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*J Immunol* 2005; 175:7805-7809; doi: 10.4049/jimmunol.175.12.7805

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Cutting Edge: Foxj1 Protects against Autoimmunity and Inhibits Thymocyte Egress

Subhashini Srivatsan1 and Stanford L. Peng2*†

Previous studies suggest that the forkhead transcription factor Foxj1 inhibits spontaneous autoimmunity in part by antagonizing NF-κB activation. To test this hypothesis, we ectopically expressed Foxj1 in the T cells of lupus-prone MRL/lpr mice by backcrossing a CD2-Foxj1 transgene against the MRL/lpr background. Strikingly, CD2-Foxj1-MRL/lpr animals showed a significant reduction in lymphadenopathy, pathogenic autoantibodies, and end-organ disease—but surprisingly, reversion of autoimmunity was not attributable to modulation of NF-κB. Instead, CD2-Foxj1 transgenic mice exhibited a peripheral T cell lymphopenia, associated with an accumulation of mature single-positive thymocytes. Transgenic thymocytes demonstrated unimpaired lymphoid organ entry in adoptive transfer studies but demonstrated impaired thymic egress in response to CCL19, apparently independently in adoptive transfer studies but demonstrated impaired thymic egress in response to CCL19, apparently independent of CCR7, S1P1, and NF-κB. These findings confirm the importance of Foxj1 in the regulation of T cell tolerance but furthermore suggest a novel and specific role for Foxj1 in regulating thymic egress. The Journal of Immunology, 2005, 175: 7805–7809.

Members of the forkhead family of “winged-helix” transcription factors are important regulators of immune cell development and effector function. For example, Foxp3 regulates the development of regulatory T cells, Foxj1 and Foxo3a regulate CD4 T cell tolerance, and Foxn1 regulates thymic epithelial differentiation. As such, immunoregulation is coordinated by these transcription factors in a variety of immune cell types, and their dysregulation likely contributes to the pathogenesis of several immunological disorders (1, 2).

We previously identified Foxj1 in a microarray screen to identify novel transcription factors involved in autoimmune disease. Foxj1 expression was significantly down-regulated in lymphocytes from lupus-prone mice. Foxj1 is a modulator of Th1 activation, with its deficiency resulting in multiorgan systemic autoimmunity due to a role in antagonizing NF-κB activity (3, 4). An analogous role for Foxj1 exists in B cells, but there Foxj1’s importance seems less critical, since only modest defects in humoral tolerance can be attributed to intrinsic defects in Foxj1−/− B cells (4). Nonetheless, Foxj1 clearly plays a critical role in maintaining lymphocyte quiescence and tolerance.

Such observations predict that ectopic Foxj1 expression in lupus-prone mice, particularly in T cells, will repress autoimmunity. Here, we tested this hypothesis by generating Foxj1 transgenic (Tg)3 animals.

Materials and Methods

Mice

C57BL/6, MRL/MpJ-Fas(+/−)1 (MRL/lpr; The Jackson Laboratory), and B6SJL-Ppr−/− Peg3−/−BoyCr (C57BL/6-CD45:1; National Cancer Institute) mice were maintained under specific pathogen-free conditions. To generate CD2-Foxj1 Tg mice, an −1.7-kb Kpn1-Xbal fragment containing the Foxj1 cDNA from pcDNA-Foxj1 (5) was cloned into the Smal site of a human CD2-based Tg construct (6) (a gift from L. Glimcher, Harvard School of Public Health, Boston, MA). A Kpn1-Xbal fragment of this resultant plasmid, containing the CD2 promoter and locus control regions flanking the Foxj1 cDNA, was submitted to the Microinjection Core Facility of the Washington University School of Medicine for the generation of C57BL/6 Tg animals. Mice were screened by PCR using primers 5′-CACCGGGAAGCCCAATGCCTC and 5′-CCCGCCGGGCCTCATCTTTTC, which yielded a ~314-bp product corresponding to the Foxj1 cDNA. Of the 56 potential founders screened, three were found positive for the transgene, two of which bred successfully (lines 5 and 13; B6-Tg(CD2-Foxj1)5TgSp and B6-Tg(CD2-Foxj1)13TgSp). Similar results seen in this study were obtained with both founder lines (our unpublished data). MRL-Fas−/− B6-Tg(CD2-Foxj1)5Sp (Foxj1−/−Tg−/−)3 animals were generated by backcrossing the original founder lines against the MRL/lpr background over five generations, using a speed congenic strategy that ensures MRL homozygosity at all 24 proposed MRL disease susceptibility loci, as well as IgH, H-2, and CD95 (summarized in Ref. 7). All experiments were performed in compliance with the relevant laws and institutional guidelines, as overseen by the Animal Studies Committee of the Washington University School of Medicine. Assessment of murine lupus parameters, as well as Western blots and transcription factor assays, were performed as described (3, 8) or via the TransFactor ELISA kit (BD Clontech).

Thymocyte adoptive transfer

In this study, we adapted a previously described method (9). CFSE-labeled C57BL/6-CD45.1 thymocytes (5 × 106) were mixed with total thymocytes from either CD2-Foxj1 Tg or non-Tg (CD45.2) recipients, correcting to comparable numbers of Qa-2+ single-positive cells. The mixture was injected i.v. into C57BL6-CD45.1 recipients, and 24 h after transfer the numbers and percentages of CFSE− vs CD45.2 − CD4+ vs CD8+ cells in the indicated tissues were identified by flow cytometry. The relative frequency of Tg vs non-Tg cells was compared by Student’s t test (9) and corrected for multiple comparisons using the Bonferroni method (9).

Received for publication August 24, 2005. Accepted for publication October 14, 2005.

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1 This work was supported by National Institutes of Health Grants AI057471 and AI061478.

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3 Abbreviations used in this paper: Tg, transgenic; Foxj1−/−Tg−/−, CD2-Foxj1−/− transgenic positive, C57BL6/Foxj1−/−Tg−/−, CD2-Foxj1 transgenic-positive, MRL/lpr; SP, single-positive thymocyte (CD4+CD8− or CD4−CD8+).

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(CD45.2+), were determined vs the cotransferred control cells (CFSE+), corrected for any differences in the input ratio of Tg or non-Tg and control cells.

**Thymocyte egress assay**

Here, we also adapted a previously described method (10). Thymocytes from Tg or non-Tg littermates were cultured at 5 × 10^7/0.1 ml in a transwell chamber with a 5-μm pore size polycarbonate membrane (Costar) inserted in 0.6 ml of culture medium, supplemented with or without the CCR7 ligand CCL19, 100 ng/ml. After 120 min, cells were recovered from the upper and lower chambers and examined for CD4 vs CD8 expression by flow cytometry.

**Results and Discussion**

**Foxj1 overexpression protects against murine lupus**

To examine the role of Foxj1 in autoimmunity, we generated Tg mice that overexpress Foxj1 in T cells via human CD2 locus control regions (Fig. 1A). To determine whether Foxj1 could protect against autoimmunity in murine lupus, we backcrossed these mice against the lupus-prone MRL/lpr background, which is relatively deficient in Foxj1 expression (3, 4). Strikingly, Foxj1-Tg-lpr mice showed a significant reduction in several disease parameters: they developed reduced lymphadenopathy, particularly of the lymph nodes (Fig. 1B, p < 0.05 and p < 0.001 comparing Tg with non-Tg spleen and lymph nodes, respectively), associated with diminished accumulation of CD3+CD4−CD8−B220+ double-negative T cells (Fig. 1C, p < 0.0001 comparing percentage of double-negative T cells in non-Tg with Tg), which accumulate in the peripheral lymphoid organs of lpr mice as a result of autoreactive T cells unable to complete activation-induced cell death (11).

Although Foxj1-Tg-lpr animals produced anti-DNA autoantibodies, as detected by ELISA, these were not high-affinity anti-dsDNA autoantibodies as determined by *Crithidia* immunofluorescence (Fig. 1D), suggesting the presence only of low-affinity anti-ssDNA, but not high-affinity anti-dsDNA activity (12, 13)—consistent with impaired T cell activation, which is generally required for the pathogenic maturation of anti-DNA autoantibodies from anti-ssDNA to anti-dsDNA specificities (13, 14). Finally, histopathological examination of Foxj1-Tg-lpr mice revealed significantly reduced inflammation of several end-organs, including salivary gland, lung, liver, and kidney, compared with non-Tg littermates (Fig. 1E; disease scores of salivary gland, lung, liver and kidney: 0.2 ± 0.4 vs 2.8 ± 0.4; 0.2 ± 0.4 vs 2.4 ± 0.9; 0.2 ± 0.4 vs 2.6 ± 0.5; and 0.4 ± 0.5 vs 3.8 ± 0.4, respectively, on a 0–4 scale; n = 5 and p < 0.001 for all comparisons). Thus, Tg overexpression of Foxj1 significantly protects against murine lupus, as judged by lymphadenopathy, autoantibody production, and end-organ disease.
CD2-Foxj1 Tg mice have peripheral T cell lymphopenia

To understand further how Foxj1 overexpression reduced autoimmunity, we examined Foxj1 Tg C57BL/6 (Foxj1-Tg-B6) mice. Surprisingly, they displayed significantly decreased lymphoid organ cellularity due to reduced T cell number of both CD4 and CD8 subtypes (Fig. 2, A and B, p < 0.0001 comparing Tg with non-Tg for total cellularity, CD4 and CD8 cells). Peripheral blood yielded similar findings, with significantly reduced circulating total lymphocyte, CD4 and CD8 T cell counts in Foxj1-Tg-B6 animals (Fig. 2, C and D, p < 0.0001 comparing Tg with non-Tg for total lymphocytes, CD4 and CD8 cells). Analogous observations were indeed obtained in the MRL/lpr background, where Tg animals contained approximately 8- to 10-fold reduced peripheral CD4 counts (peripheral blood: 0.16 ± 0.038 vs 0.93 ± 0.11 × 10⁶; lymph node: 6.9 ± 2.4 vs 62.9 ± 17.7 × 10⁶; p < 0.0001 for all comparisons). Thus, Foxj1 overexpression induces a peripheral T cell lymphopenia. Since T cells, particularly CD4⁺, are critically required for the cellular and humoral disease in MRL/lpr mice (e.g., Refs. 14 and 15), such findings further indicate that Foxj1 protects against autoimmunity via this T cell lymphopenia.

CD2-Foxj1 Tg mice display a specific defect in thymocyte egress

Interestingly, this lymphopenia did not reflect defective T cell development per se, since Foxj1-Tg-B6 thymi generally possessed increased proportions of CD4⁺CD8⁻ single-positive (SP) thymocytes—which exhibited wild-type levels of CD4, at least as judged by flow cytometry—with comparable numbers of CD4⁺CD8⁻ double-positive thymocytes to their non-Tg littermates (Fig. 3A, p < 0.01, comparing numbers and percentages of total CD4⁺ SP thymocytes between Tg and non-Tg). Indeed, larger numbers of CD4⁺CD8⁻ CD24lowQa-2high mature SP thymocytes were present in the thymi of Tg animals (Fig. 3, A and B), suggesting that SP T cell maturation was not affected developmentally in the presence of the Foxj1 transgene.

The peripheral, but not central, T cell lymphopenia could have reflected abnormalities in apoptosis, lymphoid organ entry (cell motility), recirculation, or thymic egress. However, Tg CD4 cells, central or peripheral, did not demonstrate abnormally increased apoptosis, as judged by annexin V or propidium iodide staining (Fig. 2B and data not shown). In addition, Tg thymocytes competed as, if not more, effectively as non-Tg thymocytes in lymphoid organ entry assays involving co-adaptive

FIGURE 2. Peripheral T cell lymphopenia in Foxj1 Tg C57BL/6 mice. Leukocyte populations were quantified and/or phenotyped by standard cell counting, manual differential, and/or flow cytometry in the spleen, lymph nodes (A and B), and peripheral blood (C and D) of Foxj1 transgenic vs non-Tg littermates at 6–8 wk of age. B and C demonstrate representative flow cytometric plots used for graphs in A and D.
transfer of CFSE-labeled, C57BL/6-CD45.1 thymocytes and total thymocytes from either Foxj1 Tg or non-Tg (CD45.2+/H11001) littermates into C57BL6-CD45.1 recipients (Fig. 4A). Also, the Foxj1 Tg thymocytes were unimpaired in their ability to recirculate in the blood (Fig. 4A). On the other hand, when thymic exodus of SP thymocytes was assessed in transwell assays in vitro, both CD4+/H11001 and CD8+/H11002 thymocytes from Foxj1-Tg-B6 mice displayed significantly defective migration in response to the CCR7 ligand CCL19 (10) (Fig. 4C). Analogous findings were observed in Foxj1-Tg-lpr animals, which, compared with their non-Tg littermates, contained increased numbers of mature CD4 thymocytes (total CD4 SP 23.7±1.6 vs 16.5±2.9×10⁶; CD4+ Qa-2high 16.9±0.3 vs 3.0±0.5×10⁶; p < 0.001 for both comparisons) that migrated significantly less efficiently in response to CCL19 in vitro (CCL19 induced 21.3±6.0 from 9.7±1.0×10⁵ vs 140.0±2.0 from 10.0±1.0×10⁴ cells in transwell assays, respectively; p < 0.001). Thus, Foxj1 overexpression inhibits the ability of SP thymocytes to emigrate from the thymus, causing peripheral T cell lymphopenia in both the non-autoimmune C57BL/6 and autoimmune MRL/lpr backgrounds.
The role of Foxj1 in thymocytes and T cells

The importance of forkhead transcription factors in thymocyte development has long been recognized due to the importance of Foxn1 (nude) in thymic epithelial cells (16), as well as one previous Tg study with Foxo1 demonstrating modest decreases in total thymocyte numbers in Tg mice (17). Foxj1 itself is expressed at a low level in thymocytes, especially SP CD4+ cells, though its function there remains incompletely understood (see Ref. 3 and data not shown). Thus the present findings expand the pathways by which the Fox genes regulate T cell development to control the release of thymocytes into the periphery.

Although a growing number of chemokine and molecular gradient systems has been implicated in the regulation of thymocyte migration through the thymus and emigration, including CCR7-CCL19 (10), CXCR4-SDF-1 (18), lymphotoxin-β (19), and S1P receptor 1 (9), the role of Foxj1 in the modulation of these systems remains unclear. On one hand, Foxj1 Tg mice share some of the severe defects exhibited by deficiencies in these molecules, such as peripheral lymphopenia. However, on the other hand, Foxj1 Tgs exhibit some distinguishing characteristics: e.g., peripheral lymphoid organ entry is intact in Foxj1 Tg but is defective in the absence of CCR7 (9, 20). In addition, real-time PCR, microarray studies and/or flow cytometry results indicate that Tg CD4+ Qa-2(high) SP thymocytes contain comparable levels of CCR7, CXCR4, LTβR, and S1P1, suggesting that at least their expression is not modulated by Foxj1 (data not shown). Also, Foxj1 Tg thymocytes contained expression and activity levels of NF-κB proteins, which are modulated by Fox genes in peripheral T cells (3, 21), comparable to their non-Tg counterparts (Fig. 4, C and D, and not shown). Indeed, microarray analyses of CD4+ Qa-2(high) SP thymocytes between CD2-Foxj1 Tg and non-Tg littermates indicate that the genes with the most significantly altered expression, which presumably represent direct and/or indirect targets of Foxj1, include a large panel of genes of unknown or unclear immunological function, without reflecting differences in pathways related to NF-κB activation or (chemokine-induced) cell migration, as might be predicted (data not shown). Thus the physiological mechanisms by which Foxj1 regulates thymic egress remain to be fully elucidated. Still, these data describe Foxj1 as a novel factor in thymocyte emigration and reinforce its role in the regulation of immunological tolerance.

Acknowledgments

We thank Laurie Glimcher for the CD2 Tg construct, and Mike White for assistance with Tg animal production.

Disclosures

The authors have no financial conflict of interest.

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