Cutting Edge: In the Absence of Regulatory T Cells, a Unique Th Cell Population Expands and Leads to a Loss of B Cell Anergy

Steven M. Leonardo, Jessica L. De Santis, Laurent P. Malherbe and Stephen B. Gauld

J Immunol 2012; 188:5223-5226; Prepublished online 27 April 2012;
doi: 10.4049/jimmunol.1103731
http://www.jimmunol.org/content/188/11/5223

Supplementary Material  http://www.jimmunol.org/content/suppl/2012/04/27/jimmunol.1103731.DC1
References  This article cites 20 articles, 9 of which you can access for free at:  http://www.jimmunol.org/content/188/11/5223.full#ref-list-1
Subscription  Information about subscribing to The Journal of Immunology is online at:  http://jimmunol.org/subscription
Permissions  Submit copyright permission requests at:  http://www.aai.org/About/Publications/JI/copyright.html
Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at:  http://jimmunol.org/alerts
The absence of regulatory T cells (Tregs) results in significant immune dysregulation that includes autoimmunity. The mechanism(s) by which Tregs suppress autoimmunity remains unclear. We have shown that B cell anergy, a major mechanism of B cell tolerance, is broken in the absence of Tregs. In this study, we identify a unique subpopulation of CD4+ Th cells that are highly supportive of Ab production and promote loss of B cell anergy. Notably, this novel T cell subset was shown to express the germinal center Ag GL7 and message for the B cell survival factor BAFF, yet failed to express markers of the follicular Th cell lineage. We propose that the absence of Tregs results in the expansion of a unique nonfollicular Th subset of helper CD4+ T cells that plays a pathogenic role in autoantibody production. *The Journal of Immunology, 2012, 188: 5223–5226.*

The absence of regulatory T cells (Tregs) results in immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome, a fatal disorder associated with significant autoimmunity (1, 2). Development of autoantibodies with widespread autoreactivity is a common feature of patients with IPEX syndrome (3). We recently showed that the absence of Tregs in mice leads to a loss of B cell anergy and the production of autoantibody (4). The mechanism(s) by which Tregs control B cell anergy remains unclear. Both B cell intrinsic and extrinsic factors influence B cell anergy, with Th cell-derived signals postulated to contribute to the latter (5–9). As the absence of Tregs is known to modulate the Th cell compartment, we investigated the role of Th cells in the loss of B cell anergy associated with the absence of Tregs.

In this report, we provide evidence that the absence of Tregs is associated with the expansion of a unique Th cell population that expresses the germinal center marker GL7. Although these unique cells express markers in common with the follicular Th (T\(\text{FH}\)) cell lineage, evidence suggests they are not T\(\text{FH}\) cells. Instead, these unique cells belong to an uncharacterized Th lineage, are associated with the loss of B cell anergy, and may play a key role in the pathogenicity associated with the absence of Tregs.

**Materials and Methods**

**Mice, cell suspensions, and cell isolation**

C57BL/6 (control), Foxp3\(^{\text{DTR}}\) (10), and Ars/A1 (11) mice were maintained in specific pathogen-free conditions at the Biological Resource Center of the Medical College of Wisconsin and used between 6 and 12 wk of age. All experiments were approved by the institutional animal care and use committee. Single-cell suspensions and B cell purification was performed as previously described (4). CD4+ Th cell populations were isolated using magnetic columns (Miltenyi) and flow sorting (FACSARia; BD Biosciences).

**In vivo cell depletion**

Tregs were depleted from Foxp3\(^{\text{DTR}}\) mice by intraperitoneal administration of 50\(\mu\)g/kg diphtheria toxin (DT) every other day for the time period noted. Control animals received PBS. DT administration resulted in a specific >95% reduction of Tregs in the spleen, lymph nodes, peripheral blood, and bone marrow (data not shown) and had no effect on Tregs in C57BL/6 mice (data not shown).

**Flow cytometry**

Cells were stained and analyzed as previously described (4). DAPI was used to remove dead cells from analysis, and doublets/cell aggregates were removed from analysis using forward scatter width. The following fluorescently conjugated Abs were used: mouse anti-CD4 (RM4-5), CD3ε (145.2C11), GL7 (all eBioscience), and CXCR5 (2G8) (BD Biosciences). Non-Th cells are defined as CD4+CXCR5\(^{\text{−}}\)GL7\(^{\text{−}}\) cells, germinal center T\(\text{FH}\) (GC-T\(\text{FH}\)) cells as CD4+CXCR5\(^{\text{+}}\)GL7\(^{\text{+}}\) cells, and our novel Th population as CD4+CXCR5\(^{\text{+}}\)GL7\(^{\text{+}}\) cells.

**RNA and quantitative RT-PCR**

mRNA was isolated from sorted cell populations using TRIzol (Invitrogen). cDNA was synthesized using QuantiTech Reverse Transcription kit (Qiagen). Quantitative PCR assays were performed using a StepOnePlus PCR system (Applied Biosystems) with TaqMan Fast Universal PCR Master Mix. TaqMan primer and probes for rodent GAPDH (Mm99999915_g1), IL-21 (Mm00444919_m1), Bcl6 (Mm00477633_m1), BAFF (Mm00446347_m1), and IL-4 (Mm00445259_m1) were obtained from Applied Biosystems.

Received for publication December 23, 2011. Accepted for publication April 4, 2012.

This work was supported by the Medical College of Wisconsin/Children’s Research Institute (to S.B.G.) and the Lupus Foundation of America Wisconsin Chapter (to S.B.G.).

Address correspondence and reprint requests to Dr. Stephen B. Gauld, Division of Allergy/Immunology, Department of Pediatrics, Medical College of Wisconsin, Children’s Research Institute, 8701 Watertown Plank Road, Milwaukee, WI 53226. E-mail address: sgauld@mcw.edu

The online version of this article contains supplemental material.

Abbreviations used in this article: DT, diphtheria toxin; GC-T\(\text{FH}\), germinal center follicular Th; IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked; T\(\text{FH}\), follicular Th; Treg, regulatory T cell.
In vitro TFH cell cocultures

Isolated T cell subsets were stimulated with plate-bound anti-CD3 (0.5 μg/ml) and anti-CD28 (2.5 μg/ml) at a cell density of 200 cells/μl in a 1:1 ratio with wild-type (C57BL/6) or anergic (Ars/A1) B cells. Supernatant was harvested after 7 d to determine Ab production. Total Ig concentrations were measured using ELISA plates (Corning Costar) coated with goat anti-mouse IgG (H+L). Standard curves for total Ig concentrations were created by serial dilution of purified mouse Ig. Bound Ab was detected using goat anti-mouse IgG (H+L)–HRP (Jackson ImmunoResearch) and TMB Single Solution substrate (Invitrogen). Anti-arsonate Ab titers were determined as previously described (4).

Fluorescence microscopy

Spleens were embedded in Tissue-Tec OCT compound (Sakura Finetek) and snap frozen. Four-micrometer sections were mounted onto microscope slides, air-dried, and fixed for 10 min in ice-cold acetone. Slides were washed in TBS–TWEEN and covered slides with Prolong Gold (Invitrogen) for 30 min at room temperature. Images were acquired using a Zeiss LSM510 laser scanning microscope with a ×20 objective. Colocalization analysis was performed using Carl Zeiss Axiovision software v4.8 (aqua represents the B220 signal, yellow GL7, and fuchsia for colocalized signal).

Statistical analysis

Statistical analysis of groups was performed using an unpaired Student t test (Prism version 5.0; GraphPad Software). Differences were considered significant at a p value <0.05.

Results and Discussion

Rapid expansion of CD4+CXCR5+GL7+ Th cells in the absence of Tregs

We previously reported that Foxp3-deficient mice exhibit an expanded population of GL7+ cells of non-B cell lineage (4). We now demonstrate that these cells are almost exclusively CD3+CD4+ T cells (data not shown). GL7 expression on T cells has been reported on in vitro-activated T cells and thymocyte subsets (13, 14), but the expression of GL7 on peripheral T cells is thought to be limited to GC-TFH cells (CD4+CXCR5+GL7+) (12). Using DT-treated Foxp3DTR mice, our studies indicated that the absence of Tregs resulted in the expansion of GC-TFH cells and a unique CD4+CXCR5+GL7+ T cell subset (Fig. 1A). This novel subset was present as early as 4 d after Treg depletion and continued to increase through day 12 of Treg depletion (Fig. 1A, 1B). Notably, the expansion of CD4+CXCR5+GL7+ cells occurs prior to the development of autoantibodies mediated by the depletion of autoantibodies in Foxp3DTR mice, a feature that instead requires 6–8 d (S. Leonardo and S. Gauld, submitted for publication).

To investigate further the surface phenotype of CD4+CXCR5+GL7+ cells, we examined the expression of PD-1 and ICOS, both markers expressed on TFH cells. Our

![FIGURE 1. Rapid expansion of CD4+CXCR5+GL7+ Th cells upon Treg elimination in adult mice.](http://www.jimmunol.org/Download)

A. Representative flow cytometric contour plots of CD4-gated splenocytes from PBS- (top) or DT- (bottom) treated Foxp3DTR mice. (B) Graphical analysis of the kinetics of CD4+CXCR5+GL7+ cell frequency and absolute number from PBS-treated (open circles) or DT-treated (closed circles) Foxp3DTR mice (each symbol represents a single mouse). Graphical analysis showing the fold increase in mean fluorescence intensity of PD-1 (C) and ICOS (D) relative to non-TFH cells from PBS-treated Foxp3DTR mice. Non-TFH cells are defined as CD4+CXCR5−GL7− cells. GC-TFH cells as CD4+CXCR5+GL7+ cells (12), and our novel Th population as CD4+CXCR5+GL7+ cells. Results compiled from a minimum of four mice per group and representative of at least two independent experiments from Foxp3DTR mice treated with PBS or DT for 12 d. *p < 0.05, **p < 0.005, ***p < 0.001.
data showed that whereas GC-TFH cells from both Treg-sufficient and Treg-deficient Foxp3DTR mice expressed high levels of PD-1, CD4+CXCR5−GL7+ cells expressed low levels of PD-1 (Fig. 1C). However, CD4+CXCR5−GL7+ cells from both Treg-sufficient and -deficient Foxp3DTR mice expressed increased levels of ICOS relative to non-TFH cells, with levels being comparable to those of GC-TFH cells (Fig. 1D). We also observed that ICOS levels were highly elevated on all Th cell subsets analyzed in Treg-deficient mice (Fig. 1D).

CD4+CXCR5−GL7+ cells fail to express Bel6

TFH cell differentiation is dependent on expression of Bel6 (15–17), so we investigated whether CD4+CXCR5−GL7+ cells isolated from Treg-deficient mice by cell sorting did not express Bel6 or IL-21, a cytokine highly expressed by TFH cells (Fig. 2A, 2B). The numbers of CD4+CXCR5−GL7+ cells were too small to allow for isolation by cell sorting in Treg-sufficient mice. The lack of expression of Bel6, IL-21, CXCR5, and PD-1 expression suggested that CD4+CXCR5−GL7+ cells did not belong to the TFH lineage. CD4+CXCR5−GL7+ cells expressed message for IL-4 (Fig. 2C), a cytokine produced at high levels by GC-TFH cells (12). However, all T cell subsets analyzed from Treg-deficient mice expressed high levels of IL-4 message (Fig. 2C). Our studies also indicated that TFH cells from Treg-deficient mice fail to promote loss of B cell anergy, whereas GC-TFH cells from only Treg-deficient mice support a loss of B cell anergy (S. Leonardo and S. Gauld, submitted for publication). In contrast, CD4+CXCR5−GL7+ cells from Treg-deficient mice induced the secretion of Ig from both wild-type and Ars/A1 B cells (Fig. 3A, 3B).

CD4+CXCR5−GL7+ cells are sufficient to drive loss of B cell anergy

We have previously shown that the absence of Tregs results in the loss of B cell anergy. To examine if CD4+CXCR5−GL7+ cells can lead to a loss of B cell anergy, we cocultured wild-type B cells or anergic B cells from Ars/A1 mice (11) with non-TFH cells from either Treg-sufficient or -deficient mice failed to induce substantial Ig secretion by wild-type B cells (Fig. 3A), nor did we see any Ab secretion by Ars/A1 B cells that would indicate a loss of B cell anergy (Fig. 3B). In a separate line of investigation, we have also identified that TFH cells from Treg-sufficient-or-deficient mice fail to promote loss of B cell anergy, whereas GC-TFH cells from only Treg-deficient mice support a loss of B cell anergy (S. Leonardo and S. Gauld, submitted for publication). In contrast, CD4+CXCR5−GL7+ cells from Treg-deficient mice can drive loss of B cell anergy.

To our knowledge, this is the first demonstration that a unique population of CD4+ T cells (CD4+CXCR5−GL7+) is expanded in Treg-deficient mice, and that this novel T cell population can directly lead to a loss of B cell anergy. On the basis of recent literature showing “aged” TFH cells lose Bcl6 while maintaining CXCR5 (18), our CD4+CXCR5−GL7+ cells likely do not represent an accumulation of Bcl6lo TFH cells because our population are CXCR5+.

FIGURE 2. CD4+CXCR5−GL7+ cells isolated from Treg-deficient mice do not represent a subset of TFH cells. mRNA expression of non-TFH (CD4+CXCR5−GL7−), TFH (CD4+CXCR5+GL7+), GC-TFH (CD4+CXCR5−GL7+), and CD4+CXCR5−GL7+ cells from Foxp3DTR mice for the following genes: Bcl6 (A), IL-21 (B), IL-4 (C), and BAFF (D) for 7 d. Expression levels were normalized to GAPDH message levels. Results compiled from three independent experiments with each experiment using cohorts of three mice per group. *p < 0.05, **p < 0.005.

FIGURE 3. CD4+CXCR5−GL7− cells are sufficient to promote the loss of B cell anergy. ELISA assay to determine total Ig production from wild-type B cells (A) or anti-arsonate production from Ars/A1 B cells (B) cocultured with non-TFH (CD4+CXCR5−GL7−) cells or CD4+CXCR5−GL7+ cells from Foxp3DTR mice treated with PBS or DT for 7 d. Data are representative of a minimum of four experimental replicates generated from two independent experiments. **p < 0.005.
Treg-deficient mice is due to a developmental block in the T_{FH} lineage but instead represents a distinct T cell lineage. The rapid expansion of CD4^+CXCR5^+GL7^+ cells in the absence of Tregs (4 d after Treg depletion) suggests CD4^+CXCR5^+GL7^+ precursors remain under tight homeostatic control by Tregs. To the best of our knowledge, the expansion of CD4^+CXCR5^+GL7^+ cells is specific to the loss of Tregs, and we have not observed this population in models of autoimmunity nor after immunization or viral infection (data not shown).

The mechanism by which CD4^+CXCR5^+GL7^+ cells support a loss of B cell anergy remains unclear. IL-4 and BAFF represent candidates as they may enhance autoantibody production (6, 19, 20) and are both highly expressed, albeit at the message level, by CD4^+CXCR5^+GL7^+ cells. However, our studies also showed that BAFF and IL-4 are highly expressed by non-T_{FH} and conventional T_{FH} cells from Treg-deficient mice and T_{FH} cells from Treg-sufficient mice. Because non-T_{FH} cells from Treg-deficient mice were not shown to support a loss of B cell anergy (Fig. 3), it would suggest that these factors, alone, are not sufficient to drive loss of anergy. Finally, the lack of CXCR5 expression may represent an anatomical advantage for our novel T_{FH} subset to interact with anergic B cells because anergic B cells are known to undergo follicular exclusion (21). This may contribute to the pathogenic potential of CD4^+CXCR5^+GL7^+ cells as mediators of autoantibody production in vivo. Indeed, our studies indicated that the absence of Tregs in Foxp3^DTR mice resulted in a moderate loss of B cell zone structures, with a more prominent loss of T cell enriched areas. However, this was less severe than previously reported for Foxp3^DTR mice (4) (Supplemental Fig. 1A). This defect was associated with the presence of GL7-expressing cells (non-B cells) outside of remaining B cell zones (Supplemental Fig. 1B). Confirmation that GL7-expressing cells are either of B cell or T cell origin was determined by flow cytometry (Supplemental Fig. 1C). Together, these data are supportive of the presence of a GL7^+ T cell population that lacks CXCR5 expression.

In summary, our studies suggest that the absence of Tregs drives the expansion of a novel T_{FH} subset that supports the loss of B cell anergy.

Acknowledgments
We thank Dr. Alexander Rudensky for providing us with his Foxp3^DTR mouse model. We also thank the Imaging Core of the Children’s Research Institute, Milwaukee, particularly Christine Naughton and Suresh Kumar.

Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Fig. 1: Altered splenic B and T cell zones and expression of GL7 outside of B cell zones in the absence of Tregs.

(A) Foxp3DTR mice were treated every other day with PBS or DT. At day 12, spleens were harvested and stained with antibodies specific for B220 (red) or CD3 (green). Data is representative of 2 mice per group. (B) Spleen sections from Foxp3DTR mice treated with DT for 12 days were stained with antibodies specific for B220 (green) and GL7 (red) (left panel). B220 and GL7 signals were analyzed to estimate co-localization of signals (right panel). Aqua represents the B220 signal, yellow the GL7 signal and fucia for co-localized signals. Data shown is representative from 2 independent mice. (B) Representative flow cytometric contour plots of splenocytes from PBS (top) or DT (bottom) treated Foxp3DTR mice stained with Thy1.2, B220 and GL7 antibodies. Following a live cell gate, the expression of B220 was determined on Thy1.2-GL7+ cells.