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Expression of the Autoimmune Susceptibility Gene FcRL3 on Human Regulatory T Cells Is Associated with Dysfunction and High Levels of Programmed Cell Death-1

Louise A. Swainson, Jeff E. Mold, Urmila D. Bajpai, and Joseph M. McCune

CD4+ FoxP3+ regulatory T cells (Treg) play a critical role in maintaining self-tolerance and inhibiting autoimmune disease. Despite being a major focus of modern immunological investigation, many aspects of Treg biology remain unknown. In a screen for novel candidate genes involved in human Treg function, we detected the expression of an autoimmune susceptibility gene, FcRL3, in Treg but not in conventional CD4+ T cells. FcRL3 is an orphan receptor of unknown function with structural homology to classical Fc receptors. Numerous genetic studies have demonstrated a link between a single nucleotide polymorphism in the FCRL3 promoter and both overexpression of FcRL3 and autoimmunity diseases such as rheumatoid arthritis. Given the critical role of Treg in suppressing autoimmunity, we sought to ascertain how expression of FcRL3 relates to the phenotype, differentiation, and function of Treg. We show in this study that FcRL3 is expressed on a population of thymically derived Treg that exhibits a memory phenotype and high levels of programmed cell death-1. Purified FcRL3+ Treg are less responsive to antigenic stimulation in the presence of IL-2 than their FcRL3- counterparts, despite intact proximal and distal IL-2 signaling as determined by phosphorylation of Stat-5 and upregulation of Bcl2. In vitro suppression assays demonstrated that FcRL3+ Treg have reduced capacity to suppress the proliferation of effector T cells. These data suggest that FcRL3 expression is associated with Treg dysfunction that may, in turn, contribute to the loss of self-tolerance and the development of autoimmunity. The Journal of Immunology, 2010, 184: 3639–3647.
lymph nodes (MLNs) were dissected from the fetal mesentery. Single-cell suspensions were washed in R10 medium before phenotypic analysis. MLN suspensions were first subjected to density centrifugation as above and then cultured in RPMI 1640 (Life Technologies, Rockville, MD) supplemented with 10% FBS, 50 IU/ml and 100 μg/ml anti-CD3 (anti-CD3 16-102, eBioscience, San Diego, CA). During culture, CD4+ T cells were gated and sorted on a FACSAria or Aria II (BD Biosciences) and all data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Materials and Methods

Tissues and cell isolation

Adult peripheral blood samples were collected from healthy individuals, all of whom gave written informed consent using protocols approved by the University of California at San Francisco Committee on Human Research (San Francisco, CA). Adult blood buffy coats from healthy donors were obtained from Stanford Blood Center, Palo Alto, CA. PBMCs from blood or buffy coats were isolated by density centrifugation using Ficoll-Hypaque Plus (Amersham Biosciences, Piscataway, NJ). All phenotyping and functional analyses were performed on fresh samples. Lymphocytes were cultured in RPMI 1640, according to the manufacturer's protocol. The samples were acquired on an LSRII flow cytometer (BD Biosciences), and all data were analyzed using FlowJo software (Tree Star, Ashland, OR). Doubled discrimination based on forward scatter height versus area was performed to eliminate cellular aggregates.

FACS sorting

CD4+ T cells from buffy coats were pre-enriched by negative selection using the RosetteSep tetrameric complex system (StemCell Technologies, Vancouver, British Columbia, Canada) and conventional CD4+ T cells (CD25loCD127hiFcRL3−) were added to cell-staining mixtures in conjunction with anti-CD3 and anti-CD25 PE-Cy7, anti-CCR7 PE-Cy7, anti-CD127 PE, anti-CD25 APC-Cy7, and anti-CD25 APC-PE (all from BD Biosciences; anti-ICOS PE, anti–HLA-DR APC (BD Biosciences); anti–CD3 PE, and anti–CD8 PE-Cy5.5 (Caltag Laboratories, Burlingame, CA); anti–CD4 Qdot605 (Invitrogen); anti–CD45RA ECD, and anti–CTLA-4 PE (Beckman Coulter, Fullerton, CA). Dead cells were excluded from all flow cytometric analyses by cell surface staining with Aqua Live/Dead amine reactive dead cell stain reagent (Invitrogen).

RNA isolation and RT-PCR analysis

Total RNA was extracted using the RNeasy mini kit (Qiagen, Valencia, CA), and cDNA was prepared by reverse transcription using the Omniscript RT kit (Qiagen). Quantitative PCR was performed using FcRL3, FoxP3, or hypoxanthine phosphoribosyltransferase (HPRT) TaqMan gene expression assays with TaqMan Universal PCR Mix (Applied Biosystems, Foster City, CA), according to the manufacturer’s protocol. Amplification of cDNA was performed using the AB Step One Plus instrument (Applied Biosystems); the cycling conditions included a denaturation step for 10 min at 95°C followed by 40 cycles of denaturation (95°C for 15 s) and extension (60°C for 1 min).

SNP analysis

A total of 1 × 10^6 PBMC from 29 healthy adult donors were stored as dry pellets at −80°C until analysis. DNA was isolated from the cell pellets using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer’s instructions. A PCR-based 5’ nuclelease genotyping assay was used to specifically discriminate between the −169C and −169T FcRL3 allele polymorphisms. This assay comprised unlabeled forward and reverse PCR primers (900 nM final concentration) and two allele-specific probes labeled with either VIC or FAM reporter dye (200 nM each). Amplification was performed using the AB Step One Plus instrument (Applied Biosystems). Assay components were added to 20 ng DNA in a 20-μl reaction containing TaqMan Universal PCR Mix. An AB Step One Plus instrument was used for amplification and detection, and AB system software was used for analysis (Applied Biosystems).

Abs and flow cytometric phenotyping

The anti-FcRL3 Ab was kindly provided by Genentech (South San Francisco, CA) (24). The Ab and a control irrelevant protein (human serum albumin; Sigma-Aldrich, St. Louis, MO) were conjugated to biotin using the Fluoro-reporter mini-biotin protein-labeling kit (Invitrogen, Carlsbad, CA), and then added to cell-staining mixtures in conjunction with anti-CD3 and anti-CD25 Abs (2% final concentration) and secondary detection of FcRL3 or control was performed with a streptavidin-Qdot655 conjugate (Invitrogen). For phenotypic analysis and cell sorting by flow cytometry, the following Abs were used: anti-CD3-PE, anti-CD25 PE-Cy7, anti-CD25 APC-Cy7, anti-CD28 FITC, anti–HLA-DR APC, anti-CXCR3 PE, and anti–CD45RA PE-Cy7. A total of 1 × 10^6 PBMC from 29 healthy adult donors were stored as dry pellets at −80°C until analysis. DNA was isolated from the cell pellets using the DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer’s instructions. A PCR-based 5’ nuclelease genotyping assay was used to specifically discriminate between the −169C and −169T FcRL3 allele polymorphisms. This assay comprised unlabeled forward and reverse PCR primers (900 nM final concentration) and two allele-specific probes labeled with either VIC or FAM reporter dye (200 nM each). Amplification was performed using the AB Step One Plus instrument (Applied Biosystems); the cycling conditions included a denaturation step for 10 min at 95°C followed by 40 cycles of denaturation (95°C for 15 s) and extension (60°C for 1 min).

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T cells were seeded at 100,000 cells/well in the presence of 0, 5, 12.5, or 50 ng/ml recombinant human TGF-β1 (R&D Systems). For inhibition of TGF-β signaling, the activin receptor-like kinase inhibitor SB-431542 (Sigma-Aldrich) was added at 1 μM.

**Proliferation assays and Bcl2 detection**

Ninety-six-well round-bottomed culture wells were precoated with 0.68 µg/ml each OKT3, SP34-2, and Hitt3a clones of anti-CD3 Abs (for a total of 2 µg/ml anti-CD3) in PBS for 5 h at 37°C and then washed to remove excess Ab. Soluble anti-CD28 Ab (BD Biosciences) and recombinant human IL-2 (R&D Systems) were added at a concentration of 1 µg/ml and 50 IU/ml, respectively. FACs-sorted T cell subpopulations were either stained immediately for Bcl2 as described below or seeded at 100,000 cells/well and incubated for 5 d. IL-2 was supplemented on day 3 at 50 IU/ml, respectively. FACS-sorted T cell subpopulations were either stained immediately for Bcl2 as described below or seeded at 100,000 cells/well and incubated for 5 d. IL-2 was supplemented on day 3 at a concentration of 25 IU/ml. Following 5 d of culture, cells were recovered and stained with a live/dead marker, and Abs targeted to CD3, CD4, and CD8 for flow cytometry using an anti-FcRL3 mAb (24).

**Stat-5 phosphorylation analysis**

FACS-sorted T cell subpopulations were resuspended in 100 μR10 medium and prewarmed to 37°C prior to stimulation with either R10 medium alone or with 50 IU/ml IL-2 for 30 min. Cells were immediately fixed and then permeabilized (Cytofix/ Cytoperm, BD Biosciences) prior to staining for Ki67 (BD Biosciences) or Bcl2 (DakoCytomation, Carpinteria, CA) using an isotype control.

**Statistical analysis**

Statistical analysis was performed in Prism software (GraphPad, San Diego, CA) using either paired or unpaired Student t test, as indicated in the text for relevant figures. For comparisons of FcRL3+ and FcRL3− T cells, the frequency of CFSElo cells was calculated for FcRL3+ and FcRL3− T cells. The frequency of CFSElo cells included all cells that had undergone at least one division.

**Variability in Treg FcRL3 expression is associated with the −169/T FCR L3 polymorphism**

Given evidence that a C/T SNP in the promoter region of FCR L3 (position −169) is associated with expression levels of FcRL3 mRNa (with higher levels found with the C allele) (22), we postulated that this polymorphism was associated with the extreme variability in FcRL3 protein expression on Treg observed in

**Results**

**FcRL3 is expressed on a subpopulation of Treg**

An earlier microarray study in our laboratory comparing CD4+CD25hi Treg and CD25lo naive CD4+ T cells revealed that the transcript for FcRL3 is ~100-fold more highly expressed in Treg than in naive CD4+ T cells (J.E. Mold, unpublished observations). To confirm this observation, PCR quantification of transcripts from Treg and naive CD4+ T cells was conducted for FcRL3 and for the Treg transcription factor, FoxP3 (Fig. 1A). FcRL3 transcripts were amplified within the Treg fractions (R) but not from the naive CD4+ fractions (N).

To determine the relationship between expression of FcRL3 mRNA and protein on Treg, surface expression levels of FcRL3 were detected by flow cytometry using an anti-FcRL3 mAb (24). Treg or Tconv were phenotyped by flow cytometry according to expression levels of FoxP3, CD25, and CD127 (Fig. 1B). CD4+ CD8+ T cells were initially selected from the live CD3+ population (Fig. 1B1). CD4+CD8− T cells were subsequently gated on either CD25hiCD127lo or CD25lowCD127hi (Fig. 1Bii) and then further segregated according to FoxP3 expression to document subpopulations that were either CD25hiCD127loFoxP3+ (Treg) (Fig. 1Bii) or CD25loCD127hiFoxP3− (Tconv) (Fig. 1Biv). Within the three donors analyzed, FcRL3 expression was detected on Treg but not on Tconv (Fig. 1C). Expression levels on Treg were variable, ranging from approximately half of the Treg expressing FcRL3 in donor 1 to barely detectable levels in donor 2. On the highest expressing donor 1, a separate shoulder was evident, indicative of distinct FcRL3+ and FcRL3− populations. We conclude that the autoimmune susceptibility gene FcRL3 is expressed at the protein level on the surface of a subpopulation of CD4+ Treg.
Fig. 1C. To test this hypothesis, blood samples were collected from 29 healthy adult donors and screened for FcRL3 expression levels by flow cytometry and for SNP −169C/T FCRL3 genotype by a PCR-based allelic discrimination assay. Regardless of whether the percentage of FcRL3+ Treg (Fig. 2A) or the mean fluorescence intensity (MFI) of FcRL3 within Treg (Fig. 2B) was analyzed, the data consistently showed significantly higher FcRL3 protein expression for each copy of the C allele present. Similar results have recently been reported in the case of FcRL3 expression on B cells in normal donors and patients with systemic lupus erythematosus (35). Neither the variability in Treg FcRL3 expression nor the −169C/T FCRL3 polymorphism were found to be significantly correlated with the expression of FoxP3 (Supplemental Fig. 2).

**FcRL3+ Treg are phenotypically distinct from FcRL3− Treg**

Several studies have suggested that the Treg population is heterogeneous and can be subdivided based on differential expression of markers such as CTLA-4, PD-1, CD45RA, CD62L, and CD278 (ICOS) (36–40). The expression of these and other markers was analyzed on three populations: FcRL3+ Treg, FcRL3− Treg, and Tconv (Fig. 3A). PD-1, a marker of T cell exhaustion (40–42), was found to be expressed on nearly twice as many FcRL3+ Treg as FcRL3− Treg (mean 43% versus 24%; p < 0.001; n = 8). This correlated with FcRL3+ Treg exhibiting more of a CD45RA− memory phenotype; whereas the FcRL3− subpopulation was heterogeneous for CD45RA expression, significantly fewer CD45RA+ cells were detected within the FcRL3+ subpopulation (p < 0.05). FcRL3+ Treg also expressed low levels of CCR7 and were homogeneously high in expression of CD95/Fas, whereas their FcRL3− counterparts generally exhibited higher levels of CCR7 and lower levels of CD95/Fas (p < 0.05). CD62L, which is expressed more highly on thymically derived nTreg (37), was expressed at higher levels on the FcRL3+ than on the FcRL3− subpopulation (p < 0.001). Finally, the two Treg subpopulations exhibited similar levels of CD28, CD31, ICOS, HLA-DR, and CTLA-4 (Fig. 3A, 3B). The two parameter dot plots in Fig. 3C showing coexpression of CCR7, CD45RA, and CD95/Fas within the Treg and Tconv subpopulations demonstrated that CD45RA+ FcRL3+ Treg express lower levels of CD95/Fas and higher levels of CCR7. We conclude that FcRL3+ and FcRL3− Treg exhibit distinct phenotypes; whereas FcRL3+ Treg are more heterogeneous and comprise the majority of naive Treg, the FcRL3− Treg subpopulation is skewed more toward a memory phenotype expressing higher levels of PD-1.

**FcRL3+ Treg are thymically derived and not inducible in vitro by TGF-β**

Treg can be generated either in the thymus or in peripheral lymphoid tissues (2). We evaluated fetal thymic Treg (CD25hiFoxP3+) and conventional CD4+CD8−CD3+CD25hiFoxP3+ SP CD4+ (SP4) thyocytes for FcRL3 expression (Fig. 4A) and found that the expression of FcRL3 was restricted to the Treg thyocyte population (Fig. 4Aii) with little or no expression on SP4 thyocytes (Fig. 4Aii). To determine whether FcRL3+ Treg constituted a greater fraction of Treg in the thymus than in the periphery, paired samples of thymus and either MLNs or thymus and spleen were analyzed from the same fetal donor. Consistently, thymic Treg contained a higher fraction of FcRL3+ cells than Treg found in either the MLN (Supplemental Fig. 3A) or the spleen (Supplemental Fig. 3B). Almost twice as many Treg expressed FcRL3 in the fetal thymus (32%) compared with Treg from peripheral lymphoid tissues (17%; MLN or spleen) in the six donors analyzed (p < 0.01). Peripheral FoxP3+ Treg can be induced by TCR activation in the presence of TGF-β (43). To assess whether FcRL3+ Treg can be induced from Tconv in this manner, sorted CD25hiCD127hiCD4+ T cells were stimulated for 5 d with anti-CD3 and anti-CD28 Abs (αCD3/CD28), IL-2, and increasing concentrations of TGF-β. The resulting cell cultures were then screened for FcRL3 and FoxP3 transcripts by RT-PCR. TGF-β treatment was clearly able to induce transcription of FoxP3 but not FcRL3 (Fig. 4B). In aggregate, these data indicate that FcRL3+ Treg are thymically derived and that FoxP3 expression per se is not sufficient to induce FcRL3 expression.

**FcRL3+ Treg exhibit defective proliferation in response to αCD3/CD28 activation in the presence of IL-2**

Numerous lines of evidence indicate that PD-1 expression on T cells is associated with limited proliferative capacity (40–42, 44, 45). Because the FcRL3+ Treg population expressed significantly higher levels of PD-1 compared with the population of FcRL3− Treg (Fig. 3A, 3B), we postulated that FcRL3+ Treg would demonstrate defective proliferation compared with their FcRL3− counterparts when provided TCR stimulation in the presence of IL-2 (12). FACS-sorted FcRL3+ Treg, FcRL3− Treg, and Tconv were cultured for 5 d with medium alone, IL-2, αCD3/CD28, or a combination of αCD3/CD28 and IL-2. The purity of sorted populations prior to activation is represented in Supplemental Fig. 1. As shown in the scatter plots of Supplemental Fig. 4, no blast formation was observed in any subpopulation after culture in
FcRL3+ Treg are phenotypically distinct from FcRL3− Treg. PBMCs from healthy donors were phenotyped by flow cytometry to determine the expression levels of the indicated markers on FcRL3+ and FcRL3− Treg. A, Representative histograms from individual donors showing expression of the indicated markers on FcRL3+ and FcRL3− Treg. B, Bar graph showing composite data of expression of the indicated markers on FcRL3+ (solid) or FcRL3− (striped) Treg. Data represent the mean ± SEM from between four and eight donors for each marker. Statistically significant differences by paired Student t test are marked with asterisks: * indicates that differences were significant (p < 0.05) and *** that differences were extremely significant (p < 0.001). C, Representative dot plots showing CD45RA versus CCR7 (top row), CD45RA versus CD95/Fas (middle row), and CCR7 versus CD95/Fas (bottom row) for FcRL3+ Treg, FcRL3− Treg, and Tconv.

FIGURE 4. FcRL3+ Treg are phenotypically distinct from FcRL3− Treg. A, Flow cytometric analysis of a representative second trimester human fetal thymus showing FcRL3 expression on thymic Treg. SP CD4+ (SP4) cells were gated from total live thymocytes, subsequently selected for expression of CD3, and then Treg and conventional SP4 thymocytes were defined by FoxP3 and CD25 expression (gates i and ii, respectively). FcRL3 expression (black lines) and control stains (shaded histograms) are shown for these gates. B, Treg were FACS sorted from total CD4+ T cells and then cultured for 5 d with anti-CD3 and anti-CD28 Abs (αCD3/CD28) and increasing concentrations of TGF-β. At the highest concentration of TGF-β, the TGF-β inhibitor SB-431542 was also added to the culture medium. At the end of the 5-d culture period, RT-PCR was performed to determine transcript levels of FoxP3 and FcRL3. Transcripts were normalized to HPRT and are expressed as the mean ± SD of triplicate samples.

FIGURE 3. FcRL3+ Treg are phenotypically distinct from FcRL3− Treg. PBMCs from healthy donors were phenotyped by flow cytometry to determine the expression levels of the indicated markers on FcRL3+ and FcRL3− Treg. A, Representative histograms from individual donors showing expression of the indicated markers on FcRL3+ Treg, FcRL3− Treg, or Tconv. Shaded gray histograms represent FMO control stains. B, Bar graph showing composite data of expression of the indicated markers on FcRL3+ (solid) or FcRL3− (striped) Treg. Data represent the mean ± SEM from between four and eight donors for each marker. Statistically significant differences by paired Student t test are marked with asterisks: * indicates that differences were significant (p < 0.05) and *** that differences were extremely significant (p < 0.001). C, Representative dot plots showing CD45RA versus CCR7 (top row), CD45RA versus CD95/Fas (middle row), and CCR7 versus CD95/Fas (bottom row) for FcRL3+ Treg, FcRL3− Treg, and Tconv.

medium alone or in the presence of IL-2, whereas αCD3/CD28 activation resulted in blast formation in Tconv but not in either Treg subpopulation. The addition of IL-2 to αCD3/CD28 stimulation (far right column of Supplemental Fig. 4) induced blast formation in Tconv and FcRL3− Treg but to a lesser degree in FcRL3+ Treg, which exhibited a reduced cell size compared with FcRL3− Treg as seen by the forward scatter parameter (mean forward scatter 62700 versus 67000, respectively; n = 4).

To more accurately detect the induction of cell cycling, cells were stained for the proliferation Ag Ki67 (Fig. 5A). No Ki67+ cells were detected in any subpopulation after culture in medium alone and only a small proportion (22%) of Tconv were Ki67+ after IL-2 stimulation. αCD3/CD28 activation resulted in Ki67+ expression in nearly all of Tconv (83%), whereas less than 15% of the Treg populations were Ki67+ under this condition. The addition of IL-2 to αCD3/CD28 activation was sufficient to induce Ki67 in the majority of FcRL3− Treg (64%), whereas only half this number stained positive for the proliferation Ag in the FcRL3+ subpopulation (32%). The composite data from four different donors shown in Fig. 5B indicates that the hypoproliferation of FcRL3+ Treg versus FcRL3− Treg in response to αCD3/CD28 plus IL-2 is significant (p < 0.05), confirming the hypothesis that FcRL3+ Treg are relatively defective in their proliferative responses.

FcRL3+ and FcRL3− Treg demonstrate intact proximal and distal signaling responses to IL-2.

To ascertain whether FcRL3+ Treg are refractory to a stimulus from IL-2, both proximal and distal readouts of IL-2 signaling were monitored. The transcription factor, Stat-5, is rapidly phosphorylated following IL-2 binding to the IL-2 receptor, resulting in Stat-5 dimerization and nuclear entry to facilitate transcription of IL-2 response genes, including Bcl2 (46). FACS-sorted FcRL3+ Treg, FcRL3− Treg, and Tconv were stimulated with either IL-2 or medium alone for 30 min and stained intracellularly using an Ab specific to a phosphorylated epitope at the tyrosine 694 residue of Stat-5 (P-Stat-5). Fig. 6A shows composite data of P-Stat-5 induction by IL-2 from three individual donors for each T cell subpopulation. As expected, IL-2 induced higher levels of P-Stat-5.
FcRL3+ Treg have intact proximal and distal IL-2 signaling responses.

- **Statistical Analysis:**
  - For FcRL3+ Treg and FcRL3+ Tconv, the proliferation assay was performed with cells from 14 different donors, using different ratios of sorted addback to responder cells ranging from 1:5 to 1:25 (Fig. 7B). The CFSE profiles of one representative donor (Fig. 7B) show that the proportion of Treg in the coculture diminishes, the proliferation of the responders increases. Notably, for ratios at which differences in the suppressive capacity of FcRL3+ and FcRL3− Treg can be clearly observed (i.e., at 1:10 and 1:15 addback ratios for this donor), the FcRL3+ Treg population was less able to inhibit responder T cell proliferation.

- **Discussion:**
  - We report in this study that FcRL3 is expressed on a subpopulation of human CD4+CD25+FoxP3+ Treg with interindividual variation in expression that correlates with an SNP at promoter position 169. FcRL3+ Treg appear to emanate from the thymus and express a unique phenotype characteristic of exhausted memory T cells. Although they can signal across the IL-2 receptor, FcRL3+ Treg demonstrate intact proximal and distal signaling responses to IL-2. A, FACS-sorted FcRL3+ Treg, FcRL3− Treg, and Tconv were incubated with either medium alone (Control) or IL-2 for 30 min, fixed, and then stained for Stat-5 using an Ab specific for the phosphorylated Y641 Stat-5 epitope. The specific induction of phospho-Stat-5 is shown (MFI after IL-2 stimulation − MFI after R10 medium alone); data represent mean ± SEM of three separate experiments comprising three different donors. B, FACS-sorted FcRL3+ Treg, FcRL3− Treg, and Tconv were cultured in the presence of medium alone, IL-2, αCD3/CD28 Abs or a combination of αCD3/CD28 Abs and IL-2. After 5 d, cells were fixed, permeabilized, and stained for Bcl2. The fold change in Bcl2 expression from day 0 to analysis at day 5 is shown; data represent mean ± SEM of three separate experiments comprising four different donors.

- **Conclusion:**
  - The FcRL3+ Treg appear to be dysfunctional, exhibiting a reduced capacity to suppress responder T cell proliferation compared with their FcRL3− counterparts.
FIGURE 7. FcRL3+ Treg are dysfunctional in their ability to suppress T_{eff} cell proliferation. FACS-sorted FcRL3+ Treg, FcRL3− Treg, and Tconv were added back to CFSE-labeled responder T cells (CD25-depleted PBMC). Cocultures were stimulated with αCD3 Abs for 5 d, stained with Abs directed against CD3, CD4, and CD8, and then analyzed by flow cytometry for CFSE dilution of CD8+ T cells. A, CFSE dilution of T_{eff} after 5 d coculture with the indicated addback populations. Unstimulated responder T cells are shown as a control in the left histogram. B, CFSE dilution of responder T cells after 5 d coculture with the indicated addback populations at varying ratios of sorted cells to responders. C, Composite data showing the frequency of divided (CFSE<sup>lo</sup>) responder cells at each addback ratio from seven separate experiments comprising 14 different donors. Data represent mean ± SEM of the 14 individuals for each ratio. Statistically significant differences (p < 0.05) between FcRL3<sup>+</sup> Treg and FcRL3<sup>+</sup> Treg by paired Student t test are marked with an asterisk (*).

Dysfunctional suppressive ability of FcRL3<sup>+</sup> T_{reg}

These data are consistent with those reported by Nagata et al. (48), especially with respect to the proliferative defect of FcRL3<sup>+</sup> T_{reg} in response to antigenic stimulation in the presence of IL-2. We show in this paper, however, that FcRL3<sup>+</sup> and FcRL3<sup>+</sup> T_{reg} exhibit differences in phenotype and function. Most importantly, and in contrast to the data from Nagata et al. (48), our study demonstrates that FcRL3<sup>+</sup> T_{reg} are dysfunctional and do not inhibit the proliferation of responder T cells to the same extent as do FcRL3<sup>+</sup> T_{reg}. It is possible that this difference in results is reflective of the heterogeneity of FcRL3 expression on T_{reg} and the fact that we screened cells from a large number of donors [14 as compared with 3 in Nagata et al. (48)]. However, it is also important to consider that different T_{reg} suppression assays were used in our experiments and that methodologic distinctions may have also played a role.

The studies conducted by Nagata et al. (48) used bead-coupled anti-CD3 and anti-CD28 Abs to stimulate highly purified CD4<sup>+</sup> CD25<sup>lo</sup>CD127<sup>lo</sup> T_{eff} in the complete absence of APCs. In our experiments, a more physiologic suppression assay closer to mimicking in vivo cellular interactions was employed. Specifically, CD25-depleted autologous PBMCs were used as responders, with anti-CD3 Abs supplying the exogenous stimulation. The use of CD25-depleted PBMCs provides a more physiologically representative mixture of cells, including APCs, that can directly or indirectly influence or mediate T_{reg} function (2, 13–16). Indeed, it is becoming increasingly recognized within the T_{reg} community that different in vitro suppression assays may measure different aspects of T_{reg} cell-suppressor function (15). It is also noteworthy that we analyzed suppression exclusively on CD8<sup>+</sup> T_{eff}, whereas Nagata et al. (48) measured suppression on CD4<sup>+</sup> T_{eff}. Assessing suppression on CD4<sup>+</sup> T_{eff} can be problematic as, after 4 to 5 d proliferation, the diluted CFSE signal on CD4<sup>+</sup> T_{eff} can begin to overlap with the negative CFSE signal of unlabeled CD4<sup>+</sup> T_{reg} addback cells. Contamination of the CD4<sup>+</sup> T_{eff} CFSE<sup>lo</sup> cells with CD4<sup>+</sup> T_{reg} CFSE<sup>+</sup> cells and skewing of the data may therefore occur. We analyzed exclusively CD8<sup>+</sup> T_{eff} to avoid this potential problem, such that CFSE would only be measured on pure effector cells.

It is interesting to speculate why FcRL3<sup>+</sup> T_{reg} have diminished suppressor activity. T_{reg}-mediated suppression may involve a number of mechanisms of action attributed to T_{reg} cells. These can be broadly divided into those that target T cells (e.g., suppressor cytokines, IL-2 consumption, and cytolsis) and those that primarily target APCs (e.g., decreased costimulation or decreased Ag presentation). APCs may also modulate the activity of T_{reg} directly or indirectly (15, 16). Given the discrepancies between our data and those of Nagata et al. (48), it would be logical to speculate that the presence of APC in our suppression assay likely allowed the unmasking of differences in function between FcRL3<sup>+</sup> and FcRL3<sup>+</sup> T_{reg}. Interestingly, we find that the FcRL3<sup>+</sup> T_{reg} population expresses higher levels of PD-1. PD-1 acts as an inhibitory molecule on T_{conv} and T_{reg} after interacting with its ligands, PD-L1 and PD-L2, expressed primarily on activated dendritic cells and macrophages, cell types that are present in our suppressor assay (44). PD-1 is upregulated on exhausted T cells and plays a causative role in limiting proliferation and other functions such as cytokine secretion and cytolytic activity upon ligation by PD-L1/2 (40–42, 44, 45).

Other mechanisms may be at work in preventing FcRL3<sup>+</sup> T_{reg} from suppressing T_{eff} expansion. FcRL3<sup>+</sup> T_{reg} also exhibit higher levels of the apoptosis-inducing TNF-R family member, CD95/Fas, than FcRL3<sup>+</sup> T_{reg}, suggestive of an increased predisposition to cell death. This is supported by a recent article demonstrating that high expression of PD-1 is associated with increased spontaneous and CD95/Fas induced apoptosis in T cells (49). A reduced number of active suppressor cells due to increased apoptosis could also account for the dysfunction observed in FcRL3<sup>+</sup> T_{reg}. It would be interesting to determine whether small interfering RNA knockdown of FcRL3<sup>+</sup> T_{reg} modulates the suppressive function of these cells, as it is presently unknown whether this functional defect is caused by, or merely associated with, FcRL3 expression.

*Purified FcRL3<sup>+</sup> T_{reg} exhibit defective proliferation*

Although FcRL3<sup>+</sup> T_{reg} demonstrate reduced suppressive ability in the presence of activated PBMCs, purified FcRL3<sup>+</sup> T_{reg} exhibit defective proliferation in response to αCD3/CD28 activation in the presence of IL-2 without the presence of any other cell types (48) (Fig. 5). Although Nagata et al. (48) attributed the reduced expansion of FcRL3<sup>+</sup> T_{reg} to IL-2 nonresponsiveness, we ascertained that these cells exhibit intact proximal and distal signaling responses to IL-2 (as determined by phosphorylation of Stat-5 and upregulation of Bcl2, respectively). Therefore, it is more likely that FcRL3<sup>+</sup> T_{reg} have a defect in their ability to respond to αCD3/CD28 activation. Consistent with this hypothesis, PD-1<sup>lo</sup> T cells are known to have reduced proliferative capacity and to possess shorter telomeres than PD-1<sup>hi</sup> T cells (50, 51). Moreover, our analysis of six donors showed a trend for FcRL3<sup>+</sup> T_{reg} to express...
lower levels of cyclin B2 transcripts than FcRL3− Treg (data not shown). Future experiments to determine whether blocking Abs to PD-1 restore the proliferative defect in FcRL3+ Treg may establish whether this molecule is itself a causative factor in FcRL3+ Treg defective proliferation.

Thymic development of FcRL3+ Treg

Our analysis of fetal tissues indicates that FcRL3 is expressed by a subpopulation of CD4+FoxP3+CD25hi SP4 thymocytes, but not by FoxP3+ Treg induced from conventional T cells by TCR stimulation and TGF-β. These observations suggest that FcRL3 may only be present on thymically derived nTreg and not on peripherally induced induced Treg. Despite their detection in the thymus, the FcRL3+ Treg subpopulation exhibits a memory phenotype with reduced CD45RA and CCR7 expression, suggestive of more rapid memory conversion upon migration to the periphery than FcRL3− nTreg. The switch from naive to memory phenotype in T cells is associated with proliferation and activation triggered by recognition of cognate Ag, suggesting that FcRL3+ Treg may recognize self-Ag with greater specificity or affinity than their FcRL3− counterparts. As has been observed in the case of PD-1 upregulation in the context of antigenic stimulation (40–42), chronic stimulation of FcRL3+ Treg by autoantigens could result in the exhausted phenotype and the responses observed in this study.

Perspectives: FcRL3 as a costimulator?

Given the association between various autoimmune diseases and the −169 C/T SNP that leads to overexpression of FcRL3, any discussion of FcRL3 expression on Treg must also consider the mechanisms by which this protein may have a causal impact on aberrant immune activation. In vitro signaling assays have demonstrated that ITIMs in the cytosolic domain of FcRL3 can be phosphorylated and bind to Src homology region 2 domain-containing phosphatase family phosphatases, proteins known to function as negative regulators in TCR signaling (52). In addition, FcRL5, a closely related FcRL family member, was shown to inhibit B cell activation via Src homology region 2 domain-containing phosphatase 1 tyrosine phosphatase recruitment (53). Another FcRL member, FcRL4, acts as a negative regulator of BCR signaling (54). FcRL4 expression is associated with exhaustion in dysfunctional memory B cells and poor proliferation by FoxP3+ Treg induced from conventional T cells by TCR stimulation in the context of antigenic stimulation (40–42), chronic stimulation of FcRL3+ Treg by autoantigens could result in the exhausted phenotype and the responses observed in this study.

Disclosures

The authors have no financial conflicts of interest.

References