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J Immunol published online 26 February 2010
http://www.jimmunol.org/content/early/2010/02/26/jimmunol.0903943

Supplementary Material
http://www.jimmunol.org/content/suppl/2010/02/24/jimmunol.0903943.DC1

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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

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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 autoimmune 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: 000–000.

The survival of complex organisms is dependent upon the ability of an active immune system to recognize and fend off foreign invaders while simultaneously preventing an attack on self. Several mechanisms have evolved to accomplish this feat. On the one hand, tolerance to self can be enforced upon elimination of self-reactive T and B cells; on the other, peripheral mechanisms can actively curb the proliferation and/or function of self-reactive cells that escape deletion. In the latter instance, investigations from many independent laboratories using diverse models have firmly established the involvement of CD4+ regulatory T cells (Treg). Specialized in suppressing immune responses to self-Ags, Treg play a crucial role in thwarting autoimmune disease (1, 2).

The Treg compartment is comprised of two developmentally distinct populations. Natural Treg (nTreg) originate in the thymus and are specific for self-Ags presented by thymic epithelial cells, whereas induced Treg are generated de novo in the periphery from conventional CD4+ T cells (Tconv) upon antigenic stimulation in the presence of TGF-β and dampen immune responses to foreign Ags (2, 3). Both of these Treg populations express FoxP3, a transcription factor critical for the homeostasis and suppressor functions of Treg (4) and without which dramatic autoimmune manifestations can arise (5–7).

Treg are characterized by a set of phenotypic and functional attributes that distinguish them from Tconv. In contrast to Tconv, for instance, Treg exhibit high levels of CD25 (the IL-2Rα) (8), low levels of CD127 (the IL-7Rα) (9, 10), and constitutive expression of the B7 family costimulatory receptor, CTLA-4 (11). Treg characteristically do not proliferate when stimulated via the TCR, although IL-2 can overcome this anergy (12). The maintenance of self-tolerance by Treg may involve any of several proposed immunosuppressive mechanisms, including decreasing the costimulation or Ag presenting ability of APCs (13, 14), production of suppressive cytokines, IL-2 consumption, and the direct killing of target cells (2, 15, 16).

Even though Treg have been researched extensively for over a decade, many questions about their phenotype, differentiation, and function remain unanswered. Our laboratory has conducted a number of studies investigating the biology and clinical implications of Treg (9, 17–20). During the course of this research, we discovered that FcRL3, the product of an autoimmune susceptibility gene, is expressed on Treg but not on conventional CD4+ T cells.
domains and either ITAMs, ITIMs, or both in the intracellular domains. The natural ligands of the FcRL family members remain unknown but, given their signaling domains and expression on multiple immune cell types, these members likely modulate immune cell functions by affecting signaling pathways (21). FcRL3 is expressed predominantly in B lymphocytes in lymph nodes and germinal centers (22–24), and, although its function remains unknown, numerous genetic studies have revealed an association between a single nucleotide polymorphism (SNP) in FCR3 and a variety of autoimmune diseases such as rheumatoid arthritis (RA) (22, 25–31). This C > T polymorphism at position −169 in the promoter region of FCR3 confers a higher affinity binding site for the NFκB transcription factor, resulting in increased promoter activity and upregulation of FCR3 mRNA transcription (22). This relationship implies that high expression of FcRL3 results in abnormal immune activation and loss of self-tolerance.

Early studies documented that the C allele of this dimorphic SNP was significantly associated with RA in a Japanese population (22). Many subsequent studies followed, consistently finding a significant association between RA and the FCR3 −169 C variant in Asian populations (25, 26, 30, 31). In addition, associations have been reported between the FCR3 gene and several other autoimmune diseases (e.g., systemic lupus erythematosus, Grave’s disease, and autoimmune thyroid disease) (22, 27, 29, 31). Although this association tends to be weaker in individuals of European descent, the −169 C allele was found to be significantly associated with autoimmune diseases within subgroups of patients stratified according to a functional NFκB1 polymorphism or to SNPs in other autoimmune susceptibility genes (e.g., PTPN22 or HLA-DRB1) (32–34).

The critical role of Treg in preventing autoimmunity and the genetic studies linking autoimmune diseases with the −169CT FCR3 polymorphism highlight the importance of investigating the biology of FcRL3 Treg. Specifically, we sought to ascertain how the expression of FcRL3 on Treg relates to the phenotype, differentiation, and function of this important population of cells. We demonstrate that FcRL3 Treg represent a subpopulation of thymically derived nTreg and exhibit an exhausted phenotype typically seen on chronically stimulated T cells. In accord with this phenotype, we provide evidence that, compared with their FcRL3− counterparts, purified FcRL3+ Treg are relatively nonresponsive to antigenic stimulation in the presence of IL-2 in vitro and have a reduced capacity to suppress the proliferation of effector T cells.

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 Stem Cell Food 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 (Life Technologies, Rockville, MD) supplemented with 10% FBS (Gemini Bio-Products, Woodland, CA), 1% penicillin/ streptomycin (Mediatech, Washington, DC), and 2 mM L-glutamine (Mediatech) (hereafter referred to as R10 medium).

Human fetal tissues (mesentery, spleen, and thymus) from 18–23 gestational wk specimens were obtained from San Francisco General Hospital, under the auspices of a protocol approved by the University of California at San Francisco Committee on Human Research. Mesenteric lymph nodes (MLNs) were dissected from the fetal mesentery. Single-cell suspensions were prepared by passing physically disrupted tissue through a 40 μm cell strainer (BD Falcon, BD Biosciences, San Jose, CA). Thymic suspensions were washed in R10 medium and used directly. Splenic and MLN suspensions were first subjected to density centrifugation as above and washed in R10 medium before phenotypic analysis.

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 were added to cell-sorting mixtures in conjugation with anti-CD3 staining markers. 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-AL 700, anti-CD25 PE-Cy7, anti-CD25 APC-Cy7, anti-CCR7 PE-Cy7, anti-CD127 PE, anti–programmed cell death-1 (PD-1) APC, anti-CD28 APC, anti-CD31 FITC, anti-CD95/Fas APC, anti-CD26L FITC, anti-CD278 APC, anti-ICOS PE, anti-HLA-DR-DAPC (all from BD Biosciences), anti-CD278 APC, anti–PD-L1 PE-Cy7, anti-CD62L APC, and anti-CD8 PE-Cy5.5 (Catag 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).

For phenotyping, cells were incubated with the relevant Abs diluted in PBS 2% FBS for 30 min on ice, followed by three washes with PBS 2% FBS. Secondary staining for FcRL3 detection with streptavidin-Qdot655 was then performed, followed by an additional three washes in PBS 2% FBS. Cells were then fixed in 1% paraformaldehyde (Sigma-Aldrich) for flow cytometric analysis. Intracellular detection of FoxP3 was performed using anti-FoxP3 Pacific Blue (clone PCH101); the accompanying staining kit was provided by eBioscience and was used in accordance with the manufacturer’s protocol. The samples were acquired on an LSR II flow cytometer (BD Biosciences), and all data were analyzed using FlowJo software (Tree Star, Ashland, OR). Doublet discrimination based on forward scatter height versus area was performed to eliminate cellular aggregates.

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′ nucleotide genotyping assay was used to specifically discriminate between the −169CT and −169TT 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 μM final concentration) (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).

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 density centrifugation. Following staining with Abs for detection of T cell subpopulations, sorting was performed on a BD Aria flow cytometer (BD Biosciences). A lymphocyte gate was set on the basis of forward scatter, and both dead cells and doublets were excluded. Live CD3+ CD4+CD8− T cells were gated and sorted based on expression of CD25, CD127, and FcRII to yield three populations: FcRL3− (CD25−CD127−FcRII−), FcRL3+ (CD25+CD127−FcRL3−), and conventional CD4+ T cells (CD25+CD127+FcRL3+).

In vitro induction of Treg

Ninety-six–well round-bottomed culture wells were precoated with 0.68 g/ml each OKT3, SP34-2, and Hii5a 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, Minneapolis, MN) were added at a concentration of 1 μg/ml and 50 IU/ml, respectively. Sorted conventional CD4+
T cells were seeded at 100,000 cells/well in the presence of 0, 5, 12.5, or 50 ng/ml recombinant human late TGF-β (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 Hit3α 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 a concentration of 25 IU/ml. Following 5 d of culture, cells were recovered 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 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, then fixed and permeabilized (Cytofix/Cytoperm, BD Biosciences) prior to staining for Ki67 (BD Biosciences) or Bcl2 (DakoCytonation, Carpinteria, CA) with an isotype control.

Stat-5 phosphorylation analysis

FACS-sorted T cell subpopulations were resuspended in 100 μl R10 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 and PhosFlow Perm III, BD Biosciences). The phosphorylation status of Stat-5 was assessed using an anti–phospho-STAT-5 (Y694) Ab coupled to Pacific Blue, according to the manufacturer’s instructions. Control fluorescence was analyzed using a Pacific Blue-coupled control IgG Ab. All reagents were purchased from BD Biosciences.

Treg suppression assays

For stimulation, 96-well round-bottomed culture wells were precoated with 0.083 μg/ml each OKT3 (eBioscience), SP34-2, and Hit3α (BD Biosciences) clones of anti-CD3 Abs (for a total of 0.25 μg/ml anti-CD3) in PBS for 5 h at 37°C and then washed to remove excess Ab. To obtain responder cells, PBMCs were depleted of Treg using MACS anti-CD25 Ab-conjugated microbeads (Miltenyi Biotec) followed by magnetic column removal of the CD25-expressing cells, according to the manufacturer’s protocol. CD25-depleted PBMCs were CFSE labeled by incubation in 1 μM CFSE (Invitrogen), diluted in PBS at a concentration of 10^5 cells/ml for 5 min at 37°C, washed three times with R10 medium, and then seeded in anti-CD3–coated wells at a density of 10^5 cells/well. Autologous FACS-sorted T cell subpopulations were added back at sorted cell/responder ratios varying from 1:5 (20,000 cells) to 1:25 (4,000 cells), volumes were normalized to 200 μl/well with R10 medium, and cocultures were incubated for 5 d. After this time, cells were recovered, stained with a live/dead marker, and Abs targeted to CD3, CD4, and CD8 for flow cytometric analysis. CFSE dilution (i.e., proliferation) of responder T cells was measured on live CD3+CD8+CD4+ T cells. The frequency of CFSElo responder cells included all cells that had undergone at least one division.

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− Treg statistically significant differences are indicated on the figures with asterisks. Results were considered significant at p < 0.05 (*), very significant at p < 0.01 (***), and extremely significant at p < 0.001 (****).

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. 1Bi). CD4+CD8− T cells were subsequently gated on either CD25hiCD127lo or CD25loCD127hi (Fig. 1Bii) and then further segregated according to FoxP3 expression to document subpopulations that were either CD25hiCD127loFoxP3+ (Treg) (Fig. 1Biii) or CD25hiCD127loFoxP3− (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 FcRL3hi and FcRL3lo populations. We conclude that the autoimmune susceptibility gene FcRL3 is expressed at the protein level on the surface of a subpopulation of CD4+ Treg.

Variability in Treg FcRL3 expression is associated with the −169/C FCRL3 polymorphism

Given evidence that a C/T SNP in the promoter region of FCRL3 (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

FIGURE 1. FcRL3 is expressed on a subpopulation of Treg A, CD4+ CD25hi Treg (R) or CD4+CD25loCD45RA− naive (N) T cells from three adult donors were FACS sorted, and cDNA was analyzed for expression levels of FcRL3 and FoxP3 transcripts. Expression is shown relative to transcript levels of HPRT. B, Flow cytometry gating on live CD3+ adult T cells to determine FcRL3 expression on CD4+ Treg and conventional CD4+ T cells (Tconv). Treg were defined by the combination of CD4+CD8− (i), CD25hiCD127lo (ii), and FoxP3+ (iii) gates. Tconv were defined by the opposite gates (i.e., CD25loCD127hi in ii and FoxP3− in iv). C, Expression levels of FcRL3 (black line) versus control staining (shaded histogram) on Treg or Tconv from three donors. The three donors in A do not correspond to the three donors in C.
fluorescence intensity (MFI) of FcRL3 within Treg (Fig. 2) were measured by flow cytometry as in Fig. 1 in 29 healthy donors and the percentage of FcRL3+ Treg 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 demonstrate 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) thymocytes for FcRL3 expression (Fig. 4A) and found that the expression of FcRL3 was restricted to the Treg thymocyte population (Fig. 4Ai) with little or no expression on SP4 thymocytes (Fig. 4Aii). To determine whether FcRL3+ Treg constitute 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 cultured 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 the presence of αCD3/CD28 in the absence of IL-2.
medium alone or in the presence of IL-2, whereas αCD3/CD28 activation resulted in blast formation in T_{conv} but not in either T_{reg} subpopulation. The addition of IL-2 to αCD3/CD28 stimulation (far right column of Supplemental Fig. 4) induced blast formation in T_{conv} and FcRL3^{+} T_{reg} but to a lesser degree in FcRL3^{+} T_{reg}, which exhibited a reduced cell size compared with FcRL3^{+} T_{reg} 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 T_{conv} were Ki67^{+} after IL-2 stimulation. αCD3/CD28 activation resulted in Ki67 expression in nearly all of T_{conv} (83%), whereas less than 15% of the T_{reg} 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^{+} T_{reg} (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^{+} T_{reg} versus FcRL3^{+} T_{reg} in response to αCD3/CD28 plus IL-2 is significant (p < 0.05), confirming the hypothesis that FcRL3^{+} T_{reg} are relatively defective in their proliferative responses.

**FcRL3^{+} and FcRL3^{−} T_{reg} demonstrate intact proximal and distal signaling responses to IL-2**

To ascertain whether FcRL3^{+} T_{reg} 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^{+} T_{reg}, FcRL3^{−} T_{reg}, and T_{conv} 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 in T_{conv} and FcRL3^{+} T_{reg} as shown in Fig. 6B. The hypoproliferation of FcRL3^{+} T_{reg} versus FcRL3^{+} T_{reg} in response to αCD3/CD28 plus IL-2 is significant (p < 0.05), confirming the hypothesis that FcRL3^{+} T_{reg} are relatively defective in their proliferative responses.

**FcRL3^{+} and FcRL3^{−} T_{reg} are phenotypically distinct from FcRL3^{−} T_{reg}**

PBMCs from healthy donors were phenotyped by flow cytometry to determine the expression levels of the indicated markers on FcRL3^{+} and FcRL3^{−} T_{reg}. A. Representative histograms from individual donors showing expression of the indicated markers on FcRL3^{+} T_{reg}, FcRL3^{−} T_{reg}, or T_{conv}. 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) T_{reg}. 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/ Fox (middle row), and CCR7 versus CD95/Fox (bottom row) for FcRL3^{+} T_{reg}, FcRL3^{−} T_{reg}, and T_{conv}.
in complete inhibition of Teff proliferation by both Treg populations added to CFSE-labeled autologous responders in a coculture of Tconv had no effect.

Given that FcRL3+ Treg exhibit an exhausted phenotype with high levels of PD-1 (Fig. 3) and defective proliferative responses (Fig. 5) compared with FcRL3− Treg, we postulated that they would be dysfunctional in their regulatory capacity, i.e., the ability to suppress effector T cell proliferation in vitro relative to their FcRL3− counterparts. Given the many reports linking expression of FcRL3 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.

Distal signaling responses to IL-2.

FcRL3+ Treg are dysfunctional in their ability to suppress effector T cell proliferation

Given that FcRL3+ Treg exhibit an exhausted phenotype with high levels of PD-1 (Fig. 3) and defective proliferative responses (Fig. 5) compared with FcRL3− Treg, we postulated that they would be dysfunctional in their regulatory capacity, i.e., the ability to suppress the proliferation of effector T cells (Teff). To test this hypothesis, FACS-sorted FcRL3+ Treg, FcRL3− Treg, and Tconv were FACS sorted from adult PBMCs and cultured in the presence of R10 medium alone (Control), 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 the intracellular proliferation Ag, Ki67. A. Histogram overlays of Ki67 expression from one representative donor. B. Composite data from three separate experiments comprising four different donors showing the mean ± SEM of percentage of Ki67+ cells. Statistical significance comparing FcRL3+ and FcRL3− Treg was determined using paired Student t test; * indicates that differences were significant (p < 0.05).

To assay distal IL-2 signaling responses, FACS-sorted T cell subpopulations were stimulated under the same conditions as in Fig. 5 and then stained for Bcl2. Fig. 6B shows composite data from four different donors of the fold change in Bcl2 levels from day 0 to day 5. In response to IL-2, Bcl2 was upregulated on both FcRL3+ Treg and FcRL3− Treg (p < 0.01). Both Treg subpopulations also showed significant upregulation of Bcl2 in response to the combination of αCD3/CD28 plus IL-2 compared with αCD3/CD28 alone (p < 0.01 for FcRL3+ Treg, and p < 0.05 for FcRL3− Treg). There was, however, no significant difference in Bcl2 upregulation between FcRL3+ and FcRL3− Treg (p > 0.05). These data demonstrate that FcRL3+ Treg have intact proximal and distal IL-2 signaling responses that are comparable in magnitude to FcRL3− Treg.

Discussion

We report in this study that FcRL3 is expressed on a subpopulation of human CD4+CD25+FoxP3+ Tregs, 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 Y694 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.

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FIGURE 5. FcRL3+ Treg exhibit defective proliferation in response to αCD3/CD28 activation in the presence of IL-2. A, FcRL3+ Treg, FcRL3− Treg, and Tconv were FACS sorted from adult PBMCs and cultured in the presence of R10 medium alone (Control), 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 the intracellular proliferation Ag, Ki67. A, Histogram overlays of Ki67 expression from one representative donor. B, Composite data from three separate experiments comprising four different donors showing the mean ± SEM of percentage of Ki67+ cells. Statistical significance comparing FcRL3+ and FcRL3− Treg was determined using paired Student t test; * indicates that differences were significant (p < 0.05).

FIGURE 6. FcRL3+ and FcRL3− Treg demonstrate intact proximal and distal signaling responses to IL-2. A, 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.

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Dysfunctional suppressive ability of FcRL3+ Treg

These data are consistent with those reported by Nagata et al. (48), especially with respect to the proliferative defect of FcRL3+ Treg in response to antigenic stimulation in the presence of IL-2. We show in this paper, however, that FcRL3+ and FcRL3+ Treg exhibit differences in phenotype and function. Most importantly, and in contrast to the data from Nagata et al. (48), our study demonstrates that FcRL3+ Treg are dysfunctional and do not inhibit the proliferation of responder T cells to the same extent as do FcRL3+ Tconv. It is possible that this difference in results is reflective of the heterogeneity of FcRL3 expression on Treg 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 Treg 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+ CD25(lo)CD127(lo) T cells 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 modulate Treg function (2, 13–16). Indeed, it is becoming increasingly recognized within the Treg community that different in vitro suppression assays may measure different aspects of Treg cell-suppressor function (15). It is also noteworthy that we analyzed suppression exclusively on CD8+ Teff, whereas Nagata et al. (48) measured suppression on CD4+ Teff. Assessing suppression on CD4+ Teff can be problematic as, after 4 to 5 d proliferation, the diluted CFSE signal on CD4+ Treg can begin to overlap with the negative CFSE signal of unlabeled CD4+ Treg addback cells. Contamination of the CD4+ Teff CFSElo− cells with CD4+ Treg CFSE− cells and skewing of the data may therefore occur. We analyzed exclusively CD8+ Teff to avoid this potential problem, such that CFSE would only be measured on pure effector cells.

It is interesting to speculate why FcRL3+ Treg have diminished suppressor activity. Treg-mediated suppression may involve a number of mechanisms of action attributed to Treg cells. These can be broadly divided into those that target T cells (e.g., suppressor cytokines, IL-2 consumption, and cytolyis) and those that primarily target APCs (e.g., decreased costimulation or decreased Ag presentation). APCs may also modulate the activity of Treg 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+ and FcRL3+ Treg. Interestingly, we find that the FcRL3+ Treg population expresses higher levels of PD-1. PD-1 acts as an inhibitory molecule on Tconv and Treg after interacting with its ligands, PDL1 and PDL2, 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 PDL1/2 (40–42, 44, 45).

Other mechanisms may be at work in preventing FcRL3+ Treg from suppressing T eff expansion. FcRL3+ Treg also exhibit higher levels of the apoptosis-inducing TNF-R family member, CD95/ Fas, than FcRL3− Treg, 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+ Treg. It would be interesting to determine whether small interfering RNA knockdown of FcRL3+ Treg modulates the suppressor function of these cells, as it is presently unknown whether this functional defect is caused by, or merely associated with, FcRL3 expression.

**Purified FcRL3+ Treg exhibit defective proliferation**

Although FcRL3+ Treg demonstrate reduced suppressive ability in the presence of activated PBMCs, purified FcRL3+ Treg 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+ Treg 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+ Treg have a defect in their ability to respond to αCD3/CD28 activation. Consistent with this hypothesis, PD-1+ T cells are known to have reduced proliferative capacity and to possess shorter telomeres than PD-1− T cells (50, 51). Moreover, our analysis of six donors showed a trend for FcRL3+ Treg to express

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**FIGURE 7.** FcRL3+ Treg are dysfunctional in their ability to suppress Teff 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 Teff 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 (CFSElo) 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+ Treg and FcRL3− Treg by paired Student t test are marked with an asterisk (*).
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 FcR3 is expressed by a subpopulation of CD4+FoxP3+CD25+CD45RA− SP4 thymocytes, but not by FoxP3+ Treg induced from conventional T cells by TCR stimulation and TGF-β. These observations suggest that FcR3 may only be present on thymically derived nTreg and not on peripherally induced induced Treg. Despite their detection in the thymus, the FcR3+ Treg subpopulation exhibits a memory phenotype with reduced CD45RA and CCR7 expression, suggestive of more rapid memory conversion upon migration to the periphery than FcR3− 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 FcR3+ Treg may recognize self-Ag with greater specificity or affinity than their FcR3− counterparts. As has been observed in the case of PD-1 upregulation in the context of antigenic stimulation (40–42), chronic stimulation of FcR3+ Treg by autoantigens could result in the exhausted phenotype and the responses observed in this study.

**Perspectives: FcR3 as a contrasuppressor?**

Given the association between various autoimmune diseases and the −169 C/T SNP that leads to overexpression of FcR3, any discussion of FcR3 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 FcR3 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, FcR5, a closely related FcR family member, was shown to inhibit B cell activation via Src homology region 2 domain-containing phosphatase 1 tyrosine phosphatase recruitment (53). Another FcR member, FcR4, acts as a negative regulator of BCR signaling (54). FcR4 expression is associated with exhaustion in dysfunctional memory B cells and poor proliferation in response to B cell stimuli, consistent with high-level expression of multiple inhibitory receptors (55). We observe striking similarities in the phenotype and function of the FcR3+ Treg.

Given these findings, we hypothesize that FcR3 may negatively regulate TCR signaling in Treg. A recent publication supports this hypothesis with the demonstration that coligation of the FcR3 intracellular domain in a B cell line inhibited tyrosine phosphorylation and calcium mobilization mediated by BCR signaling (56). It is, however, difficult to reconcile high expression of a potential inhibitory molecule like FcR3 on B cells with predisposition to autoimmunity, particularly as it is low expression of inhibitory molecules such as FcγRIIB on B cells that increases the risk for loss of self-tolerance (57, 58). A role for FcR3 as an inhibitory coreceptor is more readily explained in the context of its link with autoimmune manifestations if it acts as a negative regulator of otherwise immunosuppressive T cell functions. Treg can have both beneficial and deleterious effects. Although Treg prevent autoimmune responses and excessive inflammation, they can also suppress useful immunity and thus, in turn, require regulation and brakes to restrict their effects. As Walter B. Cannon (59), the father of the concept of homeostasis, declared in 1932: "When a factor is known which can shift a homeostatic state in one direction, it is reasonable to look for a factor for factors having an opposing effect." As early as 1981, Gershon (60) adopted this concept in the arena of regulatory T cells, postulating that contrasuppressor T cells may control the activity of suppressor T cells. Additional precedents exist for negative regulators of Treg, such as PD-1 and TLR8-mediated inhibition of Treg function (40, 61). We accordingly postulate that FcR3 represents a novel mechanism of contrasuppression, which, under conditions of high expression induced by the −169 C/T SNP, contributes to greater inhibition of Treg function, higher levels of immune activation, and the loss of tolerance to self.

In sum, the results of this study demonstrate that Treg expressing the FcR3 autoimmune susceptibility gene exhibit an exhausted memory phenotype and aberrant suppressive function. Future elucidation of FcR3+ Treg differentiation, signaling, and function will likely expand upon our understanding of immune tolerance and homeostasis and may create opportunities for the development of new therapeutic interventions in disease settings.

**Acknowledgments**

We are grateful to Dr. Andy Polson and Genentech for kindly providing the anti-FcR3 Ab, without which this study would not have been possible. Helpful assistance in FACS sorting was provided by Bill Hyun and Terence Ho of the DEM flow core. We also thank members of the McCune laboratory for valuable comments and suggestions throughout the course of this work and for critical review of the manuscript.

**Disclosures**

The authors have no financial conflicts of interest.

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Supplemental Figure legends

Supplemental Figure 1. Purity of sorted populations of FcRL3$^+$ T$_{reg}$, FcRL3$^-$ T$_{reg}$ and T$_{conv}$
A. Pre-FACS-sorted sample showing CD25 and CD127 expression on live CD3$^+$CD4$^+$ T cells (left panel), then CD25 and FcRL3 expression on the CD25$^{hi}$CD127$^{lo}$ cells with either anti-FcRL3 antibody or control stain. B. Post-FACS-sorted FcRL3$^+$ T$_{reg}$, FcRL3$^-$ T$_{reg}$ and T$_{conv}$ populations showing CD25 and CD27 expression (upper panel), and CD25 and FcRL3 expression (lower panel). C. Unsorted samples were analyzed for FoxP3 expression, based on gates set to equivalent levels of CD25, CD127 and FcRL3 that were obtained in the sorted populations.

Supplemental Figure 2. Variability in T$_{reg}$ FcRL3 expression is not associated with variability in FoxP3 expression
FcRL3 and FoxP3 expression were analyzed by flow cytometry as in Figure 1 in 29 healthy donors. The FcRL3 MFI, FoxP3 MFI and % FcRL3$^+$ within T$_{reg}$ were determined, together with the frequency of T$_{reg}$ within total CD4$^+$ T cells, as defined by CD25$^{hi}$CD127$^{lo}$FoxP3$^+$. PBMC from the same donors were screened for the -169 C/T FCRL3 SNP genotype by a PCR allelic discrimination assay. In (A) and (B), plots show the relationship between T$_{reg}$ FoxP3 MFI and either T$_{reg}$ FcRL3 MFI (A) or % FcRL3$^+$ within T$_{reg}$ (B). Statistical analysis indicates that neither parameter was significantly correlated with T$_{reg}$ FoxP3 MFI ($P > 0.05$). r indicates the Spearman’s rank correlation coefficient. In (C) and (D), donors are grouped according to genotype and shown in the category scatter plots against the T$_{reg}$ FoxP3 MFI (C) or % T$_{reg}$ in CD4$^+$ T cells (D);
horizontal bars show the mean of each group. Differences between groups were not statistically significant as determined by unpaired Student’s t-test ($P > 0.05$).

*Supplemental Figure 3. FcRL3$^+$ T$_{reg}$ constitute a greater fraction of T$_{reg}$ in the thymus than in the periphery*

FcRL3 expression was analyzed by flow cytometry on fetal thymus and either mesenteric lymph node (MLN, $A$) or spleen ($B$) from paired donors. The % FcRL3$^+$ within FoxP3$^+$CD25$^{hi}$ T$_{reg}$ was determined for each tissue and donor.

*Supplemental Figure 4. FcRL3$^+$ T$_{reg}$ exhibit defective blast formation in response to $\alpha$CD3/CD28 activation in the presence of IL-2*

FcRL3$^+$ T$_{reg}$, FcRL3$^-$ T$_{reg}$, and T$_{conv}$ were FACS sorted from adult PBMC and cultured in the presence of R10 medium alone, IL-2, $\alpha$CD3/CD28 antibodies, or a combination of $\alpha$CD3/CD28 antibodies and IL-2. FSC/SSC profiles of subpopulations cultured for 5 days are shown from one representative donor.
Supplemental Figure 1

A

B

C

Supplemental Figure 1
Supplemental Figure 2

A

B

C

D

\[ P = n/s \]
\[ r = 0.057 \]

\[ P = n/s \]
\[ r = -0.025 \]
Supplemental Figure 3

A

B

Donor: Thymus MLN

% Fr-BL3+

Supplemental Figure 3