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

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

Despite their importance for the functioning of the immune system, thymic development and peripheral maintenance of Foxp3+ regulatory T (T_R) cells are poorly understood. We have found that connexin 43 (Cx43), expressed by thymic T_R cells progenitors, supports T_R development. Mice with deletion of the Cx43 gene induced in T cells produce only few T_R cells and had increased proportion of activated T cells in the lymph nodes, suggesting impaired peripheral tolerance. Reduction of the T_R cell numbers was accompanied by increased presence of CD4+CD25+GITR+Foxp3− 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_R cells. We propose that a possible mechanism of Cx43 activity is by regulating Foxp3 expression in T_R lineage cells. The Journal of Immunology, 2011, 187: 000–000.

Connexin 43 is expressed in thymocytes and thymic epithelial cells, and is a component of the immunological synapse (34). Activation upregulates Cx43 in T cells, and it has been suggested that recognition of self-Ags induce the development of T_R lineage cells.

Cellular and molecular studies show that T_R lineage commitment is a multistage process often controlled by molecules expressed in other cell types that have a unique role in regulating T_R cell development. In this article, we report that connexin 43 (Cx43, Gja1) supports the development of T_R thymocytes and their precursors before the onset of Foxp3 expression. Connexins are small, transmembrane proteins that allow cells of a multicellular organism to communicate with their surrounding through small channels called gap junctions (26). More than 20 connexin genes have been found in human and mouse genomes, and disruption of connexin expression often results in developmental defects and lethality (27).

Multiple cell types of the immune system, which express Cx43, include monocytes, dendritic cells, and NK, B, and T cells (29). Cx43 is expressed in thymocytes and thymic epithelial cells, and thymocyte precursors and stromal cells communicate through the gap junctions formed by Cx43 (30, 31). Cx43 is essential for terminal differentiation of B and T cells when tested in Cx43-deficient mice; however, when Cx43 was missing in bone marrow cells alone, T cell development was normal (32, 33). Ag activation upregulates Cx43 in T cells, and it has been suggested that Cx43 is a component of the immunological synapse (34). Gap junction signaling mediated by Cx43 contributes to Ag-mediated clonal expansion but not cytokine production of activated T cells.
GENERATION OF Foxp3+ REGULATORY T CELLS

2 GENERATION OF Foxp3 + REGULATORY T CELLS, which likely contributed to impaired generation of TR cells in Cx43-deficient thymocytes were less sensitive to signaling by IL-
mice had progressively increasing numbers of activated CD4 +

Materials and Methods

Mice

Mice with conditional knockout of the Cx43 gene (Cx43 Twt and Cx43 T

T cell activation, proliferation, and Tc cell suppression assay

T cells were activated in vivo by injecting Foxp3 GFP mice with 50 µg

Cell purification, flow cytometry, and cell sorting

Single-cell suspensions were stained with Abs available commercially (BD

Production of mixed bone marrow chimeras

Bone marrow from Cx43 Twt and Cx43 T−/− mice (expressing congenic markers Ly5.1 + and Ly5.1 +) was mixed 1:1, and a total of 6 × 10⁶ cells were injected i.v. into sublethally irradiated (600 rad) RAG−/− mice. Cells were cultured for 4 d. Lymph node proliferation and Tc cell suppression assays were performed as previously described (39).

Adoptive transfer

Total CD4+ cells from Cx43 Twt, Cx43 T−/−, and 3-wk-old scurfy male mice were sorted on AutoMACs (Miltenyi). Purity of sorted cells exceeded 97%. A total of 5 × 10⁶ cells from Cx43 Twt or Cx43 T−/− were mixed with 5 × 10⁶ scurfy cells and transferred (i.v.) into TCRα− recipient mice. Recipient mice were weighted every day with weight at the day of transfer marked as 100%. Five mice of each kind were analyzed.

CD4+Foxp3 GFP+ effector cells from Cx43 Twt and Cx43 T−/− mice, CD4+CD25+Foxp3 GFP+ T cells from Cx43 T−/−, and CD4+Foxp3 GFP+ Tc cells from Cx43 Twt mouse 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 C4D+CD25+Foxp3 GFP+ T cells from Cx43 T−/− mice or CD4+Foxp3 GFP+ cells from Cx43 Twt 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′-CACAGACGTCTGA-

Western blotting

Foxp3 protein was detected in sorted (10⁶ cells/sample) CD4+CD25+ Foxp3 GFP+−, CD4+CD25−Foxp3 GFP+−, and CD4+CD25+Foxp3 GFP+− cells. Cells were lysed in the gel-loading buffer and resolved on 10% poly-

Statistical analysis

Differences between cell populations or cytokine concentrations were ana-

Results

Expression pattern of Cx43 in peripheral CD4+ T cells

To gain insight into the molecular mechanisms of Tc cell func-

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 × 10⁶ cells/well of 24-well plate) from Cx43 Twt and Cx43 T−/− mice were sorted and stimulated with plate-bound anti-CD3 (10 µg/ml) and anti-CD28 (1 µg/ml) Abs for 24 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.

YFP. Cx43 consisting of amino acids 1–228 (named Cx43NT, where NT stands for N-terminal portion) was prepared as described earlier. All con-

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activated conventional CD4+ T cells, adaptive Tr cells, and resting and activated natural Tr cells using GeneChip arrays. We have identified Cx43 as a gene differentially expressed in resting and activated effector CD4+ and Tr cells. To corroborate microarray data, we investigated Cx43 expression in resting CD44+CD62L+Foxp3GRF– and activated CD44+CD62L+Foxp3GRF+ conventional CD4+ T cells and CD4+Foxp3GRFP+ Tr cells activated in vitro without and in the presence of TGF-β (Fig. 1A). Only CD4+ 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 Tr cells sorted from Foxp3GRFP reporter mice immunized with SEB (Fig. 1C). In summary, Cx43 was expressed in conventional CD4+ T and Tr cells only after activation, and its expression is significantly augmented by TGF-β. Although Cx43 is greatly upregulated in activated Tr 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 Tr cells but could be important in T cell developmental processes requiring TCR stimulation and T cell activation.

**Cx43 is necessary for thymic generation of Tr 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 only the protein coding exon of the Cx43 gene with loxP sites (Cx43loxP mouse), were crossed to CD4-cre mice expressing cre recombinase in T cells starting at the stage of double-positive thymocytes (37, 38). In addition, we introduced Foxp3GRFP reporter transgene into CD4-c/+/Cx43loxPfloxP mice to tag Tr cells with the GFP (23). Mice transgenic for CD4-c/+/Foxp3GRFP and homozygous for the Cx43loxP allele (Cx43<sup>T/-/-</sup> mice) had normal numbers and proportions of double- and single-positive thymocytes and peripheral CD4+ and CD8α T cells, but a greatly decreased population of Tr cells (Fig. 2A–F).

**FIGURE 1.** Cx43 expression in various T cell subsets assessed by RT-PCR. A, Cx43 expression in naive conventional CD4+ T cells (Tc), CD4+ T cells activated in vitro, adaptive Tr cells (aTr), Tc cells activated in the presence of TGF-β), resting natural Tr cells (sorted Foxp3GRFP+ cells), and activated nTr cells (sorted Foxp3GRFP- cells activated in vitro). Where indicated, populations of T 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+ T cells stimulated in conditions promoting generation of Th1, Th2, Th17, aTr, and activated Tr cells. C, Cx43 expression in populations of activated CD4+ T (act. Tc), naive (Tc), and Tr cells (Foxp3GRFP+) sorted from mice immunized with SEB. Upper panels show Cx43 expression; lower panels show β-actin expression. Younger mice had more pronounced deficiency of Tr cells than older mice, increasing a possibility that Tr cells are differentially sensitive to Cx43 deficiency (Fig. 2E, 2F). Control mice (Cx43<sup>T+</sup>) used for the experiments expressed Foxp3GRFP reporter transgene and both alleles of Cx43 flanked by loxP sites, but these mice lacked CD4-c transgene, allowing for normal expression of Cx43. Young (3 wk) Cx43<sup>T/-/-</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 cells (Fig. 2G, 2H). CD4+ T cells from Cx43<sup>T/-/-</sup> mice had higher expression of activation markers CTLA-4, GITR, and ICOS, and a higher proportion of proliferating peripheral CD4+ T cells (Fig. 2I). In contrast, Cx43<sup>T/-/-</sup> mice had much lower proportion of Foxp3GRFP+ Tr cells both in the thymus and in the peripheral lymph nodes (Fig. 2B, 2D–F). The remaining Foxp3GRFP+ cells in Cx43<sup>T/-/-</sup> mice had lower expression of Foxp3 and decreased suppressor function compared with Tc 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 Tr cells. Decreased numbers of Tc cells in Cx43<sup>T/-/-</sup> mice corresponded with a higher proportion of cycling conventional CD4+ T cells with activated phenotype and suggested impaired peripheral tolerance (Fig. 2G–I; see data later in article).

Remarkably, Cx43<sup>T/-/-</sup> mice had an expanded population of CD4+CD25+Foxp3GRFP– cells in the thymus and in lymph nodes (Fig. 2B, 2D–F). These cells expressed GzT, CTLA-4, and Helios, markers characteristic of Tr cells, but did not express Foxp3 (Fig. 2J, 2K, and data not shown) (43–45). The population of CD4+CD25+Foxp3GRFP– cells resembled Tr 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 Tr lineage developmental program. Consistent with the lack of Foxp3 and Cx43, CD4+CD25+ Foxp3GRFP– cells did not inhibit proliferation of conventional CD4+ T cells in vitro (Fig. 3A). In vivo, total CD4+ T cells from Cx43<sup>T/-/-</sup> mice were less efficient in controlling effector CD4+ T cells from scurfy mice when cotransferred into recipient lymphopenic mice (Fig. 3B). The CD4+CD25+Foxp3GRFP– cells proliferated much less than conventional CD4+ 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+CD25+Foxp3GRFP– T cells in Cx43<sup>T/-/-</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 Tr cells (36). In an adaptive transfer experiment, CD4+CD25+Foxp3GRFP– cells from Cx43<sup>T/-/-</sup> mice were not able to protect mice from inflammatory bowel disease (Fig. 4A–F). In summary, the phenotype of CD4+CD25+Foxp3GRFP– cells strongly argues that they represent cells committed to Tr lineage that did not complete whole differentiation process.

Although the development and suppressor function of Tr cells are severely compromised in Cx43<sup>T/-/-</sup> mice, proliferation of conventional CD4+ 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+ 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<sup>Twt</sup> and Cx43<sup>T<sup>2</sup>/2</sup> mice. A–D, Representative flow cytometry analysis of thymocytes (A, B) and lymph node cells (C, D) of Cx43<sup>Twt</sup> and Cx43<sup>T<sup>2</sup>/2</sup> 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 Foxp3GFP expression on gated single-positive CD4<sup>+</sup> thymocytes (B) or lymph node cells (D). E and F, Total number of thymocytes and lymph node cells (left panels), and percentage of Foxp3GFP<sup>+</sup> in the populations of CD4<sup>+</sup> cells in the thymus and lymph node cells (right panels) in 1-, 3-, and 8-wk-old mice. Values for individual Cx43<sup>Twt</sup> (wt) mice (○, ◯) and for Cx43<sup>T<sup>2</sup>/2</sup> (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 Foxp3GFP<sup>+</sup> cells. G and H, Representative flow cytometry analysis of CD44 and CD62L expression on gated CD4<sup>+</sup>Foxp3GFP<sup>+</sup> lymph node cells (G), and the corresponding statistical analysis showing percentage of activated cells in the population of gated CD4<sup>+</sup> 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<sup>+</sup> T cells in Cx43<sup>Twt</sup> (wt) and Cx43<sup>T<sup>2</sup>/2</sup> (ko) mice. J, RT-PCR (left panel) and Western blot analysis (right panel) of Foxp3 expression. For RT-PCR, CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>GFP<sup>+</sup></sup> and CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>GFP<sup>+</sup></sup> cells sorted from Cx43<sup>Twt</sup> and Cx43<sup>T<sup>2</sup>/2</sup> mice were used. For Western blot, populations of naive CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>GFP<sup>+</sup></sup> and CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>GFP<sup>+</sup></sup> cells were sorted from Cx43<sup>T<sup>2</sup>/2</sup> 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<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>GFP<sup>+</sup></sup>, CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>GFP<sup>+</sup></sup>, and CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>GFP<sup>+</sup></sup> cells sorted from Cx43<sup>Twt</sup> and Cx43<sup>T<sup>2</sup>/2</sup> mice. One of two independent experiments is shown.
Cytokine production assays. Assay of CD4+Foxp3GFP+ responder cells (expressing wild type Cx43 alleles) and stimulated with soluble anti-CD3 Ab was inhibited by adding increasing numbers of CD4+CD25+ T cells sorted from Cx43Twt (λ, continuous line) and CD4+CD25Foxp3GFP+ (λ, dashed line) or CD4+CD25Foxp3GFP+ cells (x, 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 more 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 (λ) or Cx43T−/− (x) mice and transferred into TCR−/− 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+CD25Foxp3GFP− (TR-like) cells were sorted from Cx43Twt mice, and CD4+CD25Foxp3GFP− (conventional) and CD4+CD25Foxp3GFP− (TR-like) cells were sorted from Cx43T−/− mice. IL-2 was added to indicated wells. The plot shows a representative experiment of three. D, Cytokine production by T cell subsets isolated from Cx43Twt and Cx43T−/− mice. CD4+CD25 Foxp3GFP+ (conventional T cells [Tc]) were sorted from Cx43Twt (λ) and Cx43T−/− mice, and CD4+CD25Foxp3GFP− (Tc) or CD4+CD25Foxp3GFP− (Tc-like) cells were sorted from Cx43Twt or Cx43T−/− mice, respectively. Sorted cells were stimulated with plate-bound Abs anti-CD3/anti-CD28, and cytokine concentration was measured by ELISA. Brackets show statistically significant differences in cytokine concentration between cells from Cx43Twt (λ) and Cx43T−/− (λ) mice. Number above brackets shows p value. Plot shows a representative experiment of three.

FIGURE 3. Suppressor function, proliferation, and cytokine production of CD4+ T cell subsets from Cx43Twt and Cx43T−/− mice. A, Proliferation inhibition assay. Proliferation assay 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+ T cells sorted from Cx43Twt (λ, continuous line) and CD4+CD25Foxp3GFP− (λ, dashed line) or CD4+CD25Foxp3GFP− cells (x, 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 more 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 (λ) or Cx43T−/− (x) mice and transferred into TCR−/− 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+CD25Foxp3GFP− (TR-like) cells were sorted from Cx43Twt mice, and CD4+CD25Foxp3GFP− (conventional) and CD4+CD25Foxp3GFP− (TR-like) cells were sorted from Cx43T−/− mice. IL-2 was added to indicated wells. The plot shows a representative experiment of three. D, Cytokine production by T cell subsets isolated from Cx43Twt and Cx43T−/− mice. CD4+CD25 Foxp3GFP+ (conventional T cells [Tc]) were sorted from Cx43Twt (λ) and Cx43T−/− mice, and CD4+CD25Foxp3GFP− (Tc) or CD4+CD25Foxp3GFP− (Tc-like) cells were sorted from Cx43Twt or Cx43T−/− mice, respectively. Sorted cells were stimulated with plate-bound Abs anti-CD3/anti-CD28, and cytokine concentration was measured by ELISA. Brackets show statistically significant differences in cytokine concentration between cells from Cx43Twt (λ) and Cx43T−/− (λ) mice. Number above brackets shows p value. Plot shows a representative experiment of three.

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.

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+CD25Foxp3GFP+ thymocytes, but not in CD4+CD25Foxp3GFP− 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+CD25Foxp3GFP− represents immediate precursors of TR thymocytes (47).

When sorted, CD4+CD8−CD25+ double-positive thymocytes included rare CD25Foxp3GFP+ 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.
Colitis scores are shown on high-magnification panels (sacrificed after 10 wk and colon sections were examined for inflammatory formation). At least three mice in each group were analyzed. Recipient mice were transferred with CD4+Foxp3+CD25+Foxp3+ T cells from Cx43 Twt mice mixed with CD4+CD25+Foxp3– T cells isolated from Cx43 Twt mice from inflammatory bowel disease. Low (A, C, E, G; original magnification ×40) and high (B, D, F, H; original magnification ×200) magnifications of H&E-stained sections are shown. At least three mice in each group were analyzed. Recipient mice were sacrificed after 10 wk and colon sections were examined for inflammatory bowel disease. Colitis scores are shown on high-magnification panels (B, D, F, H). A and B, Recipient RAG−/− mice transferred with CD4+Foxp3+CD25+Foxp3+ T cells from Cx43 Twt mice mixed with CD4+Foxp3+Foxp3+ T cells from Cx43 Twt mice do not suffer from inflammation of the colon (grade 0 colitis). C and D, Recipient mice transferred with CD4+Foxp3+CD25− T cells from Cx43 Twt mice do not suffer from inflammation in basal and upper mucosal lamina propria (arrows). E and F, Recipient RAG−/− mice transferred with CD4+Foxp3+CD25− T cells from Cx43 Twt mice mixed with CD4+ CD25+Foxp3+ T cells from Cx43 Twt mice show moderate inflammation (grade 3 colitis). High magnification demonstrates severe inflammation in basal and upper mucosal lamina propria (arrows). G and H, The lymphocyte infiltration in mice receiving CD4+Foxp3+CD25− T cells from Cx43 Twt mice (grade 1 colitis). High magnification demonstrates mild inflammation in basal lamina propria (arrow). I, Summary of colitis scores. Colitis scores are shown for individual recipient mice. Recipient mice analyzed in A and B (○), C and D (×), E and F (△), and G and H (○) are shown. Transferred cell populations are indicated below the graph. Average values are shown by bars. Statistical differences between data are indicated by brackets, and p values are shown above brackets.

To further examine possible outcome of Cx43 deficiency on the thymic Tₚ 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 Tₚ 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+ Tₚ 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 Tₚ precursors. Our result suggests that at least one mechanism how Cx43 regulates Tₚ thymocyte generation is by increasing the sensitivity of Tₚ 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 Tₚ 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-β.

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

FIGURE 4. CD4+CD25+Foxp3+Foxp3+ T cells from Cx43 Twt mice do not protect mice receiving adaptive transfer of effector CD4+Foxp3 Foxp3+– T cells isolated from Cx43 Twt mice from inflammatory bowel disease. Low (A, C, E, G; original magnification ×40) and high (B, D, F, H; original magnification ×200) magnifications of H&E-stained sections are shown. At least three mice in each group were analyzed. Recipient mice were sacrificed after 10 wk and colon sections were examined for inflammatory bowel disease. Colitis scores are shown on high-magnification panels (B, D, F, H). A and B, Recipient RAG−/− mice transferred with CD4+Foxp3+CD25− T cells from Cx43 Twt mice mixed with CD4+Foxp3+Foxp3+ T cells from Cx43 Twt mice do not suffer from inflammation of the colon (grade 0 colitis). C and D, Recipient mice transferred with CD4+Foxp3+CD25− T cells from Cx43 Twt mice do not suffer from inflammation in basal and upper mucosal lamina propria (arrows). E and F, Recipient RAG−/− mice transferred with CD4+Foxp3+CD25− T cells from Cx43 Twt mice mixed with CD4+ CD25+Foxp3+ T cells from Cx43 Twt mice show moderate inflammation (grade 3 colitis). High magnification demonstrates severe inflammation in basal and upper mucosal lamina propria (arrows). G and H, The lymphocyte infiltration in mice receiving CD4+Foxp3+CD25− T cells from Cx43 Twt mice (grade 1 colitis). High magnification demonstrates mild inflammation in basal lamina propria (arrow). I, Summary of colitis scores. Colitis scores are shown for individual recipient mice. Recipient mice
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. 7). 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. 7). 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<sub>R</sub> cells and is necessary for the suppressor function of T<sub>R</sub> 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, and signaling molecules STAT5, CARMA-1, and c-rel, which are selectively involved in the development of T<sub>R</sub> 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<sub>R</sub> 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<sub>R</sub> development that are independent of TCR specificity of the developing thymocyte and require
FIGURE 7. Analysis of Cx43 expression in EL-4 cells. A. Mouse Cx43 protein showing transmembrane domains (TM1-4), whole-length protein (Cx43 1-382), and protein truncated at the carboxy end (Cx43NT 1-228). B. Expression of full-length wild type and N-terminal membrane domain of Cx43 in EL-4 lymphoma. Right column shows imaging for YFP-Cx43 fusion protein, middle column shows EL-4 cells in normal light, and right column shows flow cytometry analysis of EL-4 cells. EL-4 cells were transduced with empty pMSCV vector expressing rat CD2 (upper panels) or constructs encoding full-length Cx43-YFP fusion protein (middle panels) or N-terminal part of Cx43 fused with YFP (lower panels). Transduced cells (rat CD2+) were analyzed on Nikon Eclipse TE2000-S fluorescence microscope (original magnification ×100). Both wild type and truncated Cx43 incorporate into cell membrane. Green spots on microscope images are Cx43 complexes in the plasma membrane. C. Full-length Cx43 (EL-4 Cx43), but not truncated Cx43 (EL-4 Cx43NT), enhances Foxp3 expression in unstimulated EL-4 lymphoma cell line. Foxp3 expression is further increased in cells stimulated through the TCR and in cells stimulated in the presence of TGF-β. Left panels show RT-PCR reactions with Foxp3-specific primers; right panels show Western blot with Foxp3-specific Ab. Bars above images show relative quantity of Foxp3 transcript and protein standardized for β-actin with expression of Foxp3 in EL-4 cells arbitrarily set as one.

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>+</sup> thymocytes express CD25 and only a fraction of CD4<sup>+</sup>CD25<sup>+</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>−/−</sup> mice.

Despite a greatly reduced proportion of T<sub>R</sub> cells, Cx43<sup>−/−</sup> mice have an expanded population of thymic and peripheral CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> T cells. These cells do not inhibit in vitro proliferation of effector CD4<sup>+</sup> T cells but have some characteristics of T<sub>R</sub> phenotype. CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>−</sup> cells express increased levels of GITR and Helios, two molecules found on Foxp3<sup>+</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>+</sup>CD25<sup>+</sup> T<sub>R</sub> 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>+</sup>CD25<sup>+</sup>Foxp3<sup>−</sup> mice produce, at most, only very little Foxp3 transcripts. This places CD4<sup>+</sup>CD25<sup>+</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>+</sup>CD25<sup>+</sup>Foxp3<sup>−</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 latter 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 CD25+Foxp3GFP+ T cells in Cx43−/− 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 T<sub>R</sub> cells in Cx43−/− mice is currently unknown. At least some of these cells are present due to incomplete excision of the Cx43 gene (data not shown). Alternatively, CD25+Foxp3GFP+ cells may represent a T<sub>R</sub> subset that does not require Cx43 for development, which is consistent with our observation that more T<sub>R</sub> cells persist in older Cx43−/− mice. Finally, because both cre recombine, 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 recombine does not occur before Foxp3 expression. Once these cells pass the critical stage when Cx43 is required for T<sub>R</sub> lineage commitment, they progress to become Foxp3<sup>b+</sup> T<sub>R</sub> thymocytes, which do not depend on Cx43 for further development.

This last possibility is consistent with a higher proportion of Foxp3<sup>GFP+</sup> T<sub>R</sub> cells in the lymph nodes of 8-wk-old Cx43−/− mice than in younger mice, which may be because of a higher proportion of selected T<sub>R</sub> cells in older mice and expansion of the remaining T<sub>R</sub> cells in peripheral lymph nodes.

Despite severe deficiency of the T<sub>R</sub> cells, Cx43<sup>b−</sup> mice do not suffer from manifest autoimmune. Instead, the number of CD4<sup>+</sup> 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 CD25<sup>+</sup>Foxp3<sup>GFP+</sup> T<sub>R</sub> cells remain in Cx43<sup>−/−</sup> mice to control autoreactive cells. It has been shown that elimination of as much as 80–90% of T<sub>R</sub> cells does not result in rampant autoimmune (57, 58). Second, conventional CD4<sup>+</sup> T cells in Cx43<sup>−/−</sup> 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-deficient conventional CD4<sup>+</sup> T cells have higher proportion of activated CD4<sup>+</sup>CD62L<sup>+</sup> 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 Cx43<sup>−/−</sup> mice support the view that Cx43 supports T<sub>R</sub> lineage commitment and differentiation but also participates in regulating functions of mature T<sub>R</sub> and effector cells. Reported and our own data suggest that the suppressor function of T<sub>R</sub> 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

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References


