 10% polyacrylamide gel. Proteins were transferred onto a polyvinylidenefluoride membrane (Millipore). Membranes were probed with anti-Foxp3 Ab (eBio7979; 1:500, eBioscience) followed by goat anti-mouse polyclonal Ab coupled with HRP (1:5000; Bio-Rad). Membranes were developed with ECL chemiluminescence kit (Amersham) according to the manufacturer’s instructions. Detection of β-actin was performed to ensure equal loading. Primary Ab (1:8000) was from Sigma (clone AC-74). For detection of Foxp3 in EL-4 cells, lysates from 5 × 105 cells were run per lane.

Production of Cx43 expression constructs in retroviral vectors and analysis of EL-4 cells

Cx43 full-length cDNA was purchased from Open Biosystems (Huntsville, AL). cDNA lacking the stop codon was amplified by PCR and cloned into pEF1-FN1 vector (Clontech) between EcoRI and BamHI in frame with the polyhistidine tag. Recombinant baculoviruses were produced using Autographa californica nuclear polyhedrosis virus (ACNPV) in Sf9 insect cells. Cx43 expression vectors were cotransfected into Phoenix-Eco packaging cell line as described previously (41). EL-4 cell line obtained from American Type Culture Collection was then transduced, and infected cells were sorted on magnetic sorter (Miltenyi) using biotinylated Abs against rat CD2 (Cedarlane, Burlington, NC) and streptavidin beads (Miltenyi). EL-4 cells expressing Cx43 that were used for all experiments were >98% CD2+ YFP+ as assessed by flow cytometry.

Statistical analysis

Differences between cell populations or cytokine concentrations were analyzed by two-sample t test. The p values <0.05 were considered statistically significant. Colitis scores were compared using a two-sample permutation test, which is a nonparametric analog of the two-sample t test (42).

Results

Expression pattern of Cx43 in peripheral CD4+ T cells

To gain insight into the molecular mechanisms of Treg cell function, we compared global gene expression profiles of resting and
activated conventional CD4+ T cells, adaptive T<sub>R</sub> cells, and resting and activated natural T<sub>R</sub> cells using GeneChip arrays. We have identified Cx43 as a gene differentially expressed in resting and activated effector CD4<sup>+</sup> and T<sub>R</sub> cells. To corroborate microarray data, we investigated Cx43 expression in resting CD4<sup>+</sup>CD62L<sup>+</sup>Foxp3<sup>−/−</sup> and activated CD4<sup>+</sup>CD62L<sup>−</sup>Foxp3<sup>−/−</sup> conventional CD4<sup>+</sup> T cells and CD4<sup>+</sup>Foxp3<sup>−/−</sup> T<sub>R</sub> cells activated in vitro without and in the presence of TGF-β (Fig. 1A). Only CD4<sup>+</sup> T cells activated in conditions promoting generation of Th1 cells, but not Th2 and Th17 cells, express Cx43 (Fig. 1B). To corroborate in vitro data, we investigated Cx43 expression in naive, activated, and T<sub>R</sub> cells sorted from Foxp3<sup>−/−</sup> reporter mice immunized with SEB (Fig. 1C). In summary, Cx43 was expressed in conventional CD4<sup>+</sup> T and T<sub>R</sub> cells only after activation, and its expression is significantly augmented by TGF-β. Although Cx43 is greatly upregulated in activated T<sub>R</sub> cells, its expression was independent of Foxp3 because it was found in Foxp3-deficient T cells from scurfy mice (data not shown). This expression pattern suggested that Cx43 is dispensable in naive conventional and T<sub>R</sub> cells but could be important in T cell developmental processes requiring TCR stimulation and T cell activation.

**Cx43 is necessary for thymic generation of T<sub>R</sub> cells but not conventional T cells**

To further evaluate the possible role of Cx43 in T cells, we generated mice with T cell-specific deletion of Cx43. Cx43 conditional knockout mice, prepared by flanking the only protein coding exon of the Cx43 gene with loxP sites (Cx43<sup>loxP/loxP</sup> mouse), were crossed to CD4<sup>+</sup>-cre mice expressing cre recombinase in T cells starting at the double-positive thymocyte stage (37, 38). In addition, we introduced Foxp3<sup>−/−</sup> reporter transgene into CD4<sup>+</sup>-cre/Cx43<sup>loxP/loxP</sup> mice to tag T<sub>R</sub> cells with the GFP (23). Mice transgenic for CD4<sup>+</sup> and Foxp3<sup>−/−</sup> and homozygous for the Cx43<sup>loxP/loxP</sup> allele (Cx43<sup>−/−</sup> mice) had normal numbers and proportions of double- and single-positive thymocytes and peripheral CD4<sup>+</sup> and CD8<sup>+</sup> T cells, but a greatly decreased population of T<sub>R</sub> cells (Fig. 2A–F).

**FIGURE 1.** Cx43 expression in various T cell subsets assessed by RT-PCR. A, Cx43 expression in naive conventional CD4<sup>+</sup> T cells (T<sub>c</sub>), CD4<sup>+</sup> T cells activated in vitro, adaptive T<sub>R</sub> cells (aT<sub>R</sub>), T<sub>R</sub> cells activated in the presence of TGF-β (T<sub>R</sub>), resting natural T<sub>R</sub> cells (sorted Foxp3<sup>−/−</sup> cells), and activated natural T<sub>R</sub> cells (sorted Foxp3<sup>−/−</sup> cells activated in vitro). Where indicated, populations of T<sub>c</sub> cells were activated in vitro with plate-bound anti-CD3/anti-CD28 Abs and TGF-β. Experiment was repeated on cell populations sorted from three independent mice. B, Cx43 expression in CD4<sup>+</sup> T cells stimulated in conditions promoting generation of Th1, Th2, Th17, aT<sub>R</sub>, and activated T<sub>R</sub> cells. C, Cx43 expression in populations of activated CD4<sup>+</sup> T (act. T<sub>c</sub>), naive (T<sub>c</sub>), and T<sub>R</sub> cells (Foxp3<sup>−/−</sup>) sorted from mice immunized with SEB. Upper panels show Cx43 expression; lower panels show β-actin expression.

Younger mice had more pronounced deficiency of T<sub>R</sub> cells than older mice, increasing a possibility that T<sub>R</sub> cells are differentially sensitive to Cx43 deficiency (Fig. 2E, 2F). Control mice (Cx43<sup>+/+</sup>) used for the experiments expressed Foxp3<sup>GFP</sup> reporter transgene and both alleles of Cx43 flanked by loxP sites, but these mice lacked CD4<sup>+</sup>-cre transgene, allowing for normal expression of Cx43. Young (3 wk) Cx43<sup>1−/−</sup> mice had the same proportion of activated/memory cells in the lymph nodes as control mice, whereas older mice (8 wk) had an increased proportion of activated T<sub>c</sub> cells (Fig. 2G, 2H). CD4<sup>+</sup> T<sub>c</sub> cells from Cx43<sup>1−/−</sup> mice had higher expression of activation markers CTLA-4, GITR, and ICOS, and a higher proportion of proliferating peripheral CD4<sup>+</sup> T<sub>c</sub> cells (Fig. 2J). In contrast, Cx43<sup>1−/−</sup> mice had much lower proportion of Foxp3<sup>−/−</sup> T<sub>R</sub> cells both in the thymus and in the peripheral lymph nodes (Fig. 2B, 2D–F). The remaining Foxp3<sup>−/−</sup> cells in Cx43<sup>1−/−</sup> mice had lower expression of Foxp3 and decreased suppressor function compared with T<sub>R</sub> cells isolated from normal mice (see later). Altogether, phenotypic analysis of T cell populations in the thymus and peripheral lymph nodes showed that Cx43 is required for proper differentiation of T<sub>R</sub> cells. Decreased numbers of T<sub>R</sub> cells in Cx43<sup>1−/−</sup> mice corresponded with a higher proportion of cycling conventional CD4<sup>+</sup> T cells with activated phenotype and suggested impaired peripheral tolerance (Fig. 2G–I; see data later in article).

Remarkably, Cx43<sup>1−/−</sup> mice had an expanded population of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>−/−</sup> cells in the thymus and lymph nodes (Fig. 2B, 2D–F). These cells expressed GITR, CTLA-4, and Helios, markers characteristic of T<sub>R</sub> cells, but did not express Foxp3 (Fig. 2J, 2K, and data not shown) (43–45). The population of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>−/−</sup> cells resembled T<sub>R</sub> cells that develop in the absence of Foxp3 expression, in scurfy or Foxp3 knockout mice. The presence of such cells argues that Cx43 is involved in regulating Foxp3-dependent suppressor functions that could be separated from the rest of the T<sub>R</sub> lineage developmental program. Consistent with the lack of Foxp3 and Cx43, CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>−/−</sup> cells did not inhibit proliferation of conventional CD4<sup>+</sup> T cells in vitro (Fig. 3A). In vivo, total CD4<sup>+</sup> T cells from Cx43<sup>1−/−</sup> mice were less efficient in controlling effector CD4<sup>+</sup> T cells from scurfy mice when cotransferred into recipient lymphopenic mice (Fig. 3B). The CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>−/−</sup> cells proliferated much less than conventional CD4<sup>+</sup> T cells when stimulated in vitro, did not produce inflammatory cytokines, and produced only small amounts of IL-2 (Fig. 3C, 3D). The lack of suppressor function of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>−/−</sup> T<sub>R</sub> cells in Cx43<sup>1−/−</sup> mice was most likely due to the deficiency of Foxp3, but also could be because of the lack of Cx43, which has been shown to contribute directly to the suppressor function of T<sub>R</sub> cells (36). In an adaptive transfer experiment, CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>−/−</sup> cells from Cx43<sup>1−/−</sup> mice were not able to protect mice from inflammatory bowel disease (Fig. 4A–F). In summary, the phenotype of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>−/−</sup> cells strongly argues that they represent cells committed to T<sub>R</sub> lineage that did not complete whole differentiation process.

Although the development and suppressor function of T<sub>R</sub> cells are severely compromised in Cx43<sup>1−/−</sup> mice, proliferation of conventional CD4<sup>+</sup> T cells, stimulated in vitro, was not affected (Fig. 3C). These cells were able to express Th subset-specific transcription factors T-bet, GATA3, and RORγt, and consistently produced similar quantity of IL-2, lower quantities of IFN-γ, and increased quantity of IL-4 (Fig. 3D and data not shown). Cx43 is expressed only in cells stimulated to become Th1, which suggests that Cx43 modulates the outcome of activation of only some of the conventional CD4<sup>+</sup> T cell subsets. It is likely that the magnitude of this modulation depends on the level of Cx43 expression that is...
FIGURE 2. Flow cytometry and Foxp3 gene expression analysis of T cell subsets in the thymus and lymph nodes of 8-wk-old Cx43\textsuperscript{wt} and Cx43\textsuperscript{−/−} mice. 

A–D, Representative flow cytometry analysis of thymocytes (A, B) and lymph node cells (C, D) of Cx43\textsuperscript{wt} and Cx43\textsuperscript{−/−} mice. (A, C) Total thymocyte and lymph node populations were stained with Abs specific for CD4 and CD8. B and D, Flow cytometry analysis of CD25 and Foxp3 GFP expression on gated single-positive CD4\textsuperscript{+} thymocytes (B) or lymph node cells (D). E and F, Total number of thymocytes and lymph node cells (left panels), and percentage of Foxp3 spermatogenesis cells in the populations of CD4\textsuperscript{+} cells in the thymus and lymph node cells (right panels) in 1-, 3-, and 8-wk-old mice. Values for individual Cx43\textsuperscript{wt} (wt) mice (○, ●) and for Cx43\textsuperscript{−/−} (ko) mice (×) are shown. Average values and standard deviations are shown to the right of individual mice data. Statistical differences between data are indicated by brackets, and \( p \) values are shown above brackets. Seven mice of each type were analyzed for cell number comparison and four mice for each time point to compare percentages of Foxp3\textsuperscript{GFP+} cells. G and H, Representative flow cytometry analysis of CD44 and CD62L expression on gated CD4\textsuperscript{+}Foxp3\textsuperscript{GFP−} lymph node cells (G), and the corresponding statistical analysis showing percentage of activated cells in the population of gated CD4\textsuperscript{+} T cells (H). Four mice of each type were analyzed. Analysis description and symbols used are as in E and F. I, Expression of CTLA4, GITR, ICOS, and Ki-67 by peripheral CD4\textsuperscript{+} T cells in Cx43\textsuperscript{wt} (wt) and Cx43\textsuperscript{−/−} (ko) mice. Analysis description and symbols used are as above. Data from at least three mice are shown. J, RT-PCR (left panel) and Western blot analysis (right panel) of Foxp3 expression. For RT-PCR, CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{GFP+} and CD4\textsuperscript{+}CD25\textsuperscript{−} Foxp3\textsuperscript{GFP+} cells were sorted from Cx43\textsuperscript{wt} and Cx43\textsuperscript{−/−} mice. For Western blot populations of naive CD4\textsuperscript{+}CD25\textsuperscript{−}Foxp3\textsuperscript{GFP−} and CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{GFP+} and CD4\textsuperscript{+}CD25\textsuperscript{−}Foxp3\textsuperscript{GFP−} mice, cells were sorted from Cx43\textsuperscript{−/−} mice and used for analysis. Experiment was repeated three times. K, Flow cytometry analysis of Foxp3 (left panels), GITR (middle panels), and Helios (right panels) expression in populations of CD4\textsuperscript{+}CD25\textsuperscript{+}Foxp3\textsuperscript{GFP−}, CD4\textsuperscript{+}CD25\textsuperscript{−}Foxp3\textsuperscript{GFP+}, and CD4\textsuperscript{+}CD25\textsuperscript{−}Foxp3\textsuperscript{GFP+} cells sorted from Cx43\textsuperscript{wt} and Cx43\textsuperscript{−/−} mice. One of two independent experiments is shown.
A representative experiment of three.

Cx43T

transfer.

the original weight of individual mice at various time intervals after cell

mixed 1:1 with total CD4+ T cells from Cx43Twt (wt) or CD4+CD25+Foxp3GFP+ (TR) cells were sorted from Cx43 Twt mice, and CD4+CD25+Foxp3GFP+ cells (×, dotted line) sorted from Cx43T−/− mice. The percentage stimulation was calculated by dividing the proliferation reading from a particular well by the reading from a well with only Foxp3GFP+ responder cells. The plot shows a representative experiment of three. B, TR cells from Cx43T−/− mice are less efficient in controlling CD4+ T cells isolated from scurfy mice. CD4+ T cells from scurfy mice were mixed 1:1 with total CD4+ T cells from Cx43Twt (Δ, continuous line) and Cx43T−/− (×) mice and transferred into TcRa− recipient mice. The plot shows percentage of the original weight of individual mice at various time intervals after cell transfer. C, Proliferation assay of T cell subsets isolated from Cx43Twt and Cx43T−/− mice and stimulated with plate-bound anti-CD3/anti-CD28 Abs. CD4+CD25−Foxp3GFP− (conventional T cells) and CD4+CD25+Foxp3GFP+ (Treg-like) cells were sorted from Cx43Twt mice, and CD4+CD25−Foxp3GFP− (Δ) or CD4+CD25+Foxp3GFP+ (×) cells were sorted from Cx43T−/− mice. IL-2 was added to indicated wells. The plot shows a representative experiment of three. D, Cytokine production.

FIGURE 3. Suppressor function, proliferation, and cytokine production of CD4+ T cell subsets from Cx43Twt and Cx43T−/− mice. A, Proliferation inhibition assay. Proliferation of CD4+Foxp3GFP− responder cells (expressing wild type Cx43 alleles) and stimulation with soluble anti-CD3 Ab was inhibited by adding increasing numbers of CD4+CD25+ TR cells sorted from Cx43Twt (Δ, continuous line) and Cx43T−/− (×, dashed line) or CD4+CD25−Foxp3GFP− cells (×, dotted line) sorted from Cx43T−/− mice. The percentage stimulation was calculated by dividing the proliferation reading from a particular well by the reading from a well with only Foxp3GFP− responder cells. The plot shows a representative experiment of three. B, TR cells from Cx43T−/− mice are less efficient in controlling CD4+ T cells isolated from scurfy mice. CD4+ T cells from scurfy mice were mixed 1:1 with total CD4+ T cells from Cx43Twt (Δ, continuous line) and Cx43T−/− (×) mice and transferred into TcRa− recipient mice. The plot shows percentage of the original weight of individual mice at various time intervals after cell transfer. C, Proliferation assay of T cell subsets isolated from Cx43Twt and Cx43T−/− mice and stimulated with plate-bound anti-CD3/anti-CD28 Abs. CD4+CD25−Foxp3GFP− (conventional T cells) and CD4+CD25+Foxp3GFP+ (Treg-like) cells were sorted from Cx43Twt mice, and CD4+CD25−Foxp3GFP− (Δ) or CD4+CD25+Foxp3GFP+ (×) cells were sorted from Cx43T−/− mice. IL-2 was added to indicated wells. The plot shows a representative experiment of three. D, Cytokine production.

Lack of TR development in Cx43T−/− mice is TR intrinsic

The transgenic expression of cre recombinase may affect Cx43 expression in other cell types besides thymocytes. Alternatively, thymocytes expressing Cx43 could induce Cx43 expression on stromal epithelial cells or other thymocytes. To conclusively establish that a defect of TR cell lineage development in Cx43T−/− mice is T cell intrinsic, we have prepared mixed bone marrow chimeras.Recipient mice were irradiated and reconstituted with wild type bone marrow from Ly5.1−/−Cx43Twt and mutant bone marrow from Ly5.1−/−Cx43T−/− mice. The TR cells developed efficiently only from wild type bone marrow (Fig. 5). A much greater proportion of wild type TR cells (greater than in normal mice) was observed in the single-positive thymocytes, suggesting that wild type bone marrow compensates for the lack of TR cells originating from the Cx43-deficient cells, whereas TR development from mutant thymocytes remained impaired. This result argues that soluble factors or molecules produced by stromal cells and/or other thymocytes are not able to restore TR cell numbers, which argues for the intrinsic developmental defect of Cx43T−/− thymocytes.

Cx43 is expressed in TR precursors at the double- and single-positive stage

To further determine the stage of thymocytes when Cx43 is first expressed, we have sorted double-negative, double-positive, and single-positive thymocytes and investigated the presence of Cx43 transcripts by RT-PCR. Flow cytometry analysis of double-positive thymocytes shows that Cx43T−/− mice have much a lower proportion of Foxp3GFP+ T cells but a similar proportion of CD4+CD8+CD25+ cells as the wild type mice (Fig. 6A). RT-PCR analysis detected a low level of Cx43 expression in CD4+CD8+ CD25+ cells. Much greater levels of Cx43 were detected in single-positive CD4+CD25+Foxp3GFP− thymocytes, but not in CD4+CD25−Foxp3GFP+ conventional thymocytes or CD25+Foxp3GFP+ or CD25+Foxp3GFP− TR thymocytes expressing Foxp3 (Fig. 6B). Altogether, the gene expression study showed that Cx43 is detected in double- and single-positive subsets expressing CD25 but is downregulated in thymocytes expressing Foxp3. In summary, gene expression studies place Cx43 expression at the stage of thymocyte development just before the onset of Foxp3 expression, when the critical decision of the TR lineage commitment is made. Our studies are consistent with the recent report that the single-positive thymocyte subset CD4+CD25+Foxp3GFP− represents immediate precursors of TR thymocytes (47).

When sorted, CD4+CD8+CD25+ double-positive thymocytes included rare CD25+Foxp3GFP+ cells. Because single-positive Foxp3GFP+ thymocytes do not express Cx43, we did not formally determine whether Cx43 was still downregulated in double-positive TR thymocytes, following Foxp3 expression, or later at the stage of single-positive CD4+Foxp3GFP+ thymocytes. However, regardless of the exact time of onset of Foxp3 expression, all single-positive Foxp3GFP+ thymocytes downregulate Cx43. Altogether, experimental data show that Cx43 expression is tightly controlled, and that Cx43 transcripts are detected in thymocyte subsets defined as immediate precursors of Foxp3+ TR cells.

regulated by cytokines; for example, it is greatly augmented by TGF-β. In vivo, effector CD4+Foxp3GFP− cells from Cx43T−/− mice were less effective in inducing inflammatory bowel disease than the respective population from Cx43-sufficient mice (Fig. 4G, 4H). The weaker functions of effector cells from Cx43T−/− mice could result from observed Th2 shift in the cytokine expression profile or impaired migration to the intestine. Alternatively, as reported earlier, Cx43 and gap junctions are important for the initiation of the immune response and T cell activation in vivo (46). In summary, the lack of Cx43 in CD4+ T cells selectively impaired the generation of TR cells, whereas the development of conventional CD4+ cells was not affected.

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To further examine possible outcome of Cx43 deficiency on the thymic T_R precursors, we analyzed their sensitivity to IL-2 by analyzing phosphorylation of STAT5. IL-2 signaling that depends on STAT5 phosphorylation regulates both thymic generation of T_R cells and their peripheral homeostasis and function (48). We have found that IL-2 results in suboptimal phosphorylation of STAT5 in CD4^+CD25^+Foxp3^+ thymocytes, which are immediate precursors of CD4^+CD25^+Foxp3^+ T_R thymocytes (Fig. 6C, 6D). Expression of CD25 is similar in wild type and Cx43^−/− mice, and is considered to be the result of high-affinity interaction between the MHC–peptide complexes and TCRs on T_R precursors. Our result suggests that at least one mechanism how Cx43 regulates T_R thymocyte generation is by increasing the sensitivity of T_R precursors to IL-2 signaling.

Cx43 could be directly involved in signaling complexes regulating Foxp3 expression

Signaling functions mediated by Cx43 depend on the intercellular communication through gap junctions, both on transporting molecules via hemichannels (without communicating with other cells) and on channel-independent mechanisms. We have observed differences between Cx43-sufficient and -deficient CD4^+ T cells in our in vitro tests when cellular interactions probably do not involve gap junction formation. Gap junction-independent signaling is mediated by the C-terminal cytoplasmic domain, which interacts with a number of proteins regulating cell motility, intracellular protein trafficking, and components of multiple signaling pathways (26, 49). To investigate the role of Cx43 in regulating Foxp3 expression, we took advantage of the EL-4 cell line, which was recently used as a cellular model of T_R cells to identify regulatory regions of the Foxp3 gene (16). Low level of Foxp3 is spontaneously expressed in EL-4 cells, which is greatly upregulated in cells stimulated through the TCR and further enhanced in the presence of TGF-B.

Cx43 was overexpressed in EL-4 cells (which constitutively express low level of Cx43) using retroviral vectors expressing rat CD2 reporter molecule and encoding wild type Cx43 or Cx43 mutant lacking C-terminal cytoplasmic domain both tagged with YFP (Fig. 7A). Cx43 construct without C-terminal cytoplasmic tail...
is able to form functional gap junctions but lacks adaptor sites for signaling molecules (50). EL-4 cells transduced with control vector or constructs encoding wild type or mutant Cx43 and expressing similar levels of rat CD2 were sorted, and the level of Foxp3 expression was examined by RT-PCR and Western blotting (Fig. 7B, C). EL-4 cells overexpressing wild type Cx43, but not C-terminal–deficient mutant, produced increased level of Foxp3 transcript and protein. Stimulation of EL-4 transfectants through the TCR in the presence of TGF-β further increased Foxp3 expression (Fig. 7C). This result suggests that one hypothesis of how Cx43 may augment Foxp3 expression is to contribute to the assembly of a signaling complex around its C-terminal domain. This mode of Cx43 activity is consistent with the reported participation of Cx43 in the immunological synapse where signaling molecules involved in T cell activation assemble and interact (34). We may speculate that Cx43 is a component of the synapse whose role is to increase and sustain Foxp3 expression. This function of Cx43 could be important in activated, conventional CD4+ T cells, in particular, cells exposed to TGF-β, which greatly enhances Cx43 expression. In summary, Cx43 may regulate Foxp3 expression in activated CD4+ T cells independently of its membrane channel-forming function.

Discussion

We discovered that Cx43 supports generation of T_R cells and is necessary for the suppressor function of T_R cells. Thus, Cx43 joins a growing number of molecules like CD28, cytokines, and cytokine receptors CD25 (IL-2Rα), CD122 (IL-2Rβ), and CD132 (the common γ-chain), TGF-β, CARMA-1, and c-rel, which are selectively involved in the development of T_R cells (6, 10, 12, 14, 15, 51, 52). In contrast with previously defined molecules that function downstream of the TCR complex, or accessory molecules or cytokine receptors, Cx43 may represent a new autonomous molecular pathway important for T_R lineage commitment. This pathway may depend on intercellular communication through the gap junctions, which is a characteristic function of Cx43. Thus, it is likely that Cx43 regulates unique aspects of T_R development that are independent of TCR specificity of the developing thymocyte and require
exchange of signaling molecules between cells. This possibility is supported by a report that Cx43 forms gap junctions between thymocytes and stromal epithelial cells, and by our data demonstrating that Cx43 expression is tightly controlled in thymocytes and coincides with thymocyte stages immediately preceding Foxp3 expression (30). This raises a possibility that Cx43 is a marker of early T<sub>R</sub> precursors found in populations of double- or single-positive thymocytes (47, 53). Because not all Foxp3<sup>T<sub>R</sub></sup> thymocytes express CD25 and only a fraction of CD4<sup>T</sup>CD25<sup>T</sup> thymocytes eventually becomes T<sub>R</sub> cells, it is tempting to speculate that expression of Cx43 is a more specific marker of T<sub>R</sub> lineage commitment than CD25 (47).

Signaling circuits downstream of Cx43 are currently not known, but they may involve STAT5. STAT5 phosphorylation is induced by IL-2 but also by signaling through the TCR, IL-7, and thymic stromal lymphopoietin (54, 55). In particular, signaling through IL-7 and thymic stromal lymphopoietin, which likely occur at the double-positive stage, may be important for the induction of T<sub>R</sub> precursors. We have detected decreased phosphorylation of STAT5 in Cx43-deficient thymocytes exposed to IL-2 in vitro. Considering a well-known role of IL-2 in the development of T<sub>R</sub> thymocytes, it becomes possible that Cx43 facilitates T<sub>R</sub> lineage decision by temporarily increasing the sensitivity to IL-2, and perhaps TCR, signaling in thymocytes that would become T<sub>R</sub> precursors. This function of Cx43 concludes once Foxp3 is expressed, limiting the number of developing T<sub>R</sub> cells. This scenario is consistent with observed downregulation of Cx43 expression in T<sub>R</sub> thymocytes once they initiate Foxp3 expression. Deficient IL-2 signaling could also explain lower Foxp3 expression observed in the T<sub>R</sub> cells still remaining in Cx43<sup>T<sub>R</sub></sup> mice.

Despite a greatly reduced proportion of T<sub>R</sub> cells, Cx43<sup>T<sub>R</sub></sup> mice have an expanded population of thymic and peripheral CD4<sup<T</sup>CD25<sup>T</sup>Foxp3<sup>T</sup> T cells. These cells do not inhibit in vitro proliferation of effector CD4<sup>T</sup> T cells but have some characteristics of T<sub>R</sub> phenotype. CD4<sup>T</sup>CD25<sup>T</sup>Foxp3<sup>T</sup> cells express increased levels of GITR and Helios, two molecules found on Foxp3<sup>T</sup> T<sub>R</sub> cells, do not efficiently proliferate when stimulated through the TCR in vitro, and do not produce inflammatory cytokines. Considering the phenotype and functional properties of CD4<sup>T</sup>CD25<sup>T</sup> T cells, we hypothesize that this population resembles Foxp3-deficient T<sub>R</sub>-like cells that develop in the absence of Foxp3 expression. Such cells retained most functional properties of T<sub>R</sub> cells and were earlier described in scurfy mice and in mice in which the Foxp3 gene was deleted (21–23). However, as earlier described, Foxp3-deficient T<sub>R</sub> cells transcribed Foxp3 gene, whereas CD4<sup>T</sup>CD25<sup>T</sup> thymocytes in Cx43<sup>T<sub>R</sub></sup> mice produce, at most, only very little Foxp3 transcripts. This places CD4<sup>T</sup>CD25<sup>T</sup> T cells at the stage of T<sub>R</sub> cell precursors that depend on Cx43 for Foxp3 expression. In the absence of Cx43, T<sub>R</sub> precursors develop as CD4<sup>T</sup>CD25<sup>T</sup>Foxp3<sup>T</sup> cells. This shows that not only Foxp3-deficient T<sub>R</sub> cells with transcriptionally active Foxp3 locus are able to advance to mature thymocytes and leave the thymus, but also T<sub>R</sub> precursors that do not express Foxp3 are able to progress along the T<sub>R</sub> developmental path. Thus, T<sub>R</sub> cell development consists of Foxp3-independent molecular events that produce T<sub>R</sub> lineage cells with many properties of functional T<sub>R</sub> cells, but without suppressor function, and Foxp3-dependent differentiation steps that confer and regulate T<sub>R</sub> cell suppressor function. We propose that the later steps are enhanced by Cx43. It is commonly accepted that T<sub>R</sub> development and Foxp3 expression occur in the developing thymocytes because of instructive signal delivered through the TCR, which recognizes selecting MHC–peptide ligands with increased affinity (2, 3, 56). Our data show that TCR-
dependent signals are modulated by cellular interactions that are not TCR specific. We suggest that Cx43 is one of the molecules that act before or in concert with signals from the TCR to induce Foxp3 expression.

The proportion of CD25Foxp3GFP+ T cells in Cx43T−/− mice varied between 5 and 15% of the original number present in the lymph nodes of a wild type mice. The reason for the persistence of some Treg cells in Cx43T−/− mice is currently unknown. At least some of these cells are present due to incomplete excision of the Cx43 gene (data not shown). Alternatively, CD25Foxp3GFP+ T cells may represent a Treg subset that does not require Cx43 for development, which is consistent with our observation that more Treg cells persist in older Cx43T−/− mice. Finally, because both cre recombinase, controlled by the CD4 promoter, and Cx43 are expressed at the same or very close developmental stage, it is also possible that, in some double-positive thymocytes, expression of the cre recombinase does not occur before Foxp3 expression. Once these cells pass the critical stage when Cx43 is required for Treg lineage commitment, they progress to become Foxp3+ Treg thymocytes, which do not depend on Cx43 for further development. This last possibility is consistent with a higher proportion of Foxp3GFP+ T cells in the lymph nodes of 8-wk-old Cx43T−/− mice than in younger mice, which may be because of a higher proportion of selected Treg cells in older mice and expansion of the remaining Treg cells in peripheral lymph nodes.

Despite severe deficiency of the Treg cells, Cx43T−/− mice do not suffer from manifest autoimmunity. Instead, the number of CD4+ T cells with activated phenotype in the peripheral lymph nodes gradually increases, in an age-dependent manner. The lack of acute autoimmune disease in these animals could have several causes. First, sufficient CD25Foxp3GFP+ Treg cells remain in Cx43T−/− mice to control autoreactive cells. It has been shown that elimination of as much as 80–90% of Treg cells does not result in rampant autoimmunity (57, 58). Second, conventional CD4+ T cells in Cx43T−/− mice may have altered effector functions and are less prone to autoimmunity. This interpretation is supported by the data showing that Cx43-deficient conventional T cells produce less IFN-γ, and by the analysis of mixed bone marrow chimera demonstrating that Cx43-sufficient conventional CD4+ T cells have higher proportion of activated CD44hiCD62L− cells than Cx43-deficient cells (data not shown). Finally, one cannot exclude the possibility that the TCR repertoire in Cx43 mice have lower frequency of autoreactive T cells.

In summary, functional analyses of T cell subsets in Cx43T−/− mice support the view that Cx43 supports Treg lineage commitment and differentiation but also participates in regulating functions of mature Treg and effector cells. Reported and our own data suggest that the suppressor function of Treg cells is regulated by Cx43 directly, by a gap junction-dependent mechanism, or indirectly, which may include enhancement of Foxp3 expression (36).

Moreover, both Foxp3 and Cx43 expression in mature T cells depend on TCR stimulation and are greatly augmented by TGF-β. In conclusion, we speculate that Cx43 is an important component of the molecular circuitry, which includes TGF-β, and regulates peripheral tolerance.

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Disclosures
The authors have no financial conflicts of interest.


