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Fc Receptor-Like 3 Protein Expressed on IL-2 Nonresponsive Subset of Human Regulatory T Cells¹

Satoshi Nagata,²,³*† Tomoko Ise,²*† and Ira Pastan*

Fc receptor-like 3 (FCRL3) is a cell surface protein homologous to Fc receptors. The FCRL3 gene is present in humans but not in mice. We found that FCRL3 protein is expressed on 40% of human naturally occurring CD4⁺ regulatory T (nTreg) cells (CD4⁺ CD25⁺ CD127low). Sorted nTreg cells with the surface phenotype FCRL3⁺ and FCRL3⁻ were both hypoproliferative to TCR stimulation and both suppressive on proliferation of conventional T cells (CD4⁺ CD25⁻) in vitro. They both expressed forkhead box p3 (Foxp3) protein, the intracellular regulatory T cell marker. However, in contrast to FCRL3⁺ nTreg cells, FCRL3⁻ nTreg cells were not stimulated to proliferate by the addition of exogenous IL-2. In addition, Foxp3⁺ cells induced from conventional T cells by TGF-β treatment did not exhibit FCRL3 expression. These results suggest that the FCRL3⁺ subset of human nTreg cells identified in this study arise in vivo and Foxp3 expression alone is not sufficient to induce FCRL3 expression. FCRL3 may be involved in human-specific mechanisms to control the generation of nTreg cells. The Journal of Immunology, 2009, 182: 7518–7526.

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Abbreviations used in this paper: FCRL, Fc receptor-like; Foxp3, forkhead box p3; GITR, glucocorticoid-induced TNFR family-related protein; nTreg, naturally occurring CD4⁺ regulatory T; Teff, T effector; Treg, regulatory T.

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vitro hypoproliferative phenotype of cells in response to exogenous IL-2.

Materials and Methods

Production and characterization of anti-FCRL3 mAbs

The cDNAs of human FCRL3 and other FCRLs were obtained by RT-PCR as described previously (27, 28). The cDNAs were confirmed to encode the same proteins as the following reference sequences: GenBank (http://www.ncbi.nlm.nih.gov/GenBank/) accession numbers AF459634 (FCRL1), AF459633 (FCRL2), AF459027 (FCRL3), AF436580 (FCRL4), AF436664 (FCRL5), and AF513661 (FCRL6). Expression of full-length FCRLs in transfected 293T cells and expression of their extracellular domains as human IgGFc fusion proteins were conducted as described previously (27, 28). Production and characterization of anti-FCRL3 mAbs were performed according to a series of our established protocols for obtaining mAbs to membrane proteins in their native conformation (27–30). The FCRL3-specific mAbs were screened in an ELISA using FCRL3-Fc protein as the coated Ag. Ab reactivity was confirmed in a flow cytometry using FCRL3-transfected 293T cells.

Fluorochrome-conjugated Abs in flow cytometry

The anti-FCRL3, H5, produced in this study, was conjugated to PE (custom labeled by Invitrogen/Molecular Probes). mAbs against FCRL1 (E3), FCRL2 (B24), FCRL4 (A1), and FCRL5 (F56) were prepared by us (27, 28). The following Abs were purchased: biotin-labeled Abs, anti-ICOS (ISA-3; eBioscience); FITC-labeled Abs, anti-CD3 (SP34-2; BD Biosciences 552852), anti-CD4 (SK3; BD Biosciences 555459), anti-CD8 (OKT8; eBioscience 57-4776), anti-CD69 (FN50; BD Biosciences 555459), anti-CD103 (B-Ly7; eBioscience 11-0199), anti-CD25 (BC96; eBioscience 21-0259), anti-CD38 (HIT2; BD Biosciences 555459), anti-CD45RA (HI100; eBioscience), brefeldin A (Bio-Rad) at 106 cells/ml before the flow cytometry analysis or cell sorting up to 40 h in IMDM (Invitrogen) supplemented with 10% FBS by a 10-min incubation at room temperature. Suppression assays were performed by coculturing 4000 CFSE-labeled Teff cells with different isolated cells (candidates of Treg cells) were mixed with responder CD4+ CD25+ CD127high cells (effecter T (Teff) cells) that had also been sorted from the autologous PBMCs. The Teff cells were labeled with 2 μM CFSE (Invitrogen/Molecular Probes) in IMDM supplemented with 1% FBS by a 10-min incubation at room temperature. Suppression assays were performed by coculturing 4000 CFSE-labeled Teff cells with different numbers of sorted cells (typically 4000, 2000, 1000, and 250) in 200 μl of IMDM containing 5% human AB serum (Bioreclamation) in round-bottom 96-well microtiter plates. The cells were harvested in 0.3 ml of FACS buffer containing 7-amino-actinomycin D (0.5 μg/ml; BD Biosciences) and anti-CD3, and TGF-β1 (by ELISA). IL-2 was not measured in the samples of TGF-β1 (by the beads array kit), and TGF-β1 (by the beads array kit) was determined so that fewer than 0.2% of cells in the counter sample (35). Unless indicated otherwise, a positive threshold for each marker was determined by a Fixation/Permeabilization solution (eBioscience) and then stained the cells isolated by sorting were labeled with CFSE. The labeled cells were collected for three or less colors and 300,000 events for more than four-color analysis. All of the data were further analyzed by FlowJo software (Tree Star). The gating strategies included lymphocyte gating (21), CD3 side scatter gate (33), and CD4 side scatter gate (34) as described in the figure legends. To determine positive and negative events for each marker, all reagents except for the one of interest (fluorescence minus one, or “FMO” controls) were used to identify expressing cells in the fully stained sample (35). Unless indicated otherwise, a positive threshold for each marker was determined so that fewer than 0.2% of cells in the counter sample (35). Unless indicated otherwise, a positive threshold for each marker was determined so that fewer than 0.2% of cells in the counter sample (35). Unless indicated otherwise, a positive threshold for each marker was determined so that fewer than 0.2% of cells in the counter sample (35). Unless indicated otherwise, a positive threshold for each marker was determined so that fewer than 0.2% of cells in the counter sample (35). Unless indicated otherwise, a positive threshold for each marker was determined so that fewer than 0.2% of cells in the counter sample (35). Unless indicated otherwise, a positive threshold for each marker was determined so that fewer than 0.2% of cells in the counter sample (35). Unless indicated otherwise, a positive threshold for each marker was determined so that fewer than 0.2% of cells in the counter sample (35). Unless indicated otherwise, a positive threshold for each marker was determined so that fewer than 0.2% of cells in the counter sample (35). Unless indicated otherwise, a positive threshold for each marker was determined so that fewer than 0.2% of cells in the counter sample (35).

In vitro suppression assays

To measure the suppressor activity of the sorted population of cells, the isolated cells (candidates of Treg cells) were mixed with responder CD4+ CD25+ CD127high cells (effecter T (Teff) cells) that had also been sorted from the autologous PBMCs. The Teff cells were labeled with 2 μM CFSE (Invitrogen/Molecular Probes) in IMDM supplemented with 1% FBS by a 10-min incubation at room temperature. Suppression assays were performed by coculturing 4000 CFSE-labeled Teff cells with different numbers of sorted cells (typically 4000, 2000, 1000, and 250) in 200 μl of IMDM containing 5% human AB serum (Bioreclamation) in round-bottom 96-well microtiter plates. The cells were harvested in 0.3 ml of FACS buffer containing 7-amino-actinomycin D (0.5 μg/ml; BD Biosciences) for viability staining and 20,000 FITC-conjugated beads (no. 512; Bangs Laboratories) were used as the standard for cell counting. CFSE dilution by Teff cell division was analyzed in live cell populations using an LSR II flow cytometer and FlowJo software.

In vitro cell proliferation assays

The cells isolated by sorting were labeled with CFSE. The labeled cells (5,000) were incubated with or without different numbers of anti-CD3/CD28 beads (25,000, 50,000, and 100,000) in 200 μl of IMDM containing 5% human AB serum in round-bottom 96-well microtiter plates. In some cultures, exogenous recombinant human IL-2 (BioSource International/Innogenot) was added at 10 ng/ml.

Induction of Foxp3+ cells from Teff cells in vitro by treatment of TGF-β

Sorted CD4+ CD25+ CD127high cells (5 × 104) were cultured in 1 ml of IMDM containing 5% human AB serum in 24-well culture plates for 4 days with or without 10 ng/ml recombinant human TGF-β (eBioscience) or anti-CD3/CD28 beads (10/100 μl) or IL-2 (10/110 μl).

Cytokine assay

Cytokine production in culture supernatants of the suppressor and proliferation assays were measured using a human T/H2 cytokine cytoketic bead array kit (BD Biosciences) or a human TGF-β1 Quantikine ELISA kit (R&D Systems) according to the manufacturers’ instructions. The human cytokines measured were IL-2, IL-4, IL-5, IL-10, TNF-α (by the beads array kit), and TGF-β1 (by ELISA). IL-2 was not measured in the samples with exogenous IL-2.
Results

Production and characterization of an anti-FCRL3 mAb

We produced an anti-FCRL3 IgG2b mAb, H5, by use of a DNA immunization protocol established previously (29). We carefully examined undesired cross-reactivity of H5 mAb with other FCRL proteins because there are considerable homologies between the six extracellular Ig domains of FCRL3 and the same subtypes of Ig domains of other FCRL family members (2, 6, 27, 28). H5 mAb reacted with FCRL3-Fc fusion protein in ELISA but did not react with FCRL1, 2, 4, 5, and 6-Fc fusion proteins in the same assay (Fig. 1A). H5 mAb bound to the surface of FCRL3-transfected 293T cells in a flow cytometry assay but did not bind to FCRL1, 2, 4, 5, and 6-transfected cells (Fig. 1B). We concluded that H5 mAb is specific to FCRL3 and is not cross-reactive with other FCRL proteins. H5 mAb also bound to endogenous FCRL3 protein on several human B cell lines in which FCRL3 mRNA was expressed (Fig. 1C). H5 mAb did not cross-react with FcγRIIb (CD32), or FcRIIIa (CD16) in single- or double-color flow cytometry assays using appropriate cell lines or human PBMCs (our unpublished data). H5 mAb did not bind to recombinant FCRL3 proteins in Western blotting (our unpublished data), indicating that H5 mAb recognizes a conformational epitope whose structure is denatured by SDS treatment. These results indicated that H5 mAb is a highly specific Ab that recognizes an epitope of the extracellular domain of native FCRL3 on the cell surface and is an ideal probe for characterizing this protein in immune cells.

FCRL3 is expressed on T cell subsets in human PBMCs

We stained PBMCs from normal donors with Abs to surface markers for B cells, T cells, or NK cells along with the anti-FCRL3 mAb conjugated to PE and then analyzed the cell population by flow cytometry (Fig. 2A). As expected from previous reports (2, 6, 8), FCRL3 protein was expressed on B cells (CD19⁺) and NK
expressing cells in each subpopulation. One representative experiment is shown in the positive ranges. CD25 high threshold (between P6 and P7) was drawn so that P7 includes 2% of CD4 and for CD25 (between P5 and P6) were determined so that fewer than 1% of cells in the counter control without the corresponding marker were included. P4 gates were drawn according to previous reports so as to distinguish the CD25 markers and FCRL3 on CD4 cells. As summarized in Fig. 2, expression of FCRL3 on the gated cells: 1) intracellular Foxp3 expression (Foxp3+), 2) CD25-positive (dim to high) combined with low level CD127 expression (CD4+CD25low, and CD25low), and 3) the top 2% of the highest level of CD25 expression (CD25high). As shown in Fig. 4A, nTreg cells were identified as Foxp3+, CD25−CD127low, and CD25high cells.

**FIGURE 3.** A time course of the expression of different activation markers and FCRL3 on CD4+ and CD8+ cells after T cell stimulation through TCR and CD28. Human PBMCs were stimulated with anti-CD3/CD28-coated beads. The stained cells were initially gated by CD3 side scatter profile to include large activated lymphocytes and then gated for CD4 (A) or CD8 (B). The stimulation induced transient expression of CD69 followed by CD25 but did not induce FCRL3 expression. Threshold for CD25 was determined so that <0.1% of cells in the counter control without the CD25 mAb were included in the positive range. A representative data set from three similar experiments is shown.

**FIGURE 4.** nTreg cells in human PBMCs are subdivided into two subpopulations with different FCRL3 expression levels. PBMCs from normal donors were stained with CD127, FCRL3, CD4, and CD25, fixed, permeabilized, and then stained with the intracellular Foxp3. A, Three gating strategies (Foxp3+, CD25−CD127low, and CD25high) to identify nTreg-enriched cells in CD4+ cell population are shown. Positive thresholds for Foxp3 (between P1 and P2) and for CD25 (between P5 and P6) were determined so that fewer than 1% of cells in the counter control without the corresponding marker were included in the positive ranges. CD25high threshold (between P6 and P7) was drawn so that P7 includes 2% of CD4+ cells with the highest CD25 signals. P3 and P4 gates were drawn according to previous reports so as to distinguish the CD25−CD127low nTreg population in P4 from other cells in P3 (24–26). B, Frequency of gated populations in CD4+ cells in four different specimens. C, Expression of FCRL3 on the gated cells. D, The frequency of FCRL3-expressing cells in each subpopulation. One representative experiment is shown in A and C. Four replicate experiments using different specimens are summarized in B and D.
in P2, P4, and P7 gates, respectively (24–26). As the counter populations for comparison, non-Treg cells were identified as Foxp3−, CD25low, CD127high, and CD25− cells in P1, P3, and P5 gates, respectively. The P6 gate defines CD25high cells that partly contain CD25−CD127low nTreg cells. The frequency of each population in CD4+ cells is summarized in Fig. 4B.

As shown in Fig. 4, C and D, all three Treg populations (P2, P4, and P7) contained two distinctive subpopulations that correspond to FCRL3+ and FCRL3− cells. In contrast, the three non-Treg populations (P1, P3, and P5) were almost all FCRL3− cells. Thus, FCRL3 is preferentially expressed on nTreg cells in human CD4+ fractions. The incidences of FCRL3+ cells in CD4+ nTreg cells are 20–50% (Fig. 4D). Fig. 5 displays different types of views of the flow cytometry analysis that confirm the relationships among expressions of FCRL3 and other markers in CD4+ cells. Fig. 5B shows that the Foxp3 expression level is within the positive range for both FCRL3+ and FCRL3− populations in the two nTreg gates (CD25−CD127low or CD25high), although these two cell populations show slightly different distributions of Foxp3 expression levels.

We also examined the association of FCRL3 expression with other markers, including those that had been reported to subdivide CD25+ nTreg cells into two subpopulations (26, 36). As shown in Fig. 6, our results indicated that the FCRL3+ and FCRL3− nTreg cell populations are not definable by GITR, CD38, ICOS, CD31, CD62L, CD45RA, CD45RO, CTLA-4, HLA-DR, and CD103.

Both FCRL3+ and FCRL3− nTreg cells suppress T cell proliferation in vitro

We next examined the regulatory (suppressor) activity of FCRL3+ and FCRL3− subsets of CD25+ CD127low nTreg cells on the proliferation of non-Treg cells. According to the gating strategy shown in Fig. 7A, CD25− CD127high (P1) and CD25+ CD127low (P2) cells were isolated from the CD4− subset of PBMCs by cell sorting. The CD25+ CD127high (P1) cells were used as responder cells (Teff) in a proliferation assay to test candidate Treg cells isolated from autologous PBMCs. We also isolated FCRL3− (P3) and FCRL3+ (P4) cells from the CD25+ CD127low cell population.
using a second gating (Fig. 7A). The post-sorting panels confirmed that these sorted cell populations fell into the same gates used for their sorting. The isolated FCRL3\(^{-}\)/H11002 (P3) and FCRL3\(^{-}\)/H11001 (P4) cells cannot be distinguished in their CD25 vs CD127 profiles.

As shown in Fig. 7B, the addition of CD25\(^{+}\)CD127\(^{low}\) cells (P2), FCRL3\(^{-}\)CD25\(^{+}\)CD127\(^{low}\) cells (P3), or FCRL3\(^{-}\)CD25\(^{+}\)CD127\(^{low}\) cells (P4) remarkably suppressed the proliferation of the labeled T-eff cells (P1), although addition of T-eff cells (P1) did not block the proliferation of labeled cells. A time course experiment (Fig. 7C) and an experiment using various addback ratios (Fig. 7D) indicated that the tested FCRL3\(^{-}\) (P3) and FCRL3\(^{-}\) (P4) subpopulations of CD25\(^{+}\)CD127\(^{low}\) cells manifested similar levels of suppressive activity as the total CD25\(^{+}\)CD127\(^{low}\) cells (P2). When CD25\(^{high}\) gating was used to identify nTreg cells, their FCRL3 expression was also independent of suppressor activity (our unpublished data). CD25\(^{+}\)CD127\(^{low}\) cells suppressed IL-2, IL-4, IL-5, and TNF-\(\alpha\) production by the T-eff cells in the cocultures regardless of FCRL3 expression (supplemental Table S1\(^{5}\)). Inhibitory cytokines, IL-10 and TGF-\(\beta\), in the growth-suppressed cultures were detected only at the same trace levels as the no-addback cell cultures (supplemental Table S1), which confirmed that CD25\(^{+}\)CD127\(^{high}\) cells do not contain a significant number of adaptive Treg cells such as Tr1 or Th3 cells secreting IL-10 or TGF-\(\beta\) (37, 38).

**FIGURE 8.** FCRL3\(^{-}\) nTreg cells but not FCRL3\(^{-}\) nTreg cells proliferate in the presence of IL-2 upon stimulation of their TCR and coreceptor. A, Both FCRL3\(^{-}\) and FCRL3\(^{-}\) nTreg cells show reduced proliferation without IL-2. Sorted cells were labeled with CFSE and cultured for 80 h with the indicated numbers of anti-CD3/CD28 beads. Averages + SDs of division indices of triplicate cultures are shown. B, Exogenous IL-2 conferred responsiveness to stimulation of FCRL3\(^{-}\) nTreg cells but not FCRL3\(^{-}\) nTreg cells. Sorted cells were cultured for 70 h in the presence or absence of IL-2 and anti-CD3/CD28 beads. C, Time course of the proliferation of FCRL3\(^{-}\) and FCRL3\(^{-}\) nTreg cells stimulated with anti-CD3/CD28 beads (2.5 beads/cell) in the presence or absence of IL-2. D, IL-10 secretion from FCRL3\(^{-}\) nTreg cells stimulated with anti-CD3/CD28 beads in the presence of exogenous IL-2. Each cytokine in the supernatant was measured after a 70-h culture. IL-2 was not measured in the samples with exogenous IL-2 (top panel). A–D, A representative data set from three independent experiments using different human subjects.

**FCRL3 expression on nTreg cells correlates with cell hypoproliferation**

In addition to the suppressor activity, a defining characteristic of nTreg cells in vitro is their relative inability to proliferate and to produce cytokines in response to TCR stimulation. Treg cells require exogenous IL-2 in addition to TCR stimulation for proliferation. Therefore, we next examined proliferation of the sorted FCRL3\(^{-}\) and FCRL3\(^{-}\) cells with or without anti-CD3/CD28 beads in the presence or absence of exogenous IL-2 in vitro. Fig. 8A shows the cell divisions after 80 h in culture, monitored by the dilution of intracellular CFSE. As expected, FCRL3\(^{-}\) (P3), FCRL3\(^{-}\) (P4), or the total CD25\(^{+}\)CD127\(^{low}\) cells (P2) showed no proliferation by anti-CD3/CD28 bead stimulation, whereas the non-Treg CD25\(^{+}\)CD127\(^{high}\) cells (P1) proliferated. An increase in the ratio of stimulating beads to cells did not induce proliferation of any of the three nTreg populations, whereas CD25\(^{+}\)CD127\(^{high}\) T-eff cells showed similar high levels of cell proliferation at all bead:cell ratios. Fig. 8B shows the effects of exogenous IL-2 on proliferation. The anti-CD3/CD28 beads alone or IL-2 alone showed no enhancement of proliferation in the cell cultures. In

\(^{5}\) The online version of this article contains supplemental material.
sharp contrast, when cells were stimulated both with anti-CD3/CD28 beads and IL-2, FCRL3 CD25⁺CD127low (P3) cells showed marked proliferation activity. However, FCRL3⁻CD25⁺CD127low (P4) cells remained hyposensitive to stimulation. The CD25⁺CD127low (P2) cells showed moderate proliferation that approximately corresponds to the ratio of FCRL3⁺ and FCRL3⁻ cells within the CD25⁺CD127low population (Fig. 4D). Fig. 8C shows the growth curves of different cell populations in the presence of both anti-CD3/CD28 beads and IL-2. Nonparallel growth of FCRL3⁺ and FCRL3⁻ nTreg cells suggested that the difference of susceptibility to IL-2 between these two populations was maintained during the 5-day culture period. The growth of FCRL3⁺ and the nongrowth FCRL3⁺ nTreg cells were recognized in the majority of the sorted cell population in the scattering profiles showing larger forward scatter and side scatter values for growing cells (supplemental Fig. S1). In addition to the difference in the hypoproliferative state between FCRL3⁺ and FCRL3⁻ nTreg cells, IL-10 production was induced only from FCRL3⁻ nTreg cells and not from FCRL3⁺ nTreg cells by exogenous IL-2 (Fig. 8D). This illustrates an additional difference between FCRL3⁻ and FCRL3⁺ nTreg cells in terms of their response to exogenous IL-2. Fig. 8D also showed that neither FCRL3⁺ nor FCRL3⁻ nTreg cells produced significant amounts of IL-2, IL-4, IL-5, TNF-α, or TGF-β upon anti-CD3/CD28 stimulation and further supplementation with exogenous IL-2.

**FCRL3 is not expressed on the Foxp3⁺ subset of cells induced from the CD25⁺CD127high non-Treg population by TGF-β treatment in vitro**

We next examined FCRL3 expression on TGF-β-induced Foxp3⁺ cells. TCR stimulation, in addition to TGF-β treatment, is required for induction of Foxp3 in conventional T cells. IL-2 produced from the stimulated cells was also reported to contribute to the generation of Foxp3⁺ cells (15, 39). For these reasons, we stimulated non-Treg CD25⁺CD127high cells (P1 in Fig. 7A) with anti-CD3/CD28 beads, TGF-β, IL-2, and various combinations of these factors for 4 days. As shown in the first row of panels in Fig. 9, without any stimulation, CD25⁺CD127high cells retained the same phenotype throughout the incubation period. Neither Foxp3, CD25, nor CTLA-4 was induced. In contrast, as shown in the second row of panels (Fig. 9), anti-CD3/CD28 beads activated CD25⁺CD127high cells to proliferate and become granular cells with higher side scatter and markedly increased levels of CD25 and CTLA-4 expression. As expected, Foxp3 was only slightly induced by the activation. TGF-β treatment alone did not change the phenotype of CD25⁺CD127high cells compared with the untreated cells. However, a combination of TGF-β with anti-CD3/CD28 beads converted a significant fraction of the stimulated cells to Foxp3-positive status. The induced Foxp3⁺ cells showed a marker profile similar to nTreg cells from the PBMC population, such as high levels of CD25 and CTLA-4 and lower levels of CD127. The induced Foxp3⁺ cells, however, did not express FCRL3. Addition of exogenous IL-2 showed no effects on FCRL3 expression. We conclude that FCRL3 expression is not induced in Foxp3⁺ cells produced from non-Treg CD4⁺ T cells in these conditions.

**Discussion**

In this study, we found that FCRL3 protein is expressed on 20–50% of naturally occurring Treg cells in human peripheral blood. FCRL3⁺ and FCRL3⁻ nTreg cells are both suppressive on proliferation of conventional T cells (CD4⁺CD25⁺) in vitro. However, in contrast to FCRL3⁻ nTreg cells, FCRL3⁺ nTreg cells are not stimulated to proliferate by the addition of exogenous IL-2. Interestingly, the FCRL3 gene is present in humans but not in mice (5).

Dynamics and maintenance on the FCRL3 expression on nTreg cells in vivo and the mechanism leading to the generation of two subpopulations of nTreg cells remain undefined. We have not detected other markers specific to FCRL3⁻ nTreg cells or FCRL3⁺ nTreg cells. Nor have we found any in vitro culture conditions that change the level of FCRL3 expression on Foxp3⁺ cells in a survey using conditioned medium and cytokines (including 4– to 5-day culture in the presence of IL-2, IL-4, IL-7, IL-15, and their combinations; our unpublished data). In long-term cultures of T cells in the presence of IL-2, the expression of FCRL3 was gradually diluted out by an overgrowth of FCRL3-negative cells after a few weeks of culture (our unpublished data). We did not detect FCRL3 expression in three different tumor-infiltrated lymphocyte cultures (CD8⁺, obtained from Dr. S. A. Rosenberg, unpublished data). We succeeded in inducing growth of FCRL3⁻ nTreg cells by the combined stimulation of anti-CD3/CD28 and IL-2 (Fig. 8). However, we did not detect significant FCRL3 expression in the expanded cultures from FCRL3⁻ Treg cells (our unpublished data). In addition, as shown in Fig. 9, Foxp3⁺ cells induced from CD4⁺Foxp3⁻ cells by TGF-β treatment did not express FCRL3. Combined, these data suggest that unidentified factors are necessary to induce FCRL3 expression in vivo. FCRL3⁻ nTreg cells and FCRL3⁺ nTreg cells are both suppressive (Fig. 7) and show similar positive levels of Foxp3 expression (Fig. 5). Foxp3 plays a major role in the occurrence and function of Treg cells by activating or repressing many genes by forming transcription complexes with other factors (12–14). The presence of FCRL3⁻ and FCRL3⁺ subpopulations of Foxp3⁺ nTreg cells suggest that FCRL3 expression is not solely controlled by Foxp3.

Hyporesponsiveness (anergy) to TCR and coreceptor stimulation in cell culture is an established property of nTreg cells, which can be overcome by addition of exogenous IL-2 (40, 41). Although there is a remarkable difference between in vitro and in vivo conditions for Treg cell expansion, IL-2 also plays an important role for Treg biology in vivo (42, 43). Our data revealed that FCRL3⁻ nTreg cells but not FCRL3⁺ nTreg cells undergo IL-2-dependent proliferation in vitro. This suggests that nTreg expansion and maintenance by IL-2 may be associated with the FCRL3 expression in vivo in humans. Mouse nTreg cells propagated by IL-2 in...
vitro temporarily lose their suppressive activity but spontaneously regain the activity once IL-2 is removed (41). Such experiments using the expanded FCRL3\(^{-}\) Treg cells (by anti-CD3/CD28 beads plus IL-2 treatment) will be needed in future studies. The association of nonresponsiveness to TCR stimulation with FCRL3 expression was also observed in the CD8\(^{+}\) cell fraction, whereas we did not detect suppressor activities of FCRL3\(^{-}\)CD8\(^{+}\) or FCRL3\(^{-}\)CD8\(^{+}\) cells in our assay (我们的 unpublished data).

An important question is why FCRL3\(^{-}\) nTreg cells and FCRL3\(^{+}\) nTreg cells respond differently to IL-2 despite the expression of the CD25 receptor on both populations. We detected a difference in IL-10 production after IL-2 stimulation, indicating that IL-2 likely induces a different sequence of events in FCRL3\(^{-}\) nTreg cells than in FCRL3\(^{+}\) nTreg cells, leading to the dissimilarity of the response. In preliminary assays, we did not detect different levels of CD122 (IL-2Rb) or the phosphorylated form of STAT5 (by detection using anti-phospho-Ab) after IL-2 stimulation between FCRL3\(^{+}\) and FCRL3\(^{-}\) Treg cell populations (我们的 unpublished data). Analysis of intracellular signaling and profiling gene expression patterns induced by IL-2 will be needed to determine the differences between FCRL3\(^{-}\) nTreg cells and FCRL3\(^{+}\) nTreg cells. We plan to perform a comprehensive assay using a microarray platform.

FCRL3 resembles other Ig superfamily receptors in structure, including intracellular signaling through ITAM/ITIM and tyrosine phosphorylation. These phosphorylated tyrosines are predicted to serve as docking sites for Src homology 2 domain-containing molecules to initiate downstream signaling pathways (2). Such FCRL3-mediated intracellular signals potentially play important biological roles under the epigenetic conditions of FCRL3\(^{-}\) Treg cells. In preliminary experiments, we tested the effects of anti-FCRL3 mAb in PBMCs cultured in the absence/presence of secondary Ab. We also tested the anti-FCRL3 mAb coated on anti-IgG beads. Under these limited conditions, we were not able to detect significant changes in cell growth or marker phenotype of FCRL3\(^{-}\) nTreg cells (我们的 unpublished data). We plan to further examine phosphorylation of tyrosine residues in the signaling motifs that may be induced by engagement of FCRL3 by the anti-FCRL3 mAb.

Ex vivo generation of Foxp3\(^{+}\) cells from naive T cells has been actively studied because of the potential for exploiting Treg cells for clinical use, such as reestablishing self-tolerance in autoimmune diseases (39, 44–47). Extensive studies in mice have demonstrated that induction of Treg cells from naive T cells using TGF-\(\beta\) can lead to expression of suppressive activity accompanied by Foxp3 expression. However, the accumulated evidence suggests that human Foxp3\(^{+}\) cells generated in response to TGF-\(\beta\) do not always exhibit suppressive activity (15, 46). Our finding that TGF-\(\beta\)-induced Foxp3\(^{+}\) cells do not express FCRL3 revealed a difference in marker phenotype between TGF-\(\beta\)-induced Foxp3\(^{+}\) cells and nTreg cells generated in vivo in addition to suppressive activity. Although the association of FCRL3 expression with suppressive function was not observed, seeking ex vivo conditions that generate FCRL3\(^{-}\) cells may allow induction of human suppressive T cells that are closer in nature to nTreg cells. A broad repertoire of self- and non-self-Ags can be recognized by nTreg cells in the periphery. However, specific association of a limited number of the TLR family members with nTreg cells was also reported (48). Examination of the TLR repertoire expressed on FCRL3\(^{-}\) and FCRL3\(^{+}\) nTreg cells may allow elucidation of the roles of these cells in regulating the immune system. Other important populations of Treg cells are adaptive Treg cells generated in the periphery, including IL-10-producing Trl cells (37). It will be important to characterize FCRL3 expression in this population.

In preliminary experiments, we did not detect a change of FCRL3 expression in our attempts to expand IL-10-producing Treg cells by polyclonal activation of human CD4\(^{+}\) T cells in the presence of dexamethasone and vitamin D\(_3\) (49).

The FCRL3 gene is present in humans but not in mice (1, 2, 5). Genome sequence analysis suggests that the repertoire of FCRLs was shaped in evolution by an extensive recombination process including species-specific gain and loss of distinct exons or entire genes. This has resulted in remarkable diversity of surface FCRL proteins among species. There are only FCRL1, 5, and 6 in mice, whereas humans possess FCRL2, 3, 4, 5, and 6. Differences between human nTreg cells and mouse nTreg cells have been repeatedly reported, especially as to CD25 expression level and Foxp3 expression (20, 22, 23, 46). FCRL3 might be associated with human-specific immunoregulation of nTreg cells. The biological role of FCRL3 and its natural ligand are unknown but the gene locus lq21-q23 where the FCRL3 locates has been implicated in susceptibility to autoimmune diseases (50). Recent studies suggest that the up-regulation of FCRL3 transcription induced by single nucleotide polymorphisms in the promoter of FCRL3 is associated with predisposition toward several autoimmune diseases (8, 50, 51). Although the link between FCRL3 and autoimmune diseases is not clear, it will be of importance to survey FCRL3 expression on nTreg cells from patient lymphocytes in the future.

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