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Host-derived CD4⁺ T cells attenuate stem cell mediated transfer of autoimmune arthritis in lethally irradiated B6.g7 mice

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Abstract

Objective—In the KBxN mouse model of inflammatory arthritis, T cells carrying a transgenic TCR initiate disease by helping B cells to produce arthritogenic anti-GPI (glucose -6-phosphate isomerase) autoantibodies. We found that lethally-irradiated lymphocyte-deficient B6.g7 (I-A^{g7+}) Rag^{-/-} mice reconstituted with KBxN hematopoietic stem and progenitor cells (HPSC) exhibit arthritis by week 4. In contrast, healthy B6.g7 recipients of KBN HSPC show only mild arthritis, with limited extent and duration. The objective of this study was to investigate the factors responsible for the attenuation of arthritis in the B6.g7 recipients.

Methods—Antibody responses were measured by ELISA. FACs analyses were performed for testing chimerism, expression of markers of activation and suppression, tetramer binding and intracellular cytokines in CD4⁺ T cell. Suppressive activity of CD4⁺ T cells was studied by adoptive transfer.

Results—Titers of anti-GPI Abs in reconstituted B6.g7 mice are ~60 fold lower than in reconstituted B6.g7 Rag^{-/-} mice. Examination of chimerism in the reconstituted B6.g7 mice showed that B and myeloid cells in these mice are donor-derived, but CD4⁺ T cells are primarily host-derived and enriched for cells expressing the conventional regulatory markers, CD25⁺FoxP3⁺. Notably, CD25⁺FoxP3⁺ CD4⁺ T cells express markers of suppressive function, CD73 and FR4, and delay disease after adoptive transfer. Activation of donor-derived CD4⁺ T cells is reduced, and thymic deletion of these cells appears increased.

Conclusion—Despite myeloablation, host CD4⁺ T cells having a regulatory phenotype emerge in these mice and attenuate autoimmunity.

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Introduction

Autoimmune diseases (AD) occur when tolerance to self-antigen fails, and the immune system initiates attack against self-tissues. Rheumatoid arthritis (RA) is an autoimmune disease in which T cells have been proposed to recognize auto-antigen and participate in effector pathways (1, 2). Initial bone marrow transplant experiments in mice demonstrated that the ability to transfer autoimmune arthritis rests within the hematopoietic compartment (3). These findings led to the idea of using bone marrow transplantation as therapy for RA (4). Autologous hematopoietic stem cell transplantation (AHCT) has been tried as treatment for severe AD in humans (4). However, fatal infections due to insufficient recovery of T cells and relapses of autoimmunity likely due to the persistence of autoreactive clones have limited the use of this approach (5).

Studies in C57Bl/6 (Bl/6) mice demonstrated that following lethal irradiation and transplantation of BM, the myeloid leukocytes were almost completely donor-derived, but significant numbers (25%) of CD4⁺T cells were recipient-derived (6). In another study, although host-derived cells were the major constituent (60-80%) of the Treg compartment (hTreg) 5 weeks following autologous BM transplantation (7), donor-derived Tregs were detectable around 2-3 weeks post-transplant and became the major source of Tregs by 8 weeks post-transplant. The initial predominance of the hTregs in these mice was due to their proliferative expansion during the first 5 weeks post-transplant. The presence of hTreg-enriched CD4⁺ T cells in these studies has raised the hope of devising a cell-based strategy to inhibit relapse of autoimmunity in human HCT. However, more detailed information on the emergence and function of these hTregs is needed.

Here, we describe experiments with a novel HCT-based model of autoimmune disease. We used stem cells from the spontaneous KBxN model of autoimmune arthritis in which class II-restricted, transgenic T cell receptors (TCR) drive disease. The KBxN mice are a cross of KRN mice with the NOD strain; KRN mice carry a transgenic TCR that recognizes a glucose-6-phosphate isomerase (GPI) peptide bound to the NOD MHC, I-A^{g7}. KRN × NOD F1 mice show severe distal joint inflammation, with onset at ~4-5 weeks of age. The severe symmetrical polyarthritis in these mice is dependent on expression of the KRN TCR (8), and T cell help for B cells that make pathogenic anti-GPI (glucose-6-phosphate isomerase) antibodies (9). The anti-GPI antibodies form immune complexes with GPI, triggering a joint-specific inflammatory response mediated by neutrophils, macrophages, NK cells and Th-17 T cells (9, 10). In the KBxN model, CD25⁺Foxp3⁺ Tregs are selected in the thymus and enriched in the spleen (~20%) and draining lymph nodes during arthritis. The Tregs mediate suppressive function *in vitro*, but do not inhibit severe arthritis in the mice (11). However, Treg depletion in the KBxN mice results in multi-organ inflammation with persistent arthritis (12).

The crucial role of IAg7 in the KBxN arthritis model was shown by breeding experiments: B6 or BALB/c mice carrying the IA^{g7} gene when crossed into B6 mice carrying the KRN TCR yield offspring that develop arthritis similar to the KBxN mice (13). We predicted that transfer of HSPCs from the KBxN mice into myelo-ablated B6.g7 mice (I-A^{g7} expression on Bl/6 background) would result in autoimmune arthritis, but surprisingly, autoimmunity in

recipient mice was highly attenuated. Here, we identify immunosuppressive roles for host-derived CD4⁺ T cells with regulatory phenotypes, including CD25⁻ Foxp3⁻ CD73⁺ cells.

Materials and Methods

Mice

NOD (Non-obese Diabetic) mice and B6.g7 mice derived from C57BL/6J mice expressing the NOD MHC class II molecules H2 I-A^{g7} were obtained from the Jackson Laboratory, Maine USA. Recombinase activating gene (Rag) deficient B6.g7 (B6.g7 Rag^{-/-}) mice lack T and B cells (G. B., J. S., unpublished). KRN mice with transgenic TCR against GPI were kindly provided by Dr. Diane Mathis (Harvard Medical School, Boston, MA). The arthritic KBxN mice were obtained by crossing the KRN mice with NOD mice, as is standard. All animals were bred, housed, and cared for in the Stanford Veterinary Service Center under the approval of Administrative Panel for Laboratory Animal Care, protocol number 15867.

Hematopoietic stem and progenitor cell (HSPC) isolation

HSPCs were isolated as described, with some modifications (14). BM cells were harvested and selected for c-Kit⁺ cells by the MACS system (Miltenyi Biotec). The c-Kit⁺ BM cells were then sorted in a BD FACS Aria Cell Sorter for c-Kit⁺ Sca1^{hi} Lin⁻ cells after staining with antibodies (Abs) listed in supplementary table 1. All Abs were obtained from eBioscience. FACS sorting was performed at the Stanford Shared FACS Facility at Stanford.

Haematopoietic cell transplantation (HCT)

3 to 4-month old recipients (B6.g7 or B6.g7 Rag^{-/-} mice) were lethally irradiated with a Phillips Unit irradiator (250 kv, 15 milliamp) at 980 Rad (in 2 doses with 4 hr interval). Irradiated mice were reconstituted with HSPCs from 3 to 5-month old donors (KBN mice) 3 hrs after irradiation. 10,000-12,000 HSPC in 200ul PBS were given to each recipient by tail vein injection. The reconstituted mice were maintained on sulfamethoxazole and trimethoprim (suspension, Hi-Tech Pharmacal Co. Inc, Amityville, New York) in drinking water up to 3 months post-transplant. Irradiation only (without transplantation) control mice died within 2 weeks.

Antibody and tetramers staining and flow cytometry analyses

Thymus, spleen and popliteal lymph nodes were harvested from mice. Surface staining was done with various antibodies directly conjugated to fluorochromes, as shown in supplementary table 2. For the biotin labeled Abs, pacific orange conjugated streptavidin (Invitrogen, Carlsbad, CA) was added and incubated for additional 30min. For intracellular Foxp3 staining, cells were treated with fixation/permeabilization reagent (eBioscience) followed by staining with Abs (eBioscience). For cytokine stimulation and intracellular staining, spleen cells were suspended in complete IMDM (10% FBS, 1% glutamine, 0.1% β -Mercaptoethanol) and incubated for 4 hrs at 37°C with or without Leukocyte Activation Cocktail (BD Biosciences). Cell viability was assessed by staining with Live Dead Aqua (Invitrogen). Subsequently, cells were permeabilized and stained with FITC-conjugated anti-IL-17 Ab (TC11-18H10), PE-conjugated anti-IFN γ Ab (XMG1.2) and APC-conjugated

anti-Foxp3 Ab (FJK-16s). For tetramer staining, the single cell suspension was blocked with 0.5 mg/ml Fc block for 10 min on ice. Cells were then washed and stained with PE-labeled MHC/GPI-peptide tetramer (kindly provided by Dr. Luc Teyton) at a final concentration of 10ug/ml for 1 hr at room temperature (15). PE-labeled MHC/HEL-peptide tetramer was used as negative control in all experiments. Co-staining of surface markers was done as mentioned above. In all Ab staining experiments, data were collected on a BD LSR II flow cytometer and analyzed with Flowjo software (Tree Star, Inc., Ashland, OR), gating on singlet cell populations. All FACS analysis were performed in the Stanford Shared FACS Facility at Stanford.

Evaluation of autoimmune disease

Mice were assessed by clinical arthritis index and circulating anti-GPI Ab titer. The clinical arthritis index was tallied from the degree of inflammation of each ankle, based on redness and swelling measured with a pocket thickness gauge (Mitutoyo). Scores were as follows: 0, none; 0.25, mild; 0.5, moderate and 1, severe, 4 being the maximum score in mice with full-blown arthritis (16). The mean score per animal group was calculated, with standard error of the means (SEM). GPI titer was determined by ELISA. Specifically, ELISA plates were coated with GPI protein (1-4ug) and blocked with PBS containing 0.05% Tween 20 and 1% fat-free powdered milk followed by addition of diluted serum at 100ul/well. After incubation at 37°C for 2h, the plates were washed and incubated with HRP conjugated goat-anti-mouse IgG Ab (1/5000 dilution, Millipore) for 1h before adding TMB substrate (BD OptEIA™ kit). Results were read out with SpectraMax 190 Absorbance Microplate Reader (Molecular Devices, Inc.), after stopping the reaction with 50ul H₂SO₄ (1M stock). For joint histopathology, ankle joints were fixed in 4% phosphate-buffered formaldehyde, and then sectioned and stained with hematoxylin and eosin (H & E) by Priemier Laboratory, Colorado. Microscopy was performed with a Zeiss Axio-plan 2 instrument (Carl Zeiss Microscopy, USA).

Adoptive transfer

Spleen cells and LN cells were harvested from the KBxN→B6.g7 mice 3 months after HSPC reconstitution. Cells were resuspended in HBSS buffer with 2% FCS, and surface stained with Abs against CD45.2, CD45.1, CD4, CD25, FR4 and CD73, to identify host and donor-derived regulatory cells. Host-derived CD4⁺CD25^{hi} cells and CD4⁺CD25⁻CD73⁺FR4⁺ cells were sorted using FACS Aria cell sorter and injected separately (5×10⁵ cells per mouse) into B6.g7 Rag^{-/-} recipients that had been reconstituted with KBxN HSPC 20 days earlier. Mice were then monitored for onset and progress of arthritis as above.

Statistical analysis

The groups were compared using the two-tailed unpaired student t test. Prism software (version 5; GraphPad Software) was used for statistical analysis.

Results

B6.g7 mice reconstituted with HSPC from KBxN mice have mild, transient disease

In an effort to create an HCT model for autoimmune arthritis, lethally irradiated B6.g7 Rag^{-/-} mice were reconstituted with HSPCs (ckit⁺Lin⁻Scal^{hi}) isolated from KBxN mice. The recipients were followed for development of arthritis. All developed severe arthritis (Figure 1A) and had high titer anti-GPI autoantibodies (Figure 1B), comparable to those in the transgenic KBxN mice. In contrast, lethally irradiated B6.g7 mice reconstituted with HSPCs from KBxN mice developed very mild arthritis that disappeared by week 14 (Figure 1A) and had very low titer, circulating anti-GPI autoantibodies (Figure 1B). Joint histopathology showed minimal inflammatory cell infiltrates in the KBxN→B6.g7 mice, whereas the KBxN→B6.g7 Rag^{-/-} chimeras showed prominent cell infiltration and severe bone destruction (Figure 1C). Thus, transfer of KBxN HSPCs into immunocompetent B6.g7 mice resulted in attenuated autoimmunity compared to transfer into immunodeficient B6.g7 Rag^{-/-}.

Reduced donor-derived CD4⁺T cells in reconstituted B6.g7 compared to reconstituted B6.g7 Rag^{-/-} mice

In the KBxN mouse, CD4⁺ T cells bearing the KRN TCR and antigen presenting cells expressing MHC II I-A^{g7} are required for the initiation and progress of autoimmunity. To determine if disease resistance in the B6.g7 mice was due to incomplete reconstitution of T or B cells, we analyzed splenocytes from B6.g7 recipients at 12 weeks post-HSPC-transplant. Compared to KBxN mice, the KBxN→B6.g7 chimeras showed comparable ratios of CD4:CD8 T cells, and comparable proportions of B220⁺ B cells, with a lower proportion of CD11b⁺ cells (neutrophils), presumably reflecting the reduced inflammatory response in these mice (Supplementary Figure 1A). However, compared to B6.g7 mice, the proportion of CD4⁺ T cells was low in both the chimera and KBxN mice, likely due to negative selection of the self-reactive transgenic TCR (13).

To distinguish the host and donor hematopoietic cells in the chimera, we used the congenic markers CD45.1 and CD45.2 (Supplementary Figure 1B). Approximately 98% of B cells and neutrophils were of donor origin (Figure 2A), but only ~10% of splenic CD4⁺ T cells were of donor origin (Figure 2B). As pathogenic autoreactive CD4⁺ T cells undergo expansion in the draining lymph nodes (DLN) of the inflamed target organs in the KBxN mice (13), we assessed chimerism in DLN. The joint-draining popliteal LN of the KBxN→B6.g7 chimera showed comparable percentages of donor-derived CD4⁺ T cells to those in spleen (Figure 2C). The absolute number of donor-derived splenic CD4⁺ T cells (Figure 2D) and CD4⁺CD25⁻Foxp3⁻T cells (Figure 2E) were also reduced in the B6.g7 recipients, compared to B6.g7 Rag^{-/-} recipients. Thus, insufficient numbers of KRN TCR⁺, donor-derived CD4⁺ T effector cells (Teff) is one possible explanation for attenuated autoimmunity in KBxN→B6.g7 chimeras. However, the appearance of autoantibodies and joint infiltrates in the KBxN→B6.g7 chimera indicates that Teff numbers were sufficient to break tolerance.

KBxN→B6.g7 chimeras have reduced proportions of transgenic TCR⁺ or GPI/Ag7 tetramer⁺, donor-derived CD4⁺ T cells

We next analyzed the thymi of the KBxN→B6.g7 chimera and found the majority of CD4⁺ single positive (SP) T cells (CD4SP) were host-derived and absolute numbers of donor-derived CD4SP were reduced compared to thymocytes in the KBxN→B6.g7 Rag^{-/-} chimera (Figure 3A,B). The β chain of the KRN TCR in the KBxN mice is V β 6 (13). Selection of KRN⁺ CD4SP can be evaluated by the expression of V β 6 compared to other variable regions from endogenous TCR loci, like V β 8. In the thymus and spleen of the KBxN→B6.g7 Rag^{-/-} mice, we found ~50% of the CD4⁺ T cells were V β 6⁺V β 8⁻ and ~15% of T cells were V β 6^{int} V β 8⁺ (Figure 3C,D,E), an expression pattern similar to that in the KBxN mice (13). KBxN→B6.g7 mice showed substantially lower proportions of each subset, including among CD4SP thymocytes (Figure 3C,D,E). To enumerate circulating cells expressing the KRN TCR based on their recognition of I-Ag7 molecules with bound GPI₂₈₂₋₂₉₄ peptide (17), we used tetramers to stain CD4⁺ T cells in the blood obtained from KBxN→B6.g7 and KBxN→B6.g7 Rag^{-/-} mice. The frequency of donor-derived, CD4⁺CD25⁻Foxp3⁻, tetramer⁺ cells was significantly lower in the KBxN→B6.g7 compared to the KBxN→B6.g7 Rag^{-/-} mice (2.53±0.6 vs 12.26 ±1.3, P=0.0006; Figure 4A).

Donor-derived CD4⁺CD25⁻Foxp3⁻ cells in KBxN→B6.g7 mice are less activated than those in KBxN→B6.g7 Rag^{-/-} mice

The peripheral CD4⁺ T cell compartment in KBxN mice is activated, as evidenced by expression of the activation/memory marker CD44 (18). In the arthritic KBxN→B6.g7 Rag^{-/-} chimera, similar to the KBxN mice, a high proportion of the donor-derived CD4⁺CD25⁻Foxp3⁻ cells were CD44^{hi} (62.5±3.1%). In contrast, significantly fewer donor-derived CD4⁺CD25⁻Foxp3⁻ cells in the KBxN→B6.g7 chimera (28.7±2.8%; P<0.001) were CD44^{hi} (Figure 4B). To further characterize the activation state of donor-derived CD4⁺ T cells, we measured their expression of inflammatory cytokines IFN γ and IL-17 after stimulation with PMA (Figure 4 C, D). These cytokines are known to promote arthritis in the KBxN mice (10). A higher proportion of donor-derived splenic CD4⁺ T cells expressed IFN γ (21.5 ± 6.8%) and IL-17 (2.28 ± 0.17%) in the KBxN→B6.g7 Rag^{-/-} mice than in the KBxN→B6.g7 mice (IFN γ : 4.19±1.5%, p<0.0002; IL-17: 0.36±0.37%, p<0.013). Together, these data indicate reduced activation of potentially arthritogenic T cells in the KBxN→B6.g7 chimera.

Host-derived CD4⁺ T cells are enriched for CD25⁺FoxP3⁺ regulatory T cells

The presence of host-derived CD4⁺ T cells in the spleen and the thymus of the KBxN→B6.g7 chimera indicate a possible role of these cells in attenuating autoimmunity. To investigate whether these cells had regulatory properties, we assessed levels of CD4⁺CD25⁺Foxp3⁺ Tregs in the host and donor-derived CD4⁺ T cells in the KBxN→B6.g7 reconstituted mice (Figure 5A). Host-derived splenic CD4⁺ T cells were 15.2±2.6% CD25⁺FoxP3⁺ compared to 6.7±0.85% (p<0.0059) CD25⁺FoxP3⁺ among donor-derived CD4⁺ T cells; the latter is similar to the Treg portion of CD4⁺ T cells in normal B6.g7 mice (8±0.5%, Figure 5A). The proportion of CD25⁺FoxP3⁺ in the donor-derived CD4⁺ T cells is not characteristic of CD4⁺ T cells expressing the transgenic KRN TCR. On the contrary, in

the KBxN mice, Tregs constitute 30% of the CD4⁺ T cell population (11). Similarly, in the KBxN→B6.g7 Rag^{-/-} chimera, the donor-derived CD4⁺ T cells included 26.5±1.8% CD25⁺FoxP3⁺ T cells (Figure 5A).

To test their immunosuppressive capacity, we transferred host-derived CD4⁺CD25^{hi} T cells from KBxN→B6.g7 mice (3 months post HSPC transfer) into KBxN→B6.g7 Rag^{-/-} mice 20 days post KBxN HSPC transfer. Because Foxp3 is an intracellular protein and cannot be used as a marker for sorting T regulatory cells, we used cells expressing CD4⁺CD25^{hi} as a surrogate for the CD4⁺CD25⁺FoxP3⁺ T cells (19, 20). The KBxN→B6.g7 Rag^{-/-} mice were free of disease at the time of transfer. The mice were monitored for onset and progress of arthritis. We found that injected host-derived (CD45.2) CD4⁺CD25^{hi} T cells delayed onset of disease when compared to control KBxN→B6.g7 Rag^{-/-} mice that received PBS (Figure 5B). However, by day 48 post T cell transfer, arthritis scores in both groups were comparable. At this time point, 55±14.3% of the remaining host-derived CD45.2 cells were CD4⁺CD25⁺ cells (not shown).

Host-derived CD4⁺CD25⁻Foxp3⁻ cells show a memory-like phenotype and express the suppression markers, CD73 and FR4

As another potential source of suppressive host-derived CD4⁺ T cells, we assessed CD4⁺CD25⁻Foxp3⁻ T cells for evidence of a regulatory phenotype. Radio-resistant CD4⁺ T cells undergoing lymphopenia-induced proliferation in irradiated hosts acquire a memory-like phenotype, characterized by high expression of CD44 (21). These memory-like CD4⁺ T cells have suppressive effects on naïve T cell proliferation (22). We found a higher percentage of host-derived CD4⁺CD25⁻Foxp3⁻CD44^{hi} splenic T cells compared to the percentage of this subset in B6.g7 mice (Figure 5C).

FR4 and CD73 are associated with regulatory function (23, 24). Notably, the host-derived splenic CD4⁺CD25⁻Foxp3⁻ cells in KBxN→B6.g7 mice showed a significantly increased frequency ($p<0.0002$) of cells expressing both FR4 and CD73, compared to the donor-derived CD4⁺CD25⁻Foxp3⁻ cells in the KBxN→B6.g7 mice or the CD4⁺CD25⁻Foxp3⁻ cells in normal B6.g7 mice (Figure 5D). The host-derived CD4⁺CD25⁻ cells also showed higher expression (MFI) of CD73 and FR4 than the donor-derived CD4⁺CD25⁻ T cells (Figure 5E,F). We tested the immunosuppressive capacity of these cells in adoptive transfer. Host-derived CD4⁺CD25⁻CD73⁺FR4⁺ cells from KBxN→B6.g7 mice (3 months post HSPC transfer) also delayed disease onset when transferred into KBxN→B6.g7 Rag^{-/-} mice (Figure 5B). Thus, these host-derived Foxp3⁻ cells could be contributing to protection against the manifestations of autoimmunity. These cells also persist (87±9.19% of CD45.2 cells) in the recipient mice at day 48 (not shown), although their efficacy is overcome.

The phenotype of host-derived CD4⁺ T cells in the thymi of the reconstituted mice

In the KBxN→B6.g7 mice, a high proportion (47.2±2.36%) of host-derived CD4SP thymic cells were CD25⁺Foxp3⁺ (Figure 6A). Further, a higher percentage of host-derived CD4SP CD25⁻ thymic cells were CD44^{hi}, compared to donor-derived CD4SP CD25⁻ thymic cells (Figure 6B), whereas CD4SP⁺ thymocytes typically are CD44^{lo}. We also measured CD103, an alpha(E)beta(7) integrin, previously shown to be expressed on Tregs (25) and on a unique

subset of CD4⁺CD25⁻ T cells that are suppressive in function (26). We found high CD103 expression on host-derived CD4⁺CD25⁻Foxp3⁻ T cells in the thymi of the KBxN→B6.g7 mice compared to normal B6.g7 mice or to donor-derived CD4⁺CD25⁻Foxp3⁻ T cells (Figure 6C). The host-derived and donor-derived splenic CD4⁺CD25⁻Foxp3⁻ T cells expressed low levels of CD103 (data not shown). The expression of CD103 on the host-derived CD4⁺CD25⁻Foxp3⁻ T cells in the thymus suggests a regulatory phenotype for these cells.

Discussion

Transfer of HSPCs from the KBxN mice into B6.g7 Rag^{-/-} mice results in disease similar to that in the KBxN mice. However, transfer of HSPCs into B6.g7 mice results in significant attenuation of arthritis. What regulates autoimmunity in the reconstituted mice? Arthritis in the KBxN mice is thought to be initiated by the extravasation of circulating anti-GPI/GPI immune complexes into joints, followed by complement-induced tissue destruction (27). Anti-GPI autoantibody titers in the reconstituted KBxN→B6.g7 chimera are much lower than in the KBxN mice and KBxN→B6.g7 Rag^{-/-} chimeras. This is likely a key explanation for reduced arthritis in the KBxN→B6.g7 mice. This, in turn, implicates defect(s) in the T and/or B cell compartments of these mice. Data on chimerism in the KBxN→B6.g7 mice indicate that B cells and neutrophils are >98% donor (KBxN) derived, and the fact that low levels of anti-GPI antibodies are produced demonstrates that the B cell compartment is competent to develop this specificity. In contrast, only ~10% of the peripheral CD4⁺ T cells in the KBxN→B6.g7 mice are donor-derived, and the rest are host-derived CD4⁺ T cells that emerge despite irradiation, even at high (1100 RAD) doses (not shown). The few donor-derived CD4⁺ T cells in the thymus and periphery of KBxN→B6.g7 mice include a lower proportion of tetramer⁺, Vβ6⁺ cells than in KBxN→B6.g7Rag^{-/-} mice and fewer thymic Vβ6^{int}Vβ8⁺ cells as compared to the B6.g7Rag^{-/-} chimeras. This reduced level of donor-derived KRN⁺ T cells may provide only limited amounts of help to GPI-specific B cells. In KBxN mice, Vβ6^{int}Vβ8⁺ CD4⁺ T cells escape negative selection due to lower TCR affinity compared to the Vβ6⁺ CD4⁺ T cells (13). The reduced proportion of donor-derived thymic Vβ6^{int}Vβ8⁺CD4⁺ T cells in the KBxN→B6.g7 mice suggests increased negative selection of these thymocytes. Reduced frequency of circulating tetramer⁺ CD4⁺ T cells in these mice also points to a process of negative selection. The mechanistic basis of these phenomena is under investigation (also see below).

The surviving donor-derived CD4⁺ T cells are further restrained by immunosuppressive mechanisms. They are less activated in B6.g7 than in B6.g7Rag^{-/-} recipients, as reflected in a lower proportion that is CD44^{hi}, and their levels of IFNγ and IL-17, two cytokines important for the development of arthritis in the KBxN mice (10, 28), are reduced. A key contributor to this suppression is likely the CD25⁺Foxp3⁺ cells that are enriched among the host-derived CD4⁺ T cells in the KBxN→B6.g7 chimeras, persist even at 3 months post-transplant, and function to delay arthritis in adoptive transfer. Consistent with our results, residual, host-derived Tregs allow survival of myeloablated C57BL/6 recipient mice following syngeneic BM transfer, whereas C57BL/6Rag^{-/-} recipients succumb to lethal syngeneic graft-versus-host disease (29). Interestingly, host-derived CD4⁺ T cells in the

KBxN→B6.g7 mice do not expand to populate the lymphopenic CD4⁺ T cell compartment to achieve homeostatic proportions. The cause for this limitation needs to be investigated.

CD44, an activation and memory marker, is expressed at higher levels in the host-derived CD4⁺CD25⁻Foxp3⁻ T effector population than in the donor-derived CD4⁺CD25⁻Foxp3⁻ cells or in B6.g7 mice. This result also resembles observations in BM transplantation of syngenic or Rag-deficient animals, where memory-like (CD44⁺) T cells are enriched in the host-derived CD4⁺ T cell compartment (6, 21, 22). Like conventional CD4⁺CD25⁺ Tregs, a subset of memory-like CD4⁺CD44^{hi} T cells can also restrain activation and proliferation of naïve T cells (23, 24).

As a first step toward investigating immunosuppressive mechanisms of these cells in our model, we analyzed the CD4⁺CD25⁻Foxp3⁻ cells for other markers of suppressive capacity, CD73 and folate receptor 4, FR4. CD73, a GPI-linked surface protein with ecto-5'-nucleotidase activity, mediates suppression by CD25⁺Foxp3⁺ T cells (23, 30). CD39, an ecto-ATP diphosphohydrolase that is also expressed by Tregs and neutrophils rapidly converts circulating ATP and ADP to 5'-AMP (31). CD73 through its ecto-nucleotidase activity converts 5'-AMP to adenosine (32). Adenosine mediates its immunoregulatory activities through various adenosine receptors expressed on T cells, B cells, neutrophils and macrophages (33-35). In addition to Tregs, a subset of memory-like CD4⁺ T cells expresses high levels of CD73 and is suppressive by producing adenosine (23).

FR4 is one of the receptor subtypes for folic acid. FR4 is expressed at high levels on both natural and TGFβ-induced Tregs. Host- and donor-derived conventional Tregs (CD25⁺Foxp3⁺) in the KBxN→B6.g7 chimera showed high expression of CD73 and FR4 (not shown). Strikingly, the conventional Teff or CD4⁺CD25⁻Foxp3⁻ compartment also contains CD73⁺FR4⁺ cells, more so among host-derived cells, by percentage-positive cells and expression levels. Recently, Martinez et al (36) recently showed that adoptive transfer of KRN T cells into lymphopenic TCRα^{-/-} I-A^{g7} + hosts, but not I-A^{g7} + wild-type mice, results in arthritis. They demonstrate that protection against arthritis is due to induction of anergy of KRN T effector cells via enhanced CD73 and FR4 expression. This study differs from ours in that we observe host-derived CD4⁺ T cells that exhibit a CD73⁺FR4⁺ phenotype. Adoptive transfer of these host-derived CD4⁺CD25⁻CD73⁺FR4⁺ cells from the KBxN→B6.g7 mice into the KBxN→B6.g7.Rag^{-/-} mice also delayed onset of arthritis. To our knowledge, this is the first demonstration of high levels of CD73⁺FR4⁺ cells with possible regulatory properties, in the Teff compartment, persisting in mice after lethal irradiation.

Adoptive transfer of host-derived CD25^{hi} cells or the CD73⁺FR4⁺ T cells into the KBxN→B6.g7 Rag^{-/-} leads only to delayed onset of disease and not complete protection. This could be because; additive or synergistic effects of these two regulatory populations are required for long-term control (both are present in the KBxN→B6.g7 mice),

The KBxN→B6.g7 chimeras have a high frequency (65%) of host-derived thymic CD4⁺ T cells with a mature phenotype (CD44^{hi}), which is uncharacteristic of CD4SP thymocytes. The latter are CD44^{lo} and acquire the CD44^{hi} phenotype in the periphery after encountering

antigen. Thus, these CD44^{hi} CD4⁺ T cells could have re-entered the thymus from the periphery. CD4⁺ T cells are capable of re-entering the thymus from the periphery under pathological and lymphopenic conditions (22, 37-39), and most of the re-entering cells are of the activated phenotype (39). The CD4⁺CD25⁻Foxp3⁻ host-derived thymic CD4⁺ T cells in our model express CD103, an integrin that is induced at sites of inflammation and involved in the retention of T cells at these sites (40). CD103 also increases in expression in response to IL-2 (41). Expression of CD103 on host-derived CD4⁺ T cells may mediate their retention in the thymus of the KBxN→B6.g7 chimeras. Retention of circulating CD4⁺ T cells in the thymus has been shown to contribute to tolerance induction, specifically negative selection (37, 39, 42). Re-circulating peripheral T cells also contribute to proper thymic organization and regeneration of the medullary thymic epithelium in a SCID mouse model (43). These cells may be responsible, at least in part, for the increased negative selection of KRN expressing, CD4SP thymocytes in our model.

An alternative origin for host-derived CD4SP in our model can be proposed, based on data from syngenic bone marrow transplant. Here, evidence suggested the host-derived CD4⁺ T cells arose mainly from a transient wave of differentiation from radioresistant DN2 precursors within the thymus (6, 44). The host-derived thymic CD4⁺ T cells of the KBxN→B6.g7 chimera are also enriched for Tregs. In a recent BM transplantation model, host-derived Tregs required IL-2 for proliferation and IL-7 for survival (45). Foxp3 expression by the host-derived CD4SP cells in the KBxN→B6.g7 chimera may result from increased thymic production of IL-2 due to robust negative selection (46).

In summary, persistent, host-derived CD4⁺ T cells with a high proportion of CD25⁺Foxp3⁺ cells and novel CD4⁺CD25⁻Foxp3⁻CD73⁺FR4⁺ cells are likely critical in protecting mice with autoimmune potential from full-blown disease. Recently, we found similar results using transfer of HSPCs carrying a transgenic, diabetogenic TCR in to B6.g7 mice, indicating that the phenomena we observe are not unique to the KRN TCR and the arthritis model (manuscript in preparation). Our models will facilitate further study of the origin of host-derived CD4⁺ T cells and the mechanisms by which they mediate tolerance. Enrichment of these cell types is a potential therapeutic strategy in rheumatoid arthritis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Transfer of HSPCs from KBxN donors into immunocompetent B6.g7 recipients results in protection against autoimmunity

A, HSPCs isolated from KBxN bone marrow were transferred into lethally irradiated B6.g7 and B6.g7 Rag^{-/-} recipients. Onset and progress of arthritis was measured by Clinical Arthritis Index (see materials and methods). Mean scores±SEM are shown (n=3). **B**, Lethally irradiated B6.g7 Rag^{-/-} or B6.g7 mice were reconstituted with KBxN-derived HSCs. Mice were bled for sera 10 weeks post transplant and titers of GPI specific immunoglobulin were measured by ELISA. Sera from B6.g7 Rag^{-/-} and KBxN recipients was diluted at 1:500 (left) and from B6.g7 recipients at 1:15 (right). Antibody titers were normalized with the levels in B6.g7 mice set up as 1. Fold changes of titers ±SEM are shown (n=3). **C**, H&E staining of ankle sections of lethally irradiated B6.g7 (Left) and B6.g7 Rag^{-/-} (Right) reconstituted with KBxN HSCs and sacrificed at 12 weeks post transfer. A mild synovial proliferation is seen in the reconstituted B6.g7 (Arrow head in the left). Severe bone and cartilage destruction is observed in the reconstituted B6.g7 Rag^{-/-} (Arrow head in the right). Data are representative of three (**A**, **B**) or two (**C**) independent experiments.

Figure 2. Emergence of host derived CD4⁺ T cells in the reconstituted B6.g7 mice

A, Spleen cells from KBxN→B6.g7 mice sacrificed on week 12 post transplant were stained for CD11b (myeloid cells) and B220 (B cells) and analyzed to determine host (CD45.1⁺) or donor (CD45.2⁺) origin by flow cytometry. The histograms represent mean percentage of host and donor cells $\pm$ SEM from three animals. *** $P < 0.0001$ (t -test). **B**, Frequency of host and donor-derived splenic CD4⁺ T cells. **C**, Frequency of host and donor-derived cells in the draining lymph node. **D**, Absolute numbers of host and donor-derived splenic CD4⁺ T cells. Absolute numbers were determined by multiplying the total number of cells in each spleen (determined using a Coulter counter) by the percent of the host and donor-derived CD4⁺ T cells. **E**, Absolute number of CD4⁺CD25⁻Foxp3⁻ T cells in the KBxN→B6.g7.Rag^{-/-} in comparison to the donor-derived CD4⁺CD25⁻Foxp3⁻ T cells in the KBxN→B6.g7 chimera. The histograms in (**B,C,D&E**) depict mean percentage or mean number of host and donor-CD4⁺ T cells $\pm$ SEM from six animals. *** $p < 0.001$ (t -test) (**B,C,D,E**). Data are representative of three (**A,B,C**) or two (**D,E**) independent experiments.

Figure 3. Host-derived CD4 SP T cells are predominant in the thymus

A, Frequency of host and donor-derived CD4⁺ T cells in the thymus. Thymocytes from KBxN→B6.g7 sacrificed on week 12 post-transplant were stained for CD4 and CD45.2 (host) or CD45.1 (donor). **B**, Absolute numbers of host and donor-derived thymic Single Positive (SP) CD4⁺ T cells, determined by multiplying the total number of cells in each thymus (determined using a Coulter counter) by the percentage of the host and donor-derived CD4⁺ T cells. Frequency and absolute numbers shown as mean ± SEM from six animals in the KBxN→B6.g7 chimera and four from the KBxN→B6.g7 Rag^{-/-} chimera. Statistical significance in panels **A**, **B** is indicated by **** $p < 0.0002$, ** $p < 0.001$, respectively (*t-Test*). Data representative of one of two independent experiments. **C**, Expression of T cell receptors Vβ6 and Vβ8 on the donor-derived CD4⁺ T cells. Flow cytometry plots showing the frequency of Vβ6 and Vβ8 expressing cells in the donor-derived CD4⁺ CD25⁺ Foxp3⁻ cells in the thymus and spleen in the KBxN→B6.g7 mice and KBxN→B6.g7Rag^{-/-} mice sacrificed on week 12 post-transplant. Frequency of Vβ6^{int}Vβ8⁺ and Vβ6⁺Vβ8⁻ population in the Thymi (**D**) and spleen (**E**) of KBxN→B6.g7 mice and KBxN→B6.g7Rag^{-/-} mice. Frequencies are shown as mean ± SEM from six animals in the KBxN→B6.g7 group and four in the KBxN→B6.g7 Rag^{-/-} group. *** $p < 0.0004$ (*t-Test*) (**D,E**).

Figure 4. Presence of donor-derived CD4⁺ T cells with decreased activation, reduced cytokine expression and reduced fraction of MHC/tetramer⁺ cells in the KBxN→B6.g7 mice

A, % donor-derived CD4⁺CD25⁻Foxp3⁻ CD4⁺ T cells specific for GPI₂₈₂₋₂₉₄ peptide was determined by FACs staining of blood cells with I-Ag7/GPI₂₈₂₋₂₉₄ tetramer. Frequency of I-Ag7 MHC tetramer expressing cells is shown as mean ± SEM from three KBxN→B6.g7 Rag^{-/-} and four KBxN→B6.g7 mice *** $p < 0.0001$ (two-tailed, unpaired t -test). Data are representative of two independent experiments. **B**, CD44 expression on host and donor-derived CD4⁺CD25⁻Foxp3⁻ cells in the spleens of the KBxN→B6.g7 chimeric mice sacrificed at 12 weeks post-reconstitution. Frequency of CD44 expressing cells is shown as mean ± SEM from six KBxN→B6.g7 Rag^{-/-} and four KBxN→B6.g7 mice. *** $p < 0.0001$ (two-tailed, unpaired t -test). Data are representative of three independent experiments. **C**, Frequency of IFN-γ expressing cells in the donor-derived CD25⁻Foxp3⁻ cells in the KBxN→B6.g7 and KBxN→B6.g7 Rag^{-/-} chimeras. **D**, Frequency of IL-17 expressing cells in the donor-derived CD25⁻Foxp3⁻ cells in the KBxN→B6.g7 and KBxN→B6.g7 Rag^{-/-} chimeras. Splenocytes from KBxN→B6.g7 (n=6) and KBxN→B6.g7 Rag^{-/-} (n=4) mice sacrificed on week 12 post-transplant were evaluated. Frequency shown as mean ± SEM. ** $P < 0.0002$ & * $p < 0.01$, (t -Test). Data are representative of three independent experiments.

Figure 5. Foxp3 expression in CD4⁺ CD25⁺ T cells in the reconstituted mice and memory-like phenotype (CD44⁺) in host-derived CD4⁺CD25⁺Foxp3⁺ cells

A, Frequency of CD4⁺CD25⁺Foxp3⁺ cells in the host and donor-derived splenocytes of the KBxN→B6.g7 chimeras sacrificed 12 weeks post-transplant. Frequency shown as mean ± SEM, n=9, combined data from two experiments; ** $P < 0.0059$ (t -test). **B**, Adoptive transfer of regulatory cells as described in the materials & methods. Development of arthritis represented by ankle thickness, is shown until 52 days post T cell transfer. Statistical analysis compared the ankle thickness of the KBxN→B6.g7 Rag^{-/-} mice (n=4) with mice transferred with host-derived CD25^{hi} cells (n=3) or with those transferred with host-derived CD25⁺CD73⁺FR4⁺ cells (n=3). * $p < 0.02$ (t -Test). Representative data of one of two independent experiments. **C**, CD44 expression on CD4⁺CD25⁺Foxp3⁺ cells in the spleens of B6.g7 and host-derived CD4⁺CD25⁺Foxp3⁺ cells in the spleens of the KBxN→B6.g7 chimeric mice sacrificed 12 weeks post-reconstitution. Frequency is shown as mean ± SEM from four KBxN→B6.g7 and three B6.g7 mice. ** $p < 0.005$ (t -test). Representative data of one of three independent experiments with four mice per group. **D**, Expression of FR4 and CD73 on spleen cells in the CD25⁺Foxp3⁺ population. Representative profiles from one out of three mice are shown. Average frequencies from all 3 mice ± SEM: B6.g7 CD73⁺FR4⁺: 32±4.6%, KBxN→B6.g7 host CD73⁺FR4⁺: 62±3.4%; KBxN→B6.g7 donor CD73⁺FR4⁺: 30.2±2.8%. Statistical significance (t -test) -B6.g7 vs KBxN→B6.g7 host $P < 0.0129$. KBxN→B6.g7 host vs KBxN→B6.g7 donor $P < 0.0021$. **E**, CD73 and **F**, FR4 Mean fluorescence intensity (MFI) from the host and donor-derived CD4⁺CD25⁺Foxp3⁺ cells in the two chimeras. MFI is shown as mean ± SEM from three mice. *** $P < 0.0009$, ** $P < 0.004$ (t -Test). Representative data of one of three independent experiments.

Figure 6. Thymocytes from the KBxN→B6.g7 have higher frequency of host derived Tregs
A, Frequencies of CD4⁺CD25⁺Foxp3⁺ cells in the host and donor-derived CD4SP thymocytes of the KBxN→B6.g7 chimera. Data are representative of one of three independent experiments. Frequency shown as mean ± SEM from three animals in each group. *** $p < 0.0001$ (t -Test). **B**, CD44 expression on host or donor-derived CD4⁺CD25⁺Foxp3⁻ cells in the thymi of the chimera sacrificed on week 12 post-reconstitution. Data are representative of one of three independent experiments. Frequency shown as mean ± SEM from three animals in each group. *** $p < 0.0005$ (t -Test). **C**, Frequency of CD103⁺ cells among the host and donor-derived CD25⁺Foxp3⁻ cells in the KBxN→B6.g7 mice and in the B6.g7 and KBxN→B6.g7 Rag^{-/-} mice. Frequency shown as mean ± SEM from five animals in each group. *** $p < 0.0003$ (t -Test).