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Arthritogenic Self- Reactive CD4+ T Cells Acquire an FR4hi CD73hi Anergic State in the Presence of Foxp3+ Regulatory T Cells

Ryan J. Martinez,*† Na Zhang,**† Stephanie R. Thomas,**† Sarada L. Nandiwada,**† Marc K. Jenkins,‡† Bryce A. Binstadt,‡§ and Daniel L. Mueller*,‡†

Rheumatoid arthritis develops in association with a defect in peripheral CD4+ T cell homeostasis. T cell lymphopenia has also been shown to be a barrier to CD4+ T cell clonal anergy induction. We therefore explored the relationship between clonal anergy induction and the avoidance of autoimmune arthritis by tracking the fate of glucose-6-phosphate isomerase (GPI)-reactive CD4+ T cells in the setting of selective T cell lymphopenia. CD4+ T cell recognition of self-GPI peptide/MHC class II complexes in normal murine hosts did not lead to arthritis and instead caused those T cells to develop a Folate receptor 4 hiCD73 hi anergic phenotype. In contrast, hosts selectively depleted of polyclonal Foxp3+CD4+ regulatory T cells could not make GPI-specific CD4+ T cells anergic and failed to control arthritis. This suggests that autoimmune arthritis develops in the setting of lymphopenia when Foxp3+CD4+ regulatory T cells are insufficient to functionally inactivate all autoreactive CD4+ T cells that encounter self-Ag. The Journal of Immunology, 2012, 188: 170–181.

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We previously demonstrated that following a partial reconstitution of the CD4+ T cell compartment in lymphopenic hosts, CD25Foxp3+CD4+ regulatory T cells play an important role in promoting Ag-specific tolerance within CD4+ T cells through the induction of clonal anergy. In the absence of infection or adjuvant, naive CD4+ T cells recognizing an experimental Ag for the first time lost their capacity to produce IL-2 when regulatory T cells were present, whereas anergy could not be induced in the absence of regulatory T cells (9). To further explore the relationships among the homeostasis of the CD4+ regulatory T cell compartment, the induction of clonal anergy to an arthritogenic self-Ag, and the avoidance of autoimmune arthritis, we have taken advantage of GPI-specific KRN TCR-transgenic (Tg) CD4+ T cells and their adoptive transfer into either wild-type (WT) or lymphopenic hosts to probe the development of autoimmunity because they lack the polyclonal CD25Foxp3+CD4+ regulatory T cells necessary to induce anergy in pathogenic CD4+ T cells that recognize self-Ag for the first time in the peripheral immune system. Furthermore, the adoptive transfer of CD25Foxp3+CD4+ regulatory T cells into lymphopenic hosts restores high-level expression of two novel anergy markers Folate receptor 4 (FR4) and CD73 on the autoreactive Foxp3+CD4+ T cells, reduces the potential for autoreactive CD4+ T cells to undergo clonal expansion and effector cell differentiation, and limits the capacity of these autoreactive T cells to break natural immunological tolerance within the GPI-reactive B cell repertoire.

Materials and Methods

Mice
B6.g7 (H-2b congenic) mice as well as B6 strain KRN mice that express a TCR transgenic specific for GPI-A$^b$ were gifts from Drs. Diane Mathis and Christophe Benoist (Harvard Medical School, Boston, MA) and the Institut de Genetique et de Biologie Moleculaire et Cellulaire (Strasbourg, France) (20). B6 mice were purchased from Charles River Breeding Laboratories under a contract from the National Cancer Institute (Frederick, MD). B6 TCRα−/− (B6.129S7- TCRαtm1Mom), Rag1−/− (B6.129S7- Rag1tm1Mom), and CD45.1+ KRN spleen and lymph node cells were enriched for naive CD4+ T cells by negative selection using biotin-conjugated reagents allophycocyanin-eFluor780–conjugated anti-CD4 (RM4-5), anti–CD45.1+ (A20; Becton Dickinson, San Jose, CA), and then KRN T cells were reisolated by positive selection with Foxp3 Fixation/Permeabilization Concentrate and Diluent (eBioscience), and then KRN T cells were reisolated by positive selection using the CD4+ T cell isolation kit and an LS column (Miltenyi Biotec, Auburn, CA).

Flow cytometric analysis
For the analysis of CD4+ T cells in adoptive transfer recipient spleen, CD45.1 mouse B cells were stained with PE-conjugated Ab to CD45.1 (B6.129S7-CD45.1m1, BD Biosciences), and lymph node cells were stained with fluorescein-labeled anti-CD4 and anti–CD45.1+ (A20; Becton Dickinson, San Jose, CA).

In vivo adoptive transfer and NK cell depletion
NK cells were depleted from WT and TCRα−/− B6 × B6.g7 recipients of B6 strain KRN T cells using anti-asialo GM1 Ab (Wako Chemicals USA, Richmond, VA) at a dose of 25 μg/mouse i.v. injection on days −1, 4, 9, and 14 relative to the day of KRN CD4+ T cell adoptive transfer. Donor CD45.1+ KRN spleen and lymph node cells were enriched for naive CD4+ CD25+ CD4+ T cells by negative selection using biotin-conjugated anti-CD25 (PC61.5) and anti–CD44 (IM7) from eBioscience (San Diego, CA), together with a biotin-conjugated Ab mixture for CD4+ T cell isolation and an LS column (Miltenyi Biotec, Auburn, CA). Purified naive CD4+ T cells were adoptively transferred to recipients by tail vein injection of 5 × 106 cells, and animals were then treated with CFSE prior to adoptive transfer, and the average cell division rate for CFSE-labeled KRN CD4+ T cell populations was determined as previously described (21).

Regulatory T cell transfer and Foxp3+ T cell ablation
Spleens and lymph nodes from either WT or Foxp3Δ donor B6 × B6.g7 mice were harvested, and polyclonal CD4+ T cells were purified by negative selection using the CD4+ T cell isolation kit and an LS column (Miltenyi Biotec). To deplete Foxp3-expressing cells from reconstituting polyclonal Foxp3Δ B6 × B6.g7 T cell populations, DT (List Biological, Campbell, CA) was injected i.p. at a concentration of 50 μg/kg body weight on days 8, 10, and 12 after CD4+ T cell reconstitution (19). In some experiments, donor Foxp3Δ B6 × B6.g7 spleen and lymph node cells were stained with fluorochrome-labeled anti-CD4 and anti–CD25 Ab prior to physical cell sorting to purify CD25+ GPF CD4+ and CD25−GPF+ CD4+ populations using an FACSAria flow cytometer (BD Biosciences, San Jose, CA).

Reverse transcriptase real-time quantitative PCR
CD45.1+CD4+ KRN T cells were recovered from primary adoptive transfer hosts by a combination of CD45.1 MACS microbead positive selection and BD FACSAria (BD Biosciences) flow cytometric cell sorting. Purified KRN T cells were then lysed and homogenized using QiShredder and total RNA extracted using an RNeasy Micro or Mini Kit (Qiagen, New York, NY) and then assessed for gene expression using reverse transcription real-time quantitative PCR (qPCR). cDNA was produced using the qScript CDNA SuperMix (Quanta BioSciences, Gaithersburg, MD) and examined for gene expression using real-time quantitative PCR (qPCR). Applied Biosystems Real-Time PCR system [Life Technologies Corporation, Carlsbad, CA] or PerfeCTa SYBR Green FastMix (Quanta BioSciences). All qPCR primer sets (Supplemental Fig. 4) were designed to use identical qPCR settings.

H&E staining
For histological analysis, ankles and feet were dissected, frozen in OCT medium (Sakura Finetek, Torrance, CA), cryosectioned to a thickness of 10 μm, and stained with H&E.

Arthritis scoring
Ankle thickness was measured with a Quick-Mini Series 700 comparator (Mitutoyo, Aurora, IL) and reported as the percent change in ankle thickness from day 0. Arthritis severity was also assigned a score from 0–3 for each paw based on swelling and erythema, resulting in a maximum Arthritis Clinical Index score of 12 for each mouse (23).
Anti-GPI IgG1 Ab measurement
Serum was isolated from recipient mice on specified days and measured for anti-GPI IgG1 Abs by ELISA using recombinant mouse GPI together with IgG1-specific anti-mouse Ig reagents.

Statistical analysis
Mean Arthritis Clinical Index scores were compared using the Mann–Whitney U test. Other tests of significance shown represent the results of an unpaired one-tailed Student t test. R^2 values shown were calculated using log-transformed data.

Results
Induction of autoimmune arthritis in T cell-deficient hosts by an adoptive transfer of GPI-reactive KRN CD4+ T cells
To explore the role of the clonal anergy tolerance mechanism in protection from autoimmune disease, we made use of B6 KRN TCR-Tg mice for which CD4+ T cells recognize the self-peptide GPI282–294 bound to the MHC class II (MHC II) molecule I-A^d (20, 22, 24). KRN CD4+ T cells are known to cause autoimmune arthritis when transferred into I-A^d-expressing T cell-deficient hosts, whereas WT recipients remain free of disease (25). For our experiments, 10^4 naive GPI-specific B6 strain KRN CD4+ T cells were adoptively transferred into I-A^d-expressing WT or TCRα^−/− B6 × B6.g7 mice. B6 × B6.g7 recipient mice (or B6 controls) were then monitored for development of systemic inflammatory disease, including arthritis and weight loss.

In preliminary experiments, we determined that an adoptive transfer of 10^5 CD25^−CD44^loCD4+ T cells was sufficient to park ~10^3 naive T cells in recipient mice (21 and data not shown). This small number of naive GPI-reactive T cells caused no disease in either B6 or B6 × B6.g7 WT recipients, consistent with maintenance of peripheral self-tolerance to GPI-I-A^d complexes in the WT B6 × B6.g7 mice (Fig. IA–C). In contrast, T cell lymphopenic TCRα^−/− B6 × B6.g7 recipients of KRN CD4+ T cells developed swelling and erythema involving the proximal synovial joints of all four paws beginning on about day 8 after adoptive transfer and progressing to maximal disease by days 12–14 (Fig. IA–C and data not shown). Arthritis signs in these paws were accompanied by loss of articular cartilage, intense periarthritis and bone marrow immune cell infiltration (both mononuclear and polymorphonuclear), marrow fat cell replacement, and thinning/erosion of marginal cortical bone tissue (Supplemental Fig. 1 and data not shown). Beginning on day 7 after adoptive transfer, TCRα^−/− B6 × B6.g7 hosts also lost significant body weight (Fig. 1D). The development of disease in these lymphopenic mice was entirely dependent on the GPI-specific KRN Tg TCR, as 10^4 naive polyclonal B6 CD4+ T cells demonstrated no ability to promote arthritis in TCRα^−/− B6 × B6.g7 hosts (Fig. 1C). Furthermore, the recognition of GPI-I-A^d complexes by the KRN Tg TCR appeared to be sufficient for T cell activation and arthritis induction, as KRN T cells on the Rag1^−/− background that lacked endogenous TCR gene rearrangements also caused joint disease (albeit with slower kinetics; data not shown) when transferred into the T cell-lymphopenic mice.

Autoimmune arthritis develops in association with a breakdown in natural B cell tolerance to GPI
To assess autoreactivity to GPI in the B cell compartment during the induction of arthritis, serum samples were taken from animals at various times after the adoptive transfer and examined for the presence of anti-GPI Abs of the IgG1 isotype by ELISA. Interestingly, both normal WT and TCRα^−/− B6 × B6.g7 hosts began to produce some anti-GPI IgG1 by 8 d after the KRN CD4+ T cell adoptive transfer, whereas control B6 recipients produced none (Fig. 2A). Beyond day 8, TCRα^−/− B6 × B6.g7 hosts eventually accumulated much higher levels of anti-GPI IgG1 Ab compared with WT recipients. Note that the time at which anti-GPI IgG1 was first detected in lymphopenic hosts coincided with their onset of arthritis (Fig. 2A, 2B). In contrast, the anti-GPI IgG1 autoantibody response observed in WT B6 × B6.g7 hosts never appeared sufficient to elicit joint inflammation.

Autoimmune, anti-GPI IgG1 Ab-producing B cells were investigated by flow cytometry using biotinylated GPI molecules and streptavidin-conjugated allophycocyanin (Supplemental Fig. 2). A greatly expanded population of GPI-specific FSC^hiIgH^L^hiB220^lo CD38^+ GL7^+ IgG1-switched plasmablasts was observed at day 10...
ELISA for B6 (KRN CD4+ T cells develop an anergic phenotype during of arthritis in this model system, as previously described (22). animals per group). Mean disease activity scores in the same experiment. C, Polyclonal spleen and lymph node B cells either from normal naive mice (left panels) or from day 10 KRN T cell adoptive transfer recipient WT B6 × B6.g7 (middle panels) and TCRα−/− B6 × B6.g7 (right panels) mice stained for intracellular Ig H+L chain accumulation and GPI-binding capacity. Plots and tracings are representative of multiple mice (more than six animals per group).

FIGURE 2. KRN CD4+ T cells cause an increase in the amount of anti-GPI Ab and numbers of GPI-specific B plasmablasts in hosts expressing the I-A^d^ allele. A, Mean anti-GPI IgG1 titers ± SEM were determined by ELISA for B6 (●), WT B6 × B6.g7 (■), and TCRα−/− B6 × B6.g7 (□) animals. B, Mean disease activity scores in the same experiment. C, Polyclonal spleen and lymph node B cells either from normal naive mice (left panels) or from day 10 KRN T cell adoptive transfer recipient WT B6 × B6.g7 (middle panels) and TCRα−/− B6 × B6.g7 (right panels) mice stained for intracellular Ig H+L chain accumulation and GPI-binding capacity. Plots and tracings are representative of multiple mice (more than six animals per group).

in arthritic TCRα−/− B6 × B6.g7 hosts (Fig. 2C and data not shown). In contrast, only a modest number of GPI-specific plasmablasts appeared in WT B6 × B6.g7 recipients. I-A^d^−/− expressing mice that received no KRN CD4+ T cells, or animals lacking the I-A^d^ allele, had no detectable anti-GPI Ab or GPI-specific plasmablasts, further demonstrating that the GPI-specific CD4+ T cells and GPI-I-A^d^ complexes were important to the breakdown of immunological tolerance to GPI and the induction of arthritis in this model system, as previously described (22).

KRN CD4+ T cells develop an anergic phenotype during self-Ag recognition in the normal peripheral immune system

The failure of naive KRN CD4+ T cells to completely break tolerence to GPI in the B cell compartment of WT mice led us to hypothesize that T cell clonal anergy develops before these T cells can expand to a effector population size that is sufficient to support autoreactive B cell growth and differentiation. It has previously been shown that naive CD4+ T cells recognition of soluble peptide Ag in normal mice is suboptimal in the absence of infection or adjuvant and is limited by the development of clonal anergy (26–28). In contrast, anergy induction in lymphopenic hosts can be defective due to the absence of CD25Foxp3 regulatory T cells (9, 29). Therefore, we investigated naive CD45.1+ KRN CD4+ T cell clonal expansion and phenotypic change in WT and TCRα−/− B6 × B6.g7 hosts. For these experiments, lymph node and spleen cells were isolated from CD45.2+ recipient mice, and then the donor-derived CD45.1+ KRN CD4+ T cells were enriched by positive selection and analyzed by multiparameter flow cytometry (Supplemental Fig. 3).

Naive KRN CD4+ T cells were observed to proliferate to significantly higher numbers in TCRα−/− T cell lymphopenic hosts and maintained this difference from WT recipients throughout a slow clonal contraction phase that occurred beyond day 5 (Fig. 3A). This enhanced KRN T cell clonal expansion in the lymphopenic hosts appeared to relate at least in part to an increased rate of cell cycle progression, based on the degree of CFSE dye dilution observed for KRN T cells on day 3 of the response (data not shown). By day 10, the expanded KRN CD4+ T cell populations demonstrated an Ag-experienced phenotype (CD44+) in both hosts (Fig. 3B). However, in the TCRα−/− B6 × B6.g7 mice, KRN CD4+ T cells maintained higher forward scatter profile, suggesting that they were still responding to self-peptide/MHC II presentation with growth. Most GPI-reactive KRN CD4+ T cells in T cell lymphopenic hosts were also found to have dimmed their CCR7 expression, and some additionally lost CD27, consistent with intense and prolonged/repeated TCR stimulation and progressive effector cell differentiation (30). In contrast, KRN CD4+ T cells in WT hosts frequently dimmed their expression of CD27 yet maintained a high-level CCR7 expression. These GPI-specific KRN CD4+ T cells in tolerant hosts were also observed to express high levels of the FR4 and CD73 surface markers, whereas KRN CD4+ T cells in T cell lymphopenic hosts expressed either an intermediate (int) or low level of both FR4 and CD73, often in a bimodal fashion.

Coordinate expression of both FR4 and CD73 was previously shown to occur on CD4+ regulatory T cells (31, 32). However, KRN CD4+ T cells in WT tolerant hosts did not differentiate down a regulatory T cell pathway, as Foxp3, Il2ra, and Il10 mRNA expression levels remained very low in pure KRN T cells (Fig. 3C, 3D). Instead, a modest upregulation of Gata3 and Tbx21 gene expression was observed in these T cells, with Gata3 > Tbx21. Consistent with this, mRNA extracted from this population of KRN CD4+ T cells in healthy WT hosts also contained Il4 > Ifng mRNA. In contrast to these results, KRN CD4+ T effector cells in T cell-deficient hosts expressed Tbx21 > Gata3 and Ifng > Il4 mRNA as well as high levels of GzmB and Cxcr3. Thus, in addition to differences in clonal expansion, the KRN CD4+ T cells in normal WT hosts appeared to divert their differentiation to a distinct helper phenotype more reminiscent of Th2, whereas KRN T cells in TCRα−/− B6 × B6.g7 mice destined to become arthritic expressed genes in a more Th1-like pattern. Although Th17 differentiation and IL-17a production have been implicated in the development of GATA-dependent autoimmune arthritis in mice (33), we were unable to detect significant levels of either Rorc or Il17a mRNA at any time point in our adoptive transfer experiments, and only infrequent IL-17a–producing KRN CD4+ T cells were observed by flow cytometry (Fig. 3C and data not shown).

GPI autoantibody-dependent arthritis in mice has also been shown to depend on IL-4 production by CD4+ Th cells (34); therefore, the expression of Gata3 and Il4 may have been essential to the differentiation of KRN CD4+ T effector cells capable of interacting with tolerant GPI-specific B cells and inducing the production of anti-GPI autoantibody. In both WT and TCRα−/− B6 × B6.g7 hosts, the KRN CD4+ T cells expressed similar
FIGURE 3. KRN CD4+ T cell number, phenotype, and function following adoptive transfer into WT or TCRα−/− B6 × B6.g7 hosts. A, Mean ± SEM KRN CD4+ T cell number in combined spleen and lymph nodes from WT B6 × B6.g7 (■) and TCRα−/− B6 × B6.g7 (□) recipients. The open square at T = 0 represents historical postadoptive transfer cell count; asterisks indicate significant differences between groups (p < 0.05). B, KRN CD4+ T cell FSc, CD44, CCR7, CD27, FR4, and CD73 expression at day 10. Plots are representative of more than six animals per group. Purified KRN T cell mRNA expression levels on days 5 (C) and 10 (D) after adoptive transfer into either WT (red) or TCRα−/− (green) B6 × B6.g7 hosts. Freshly isolated naive KRN T cells are also shown as a control (blue). E, Purified KRN CD4+ T cells were exposed for 7 d to GPI/I-A^g7 in WT (shaded tracing) or TCRα−/− (open tracing) B6 × B6.g7 hosts, recovered, labeled with CFSE, and then examined for CFSE dye dilution 3 d after adoptive transfer into B6 × B6.g7 secondary hosts. Digits above the tracing indicate the number of times a cell has divided. F, Average cell division rate ± SEM (n = 7) for groups as indicated. The p values were based on a Student t test.

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amounts of Bcl6 and Maf mRNAs, two gene regulators known to be important to the differentiation of T follicular helper (Tfh) cells and the induction of Il21 gene transcription, as shown in Fig. 3C (35–37). Nevertheless, the KRN T cells in TCRα−/− B6 × B6g7 mice expressed more of the Cxcr4, Cxcr5, Cd40lg, and Icos mRNAs that are important to Tfh function (Fig. 3C, 3D).

Based on all of these results, we speculated that the proliferative arrest and subsequent failure of KRN CD4+ T cells to fully differentiate down Th1 or Tfh pathways in normal hosts was the result of clonal anergy induction during their encounter with self-Ag. Perhaps consistent with this hypothesis, KRN T cells recovered from healthy WT mice expressed higher amounts of Ctlab and Rnf128 (GRAIL) mRNA; nevertheless, two other genes thought to be important to the development or maintenance of clonal anergy (Cblb and Pdcd1) were actually expressed at higher levels in chronically activated KRN CD4+ T cells from arthritic TCRα−/− mice. To more formally test the question of whether KRN CD4+ T cells became anergic in normal hosts, T cells were recovered from either WT or TCRα−/− B6 × B6g7 mice, CFSE-labeled, and then examined for their capacity to divide following re-exposure to GPI/I-A\textsuperscript{a} labeled, and then examined for their capacity to divide following TCRα(35–37). Nevertheless, the KRN T cells in TCRα−/− B6 × B6g7 mice expressed more of the Cxcr4, Cxcr5, Cd40lg, and Icos mRNAs that are important to Tfh function (Fig. 3C, 3D).

FIGURE 4. Partial reconstitution of TCRα−/− B6 × B6g7 mice with polyclonal CD4+ T cells promotes clonal anergy induction and protects against autoimmune arthritis

The partial reconstitution of lymphopenic mice with Ag-nonspecific CD4+ T cells was previously shown to inhibit Ag-dependent CD4+ T cell clonal expansion in the absence of adjuvant or infection and to promote the development of clonal anergy (9). Therefore, we wished to test whether reconstitution of TCRα−/− B6 × B6g7 hosts with bulk polyclonal CD4+ T cells could prevent the exaggerated clonal expansion and differentiation of adoptively transferred naive GPI-reactive CD4+ T cells and protect the mice from autoimmune arthritis. TCRα−/− B6 × B6g7 hosts were initially given varying numbers of B6 × B6g7 CD4+ T cells, followed 10 d later by an adoptive transfer of 10\textsuperscript{5} naive KRN CD4+ T cells to elicit the onset of autoimmune arthritis. Lymphopenic hosts reconstituted with 2 × 10\textsuperscript{6} CD4+ T cells were found to be fully protected from arthritis induction; however, transfer of 2 × 10\textsuperscript{5} or 2 × 10\textsuperscript{6} polyclonal CD4+ T cells proved insufficient to prevent the development of at least a modest degree of arthritis in some animals (Fig. 4A, 4B). Additional study of reconstituted hosts showed that the KRN CD4+ T cell clonal expansion was inversely correlated with the number of polyclonal
CD4+ T cells transferred into these mice (Fig. 4C). FR4 and CD73 expression on the GPI-specific CD4+ T cells also increased in proportion to the number of polyclonal CD4+ T cells used in the reconstitution, suggesting that CD4+ T cells are sufficient to blunt the growth and differentiation of GPI-specific T cells and restore clonal anergy induction in TCRαβ B6 × B6.B7 mice (Fig. 4D). Consistent with this, KRN CD4+ T cells recovered from animals reconstituted at high CD4+ T cell number and then stimulated ex vivo with PMA plus ionomycin, less frequently produced IL-2, IFN-γ, and/or TNF-α (Fig. 4D and data not shown). Using a Boolean gating strategy as previously described (38), we also found that the KRN CD4+ T cells from TCRαβ B6 × B6.B7 hosts had a large number of triple cytokine producers, whereas KRN CD4+ T cells in partially reconstituted hosts had a larger percentage of cells that produced no cytokines (Fig. 4E). These experiments confirmed that the development of autoimmune arthritis can be associated with the differentiation of self-Ag–specific CD4+ T cells into cytokine triple producers. Furthermore, protection from arthritis could be achieved in T cell-deficient hosts through partial reconstitution of the CD4+ T cell polyclonal repertoire.

Reconstitution of a polyclonal CD25+Foxp3+CD4+ regulatory cell repertoire is associated with decreased KRN CD4+ T cell differentiation to an FR4+CD73+ phenotype

In a number of animal models, polyclonal CD25+CD4+ regulatory T cells have been shown to support the induction and maintenance of peripheral self-tolerance and prevent the development of autoimmune disease (8, 19, 39, 40). Our own experiments have also previously demonstrated a role for CD25+Foxp3+CD4+ regulatory T cells in the induction of clonal anergy in vivo (9). Consequently, we thought that the CD25+Foxp3+CD4+ regulatory T cells within the reconstituting polyclonal CD4+ T cell population might be most important for limiting the expansion and differentiation of FR4+CD73+ KRN effector CD4+ T cells and promoting anergy in the GPI-specific T cells.

The partial reconstitution of TCRαβ B6 × B6.B7 mice with an adoptive transfer of bulk polyclonal CD4+ T cells led to a restoration of the CD25+Foxp3+CD4+ regulatory T cell compartment and protection from arthritis (Figs. 4B, 5A, 5B). This reconstitution was not accompanied by any preferential differentiation of the KRN CD4+ T cells to a Foxp3+ regulatory T cell phenotype, despite their high-level FR4 and CD73 expression (Figs. 4D, 5A). Interestingly, the total number of KRN CD4+ T cells recovered from normal and reconstituted mice showed no correlation with the number of polyclonal CD25+Foxp3+CD4+ regulatory T cells present (Fig. 5C); however, the number of nontolerant/effector phenotype FR4+CD73− KRN CD4+ T cells was observed to correlate inversely with the number of CD25+Foxp3+CD4+ regulatory T cells generated (Fig. 5D). Thus, polyclonal CD4+ T cells in these reconstituted mice did not induce the KRN naive CD4+ T cells to take on a CD25+Foxp3+ regulatory T cell phenotype. Rather, CD25+Foxp3+CD4+ regulatory T cells within the polyclonal CD4+ T cell compartment appeared to suppress the expansion and differentiation of GPI-specific FR4+CD73− CD4+ effector T cells that cause arthritis.

**FIGURE 5.** Polyclonal CD25+Foxp3+CD4+ regulatory T cells inhibit the accumulation of FR4+CD73+ GPI-specific CD4+ effector T cells. A, Identification of endogenous (top panel) and KRN (bottom panel) CD25+Foxp3+CD4+ regulatory T cells following partial polyclonal CD4+ T cell reconstitution of TCRαβ B6 × B6.B7 mice. B, Absolute number of endogenous Foxp3+CD4+ regulatory T cells identified in partially reconstituted hosts. Correlation between endogenous Foxp3+ regulatory T cells and either total KRN CD4+ T cell number (C) or FR4+CD73− KRN CD4+ T cell number (D). In B, filled symbols represent animals developing at least one arthritic joint; open symbols are animals that remained free of disease. Bars indicate the mean cell count, and p values are as indicated using Student t test.
Purified CD25⁺Foxp3⁺CD4⁺ regulatory T cells protect T cell-lymphopenic animals from severe autoimmune arthritis

To explore the hypothesis that CD25⁺Foxp3⁺CD4⁺ regulatory T cells control the number, phenotype, and function of KRN CD4⁺ T cells in WT B6 × B6.g7 mice, we made use of B6 × B6.g7-strain Foxp3⁺DTR mice for which Foxp3⁺ T cells all express GFP together with a DT receptor (DTR). Foxp3⁺DTR mice were used to sort CD25⁺GFP⁺ and CD25⁺GFP⁻ polyclonal CD4⁺ T cells, and then the sorted populations (10⁶ cells) were transferred into TCRα⁻/⁻ B6 × B6.g7 animals (19). We observed that mice reconstituted with purified CD25⁺GFP⁺CD4⁺ T cells had significantly reduced clinical scores as well as preferential differentiation of the KRN CD4⁺ T cells to an FR4⁺CD73⁺ anergic phenotype (Fig. 6A, 6B). Furthermore, the absolute number of FR4⁺CD73⁺ effectors among the GPI-reactive CD4⁺ T cells was significantly reduced (Fig. 6C). In contrast, hosts that received CD25⁻GFP⁻ polyclonal CD4⁺ T cells behaved much like the T cell-lymphopenic controls.

Polyclonal CD25⁺Foxp3⁺CD4⁺ regulatory T cells are necessary to promote the induction of clonal anergy and achieve protection against the development of autoimmune arthritis

In a final series of experiments, we sought to formally test the hypothesis that polyclonal CD25⁺Foxp3⁺CD4⁺ regulatory T cells are necessary to allow for the induction of clonal anergy in self-Ag–specific naive CD4⁺ T cells, prevent the accumulation of FR4⁺CD73⁺ autoreactive effector T cells, and protect against arthritis development. Use of B6 × B6.g7-strain Foxp3⁺DTR mice allows for the specific ablation of Foxp3⁺ regulatory T cells from within the polyclonal CD4⁺ T cell compartment in vivo using DT. To carry out these experiments, TCRα⁻/⁻ B6 × B6.g7 hosts were reconstituted with 10⁶ bulk CD4⁺ T cells from B6 × B6.g7 Foxp3⁺DTR donor mice, with DT administered on days 8, 10, and 12 to deplete Foxp3⁺CD4⁺ regulatory T cells from the reconstituting population. To evaluate the effect on arthritis, naive KRN CD4⁺ T cells were adoptively transferred into the mice at day 10, and then mice were monitored throughout the remainder of the experiment for evidence of arthritis, with the final analysis of KRN CD4⁺ T cells performed at day 20.

Remarkably, nearly all TCRα⁻/⁻ B6 × B6.g7 mice reconstituted with polyclonal Foxp3⁺DTR CD4⁺ T cells, and subsequently treated with DT to eliminate Foxp3⁺expressing CD4⁺ regulatory T cells, developed an autoimmune arthritis with a severity similar to that seen in the nonreconstituted TCRα⁻/⁻ B6 × B6.g7 hosts (Fig. 7A). KRN CD4⁺ T cells in these regulatory T cell-depleted hosts also failed to develop the anergic FR4⁺CD73⁺ phenotype (Fig. 7B). Rather, DT-treated mice accumulated to high number the highly differentiated FR4⁺ and CD73⁺ subpopulation of KRN CD4⁺ T cells shown to be associated with autoimmune arthritis development (Fig. 7C). As expected, CD4⁺ T cell-reconstituted TCRα⁻/⁻ B6 × B6.g7 mice not given DT suppressed the clonal expansion of FR4⁺CD73⁻ CD4⁺ KRN T cells and instead drove these GPI-specific T cells into an anergic (FR4⁺CD73⁻) state, with the eventual result being development of the mildest signs of arthritis (Fig. 7A, 7B).

Flow cytometry confirmed the efficacy of the Foxp3⁺CD4⁺ T cell depletion (Fig. 7D). Note that we did observe some deaths (n = 3) in Foxp3⁺DTR CD4⁺ T cell-reconstituted and DT-treated hosts, presumably as a consequence of unregulated autoimmunity from within the remaining polyclonal CD4⁺ T cell repertoire (19). However, the worsening of arthritis in reconstituted and DT-treated mice cannot be simply explained by such a nonspecific loss of immunological tolerance, because reconstituted and DT-treated hosts that were not given KRN CD4⁺ T cells failed to show signs of arthritis. DT itself also had no capacity to directly enhance the responsiveness of the KRN CD4⁺ T cells to GPI, as exacerbation of arthritis with DT required the presence of the polyclonal Foxp3⁺DTRCD4⁺ T cells and did not occur in the normal B6 × B6.g7 hosts. Thus, the arthritis-inducing effects of DT in these experiments were tightly coupled to the ablation of polyclonal CD25⁺Foxp3⁺CD4⁺ regulatory T cells, the resultant inhibition of clonal anergy induction in the naive KRN CD4⁺ T cells, and the pathological outgrowth of the FR4⁺CD73⁻ subset of Ag-experienced, GPI-specific CD4⁺ T cells that cause autoimmune arthritis.
Discussion

These data provide important evidence that the clonal anergy peripheral self-tolerance mechanism can serve as the basis for protection against the development of severe autoimmune arthritis. We note that Singh et al. (41) previously showed a role for in vivo anergy (their adaptive tolerance) to modulate the induction of chronic arthritis by CD4+ T cells responding to a Tg self-Ag: membrane-targeted pigeon cytochrome c (PCC). Similar to our model system, their adoptive transfer of PCC-specific 5C.C7 CD4+ T cells into lymphopenic CD3ε2/2PCC Tg mice led to a deregulated clonal expansion response, the production of multiple autoantibodies (e.g., anti-dsDNA, -histone, and -GPI), and the development of a destructive chronic polyarthropathy, whereas adoptive transfer into normal Tg hosts led to only an abortive clonal expansion response and no disease. In contrast to our KRN CD4+ T cells responding to GPI/I-Ag7 complexes in lymphopenic TCRα2/2 mice (as reported in this study), their 5C.C7 T cells became desensitized to self-Ag in both the lymphopenic and T cell-replete hosts. Nevertheless, the peak 5C.C7 CD4+ T cell number was observed to be reduced by >10-fold in the normal Tg recipients because of a slowed rate of cell-cycle progression and an apparent decrease in survival time. Therefore, their studies as well as those of others suggest that in vivo anergy can be an effective barrier to autoimmune disease only if self-reactive CD4+ T cell numbers are held below a threshold frequency (28, 42). Likewise, it is now clear that in the absence of a normal peripheral T cell compartment, self-Ag-reactive CD4+ T cell numbers can rise above this threshold despite any adaptation to constant TCR signaling and can retain the capacity to cause immunopathology (41, 43).

In lymphopenic hosts, GPI-specific CD4+ T cells expanded and differentiated into two separate Ag-experienced subpopulations in association with arthritis development: one FR4intCD73int and one FR42CD732. Our preliminary experiments suggest that the FR42CD732 subpopulation in lymphopenic mice is a Tbx21 (T-bet)-expressing effector T cell population with a high capacity to proliferate and produce IL-2, TNF-α, and IFN-γ upon restimulation. The FR42CD732 population appears similar to CXCR5-expressing Tfh cells, has lost much of its proliferative capacity, and does not demonstrate the same commitment to an inflammatory cytokine production (R.J. Martinez, unpublished observations). IFN-γ has been shown unnecessary for the development of spontaneous arthritis in K/BxN mice; therefore, it seems unlikely that the inability of anergic KRN CD4+ T cells to produce...
IFN-γ is important to the avoidance of arthritis in our system (33). Nevertheless, loss of some other Tbx21-dependent effector T cell activity may contribute to protection from arthritis in the normal hosts. Of note, IL-4 production by GPI-reactive T cells has previously been implicated in the breakdown of B cell tolerance and production of anti-GPI IgG1 autoantibody in K/BxN mice (34). We, too, have observed an induction of Il4 mRNA synthesis in our system, even in anergic KRN CD4+ T cells responding to GPI/I-A^d complexes in normal mice. KRN CD4+ T cell production of IL-4 may indeed be important to the activation of previously tolerant GPI-specific B cells in both normal and lymphopenic hosts, but it clearly is not sufficient for arthritis development.

In our experimental system, a relatively modest yet significant amount of anti-GPI IgG1 autoantibody could be found even in the healthy tolerant mice that had been given 10^5 naive GPI-specific CD4+ T cells. Nevertheless, these animals demonstrated no signs of arthritis or weight loss. Perhaps the numbers of GPI-reactive T cells rose sufficiently quickly to allow for at least some collaborative interaction with the GPI-specific B cells that promote their growth and differentiation prior to T cell anergy induction. Alternatively, anergic KRN CD4+ T cells that survive in normal hosts may retain the capacity to promote anti-GPI B cell responses, but their low frequency does not threaten the continued maintenance of self-tolerance and avoidance of immunopathology.

For either case, we would predict that CD4+ T cell expression of Gata3, Maf, Bcl6, Il4, Il21, Cxcr5, Cd40lg, and Icos is important to this B cell helper activity. Our previous investigation of chicken OVA-dependent immunity indicated that OVA-specific CD4+ T cells induced into clonal anergy in vivo retain the capacity to stimulate anti-OVA IgG2a class switching and Ab production, despite their continued inability to produce IL-2 (28). Unlike the results shown in this study for KRN CD4+ T cells induced into anergy in normal B6 × B6.g7 recipients, anergic OVA-specific Th1-like T cells never effectively reduced their IFN-γ production and also remained capable of responding in a delayed-type hypersensitivity assay. Nonetheless, the continuous recognition of GPI/I-A^d complexes in normal hosts (and in the absence of infection or adjuvant) may not be sufficient to induce the differentiation of KRN CD4+ T cells to an IFN-γ-producing effector T cell phenotype, even though it was sufficient to induce the expression of NFs thought important to differentiation down Th2 and Th1 pathways. Perhaps the low-frequency anergic GPI-specific CD4+ T cell population that remains in normal mice retains some capacity to stimulate anti-GPI B cells to undergo clonal expansion and IgG1 class switching; however, arthritis development requires additional effector T cell differentiation events that either fail to occur or are disrupted by the development of clonal anergy.

We show in this study that KRN CD4+ T cell proliferative responsiveness to continuous GPI/I-A^d complex presentation eventually wanes in association with an upregulation of FR4 and CD73. Rechallenge experiments confirmed that these T cells underwent a proliferative arrest and lost (or failed to gain) much of their capacity to produce IL-2, TNF-α, and IFN-γ. Therefore, high-level coexpression of FR4 and CD73 (in the absence of Foxp3) appears to mark autoreactive CD4+ T cells that have entered an anergic state. FR4 and CD73 have previously been shown to be highly expressed on Foxp3^+CD4+ regulatory T cells, and we also confirmed this in our own study (data not shown) (31, 32, 44). CD73 is an ecto-5'-nucleotidase (encoded by Nts5e) responsible for extracellular conversion of 5'-AMP to adenosine. CD73 is thought to catalyze adenosine receptor- and cAMP-mediated antiproliferative effects at sites of intense cell injury or ischemia, where adenosine nucleotide release into the extracellular space is prevalent (44, 45). Interestingly, CD73 ecto-5'-nucleotidase enzyme activity mediates at least part of the antiarthritic effects of methotrexate and sulfasalazine (46–48).

The pharmacologic effects of methotrexate also relate to folate metabolism. Through its inhibition of dihydrofolate reductase, methotrexate blocks the regeneration of tetrahydrofolate cofactors responsible for the synthesis of DNA and other purines important to T cell proliferation and survival (48, 49). FR4 mediates the transport of folic acid into T cells, and it has been suggested that metabolically active regulatory T cells are highly dependent on this vitamin to maintain cellular health (32). Although experiments shown in this study do not test the role of either molecule in the development or maintenance of CD4+ T cell anergy, the up-regulation of both FR4 and CD73 reinforces the notion that the continued survival of anergic CD4+ T cells in a nonproliferative state is actively reinforced. Whether FR4 and CD73 expression also provide these anergic GPI-reactive Foxp3+ CD4+ T cells with some suppressive or regulatory capacity in vivo remains unknown.

The mechanisms by which polyclonal Foxp3^+CD25^+ CD4+ regulatory T cells might control the clonal expansion of GPI/MHC II-specific CD4+ T cells and promote their acquisition of an FR4^hi CD73^hi anergic phenotype remain unclear. It is formally possible that GPI/MHC II-induced effector T cell differentiation and/or survival are inhibited by regulatory T cells, and only the least responsive (FR4^hiCD73^hi) KRN T cells are allowed to persist in the normal hosts. This seems unlikely, because we also observed that by day 3 after naive KRN CD4+ T cell adoptive transfer, clonal expansion and survival were similar in the WT and TCRα^-/- B6 × B6.g7 hosts, yet ~50% of the GPI/MHC II-specific T cells in the WT mice (versus ~5% in the lymphopenic recipients) had already developed an FR4^hiCD73^hi anergic phenotype (data not shown). Therefore, we favor the model that Foxp3^+ regulatory T cells drive self-reactive CD4+ T cells into an anergic state that eventually aborts their clonal expansion. Consistent with this notion, CD25^+CD4+ regulatory T cells have been shown to induce anergy in CD25^- CD4+ T cells undergoing 4 to 5 d of in vitro stimulation with CD3 mAb and allopurinol (50). Interestingly, IL-10 and TGF-β secretion by these CD25+ regulatory T cells was unnecessary for in vitro anergy induction. Nevertheless, a number of other molecules expressed by Foxp3^+ regulatory T cells (e.g., CTLA4, CD73, CD39, LAG3, and CD25) have been implicated in the control of CD4+ T cell proliferation and resistance to autoimmune disease and may have acted in this study to promote in vivo clonal anergy either through direct effects on the KRN T cells or via counterregulation of APC costimulatory molecule expression (51).

What might be the relevance of clonal anergy induction to patients with RA? The development of RA has previously been shown to be associated with a defect in peripheral T cell homeostasis (2). The exaggerated T cell homeostatic proliferative rate observed in RA patients is reminiscent of NOD mice, in which the development of insulitis and pancreatic islet cell destruction is associated with mild T cell lymphopenia, enhanced T cell homeostatic proliferation, and increased T cell turnover rate (7). Young K/BxN mice also demonstrate reduced peripheral CD4+ T cell numbers prior to their onset of disease, and an infusion of syngeneic polyclonal CD4+ T cells reduces their drive for homeostatic T cell proliferation and protects them from arthritis (52). Our data indicate that polyclonal CD25^-Foxp3^-CD4+ regulatory T cells protect lymphopenic individuals from autoimmune arthritis by inducing clonal anergy in their GPI-specific CD4+ T cells. Consistent with our results, the elimination of all Foxp3-dependent T regulatory cell function in K/BxN mice also leads to significantly increased numbers of GPI-reactive CD4+
T cells and GPI-specific Ab-forming cells, as well as the accelerated production of anti-GPI IgG1 autoantibody (4, 14). Thus, lymphopenia together with its attendant reduction in the number of CD25+Foxp3+CD4+ regulatory T cells is itself a risk factor for deregulated Th cell proliferation and differentiation, as well as resistance to T cell clonal anergy and apoptosis (53).

Patients with chronic inflammatory arthritis have relatively normal percentages of CD25+CD4+ T cells in the peripheral blood and, in fact, have increased percentages within their affected synovial joints (11, 54, 55). Although such CD25+CD4+ T cells can effectively suppress in vitro T cell proliferation assays, there is now considerable evidence to suggest that they have reduced function, particularly with regard to their capacity to inhibit CD4+ Th cell differentiation and effector cytokine generation (10, 54). TNF-α accumulation within the inflamed RA joint appears, in part, to be responsible for reduced CD25+CD4+ regulatory T cell Foxp3 expression and IL-10 production in patients (10, 56). In addition, cytokines produced by activated CD25+CD4+ T cells and/or synoviocytes present within the affected joint (e.g., IL-7, IL-15, and TNF-α) may make CD25+ CD4+ responder T cells resistant to the suppressive effects of CD25+CD4+ regulatory T cells (54). Therefore, it is conceivable that a primary defect in CD25+Foxp3+CD4+ T regulatory cell function underlies the loss of self-tolerance that causes RA. Alternatively, decreased regulatory T cell function may be a consequence of poorly controlled systemic inflammation at the onset of disease triggering, thus leading to a failure of CD4+ T cell anergy induction. Regardless, therapeutic strategies designed to restore the full functional capacity of CD25+Foxp3+CD4+ regulatory T cells in RA patients have the potential to reinstitute immunological tolerance to self-Ag within the pathogenic CD4+ T cell repertoire through the induction of clonal anergy.

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Disclosures

The authors have no financial conflicts of interest.

References


