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Higher expression of Bax in regulatory T cells increases vascular inflammation

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Abstract

This study is to examine our hypothesis that CD4+CD25^{high}Foxp3+ regulatory T cells (Tregs) have an interleukin-2 (IL-2) withdrawal-triggered apoptosis pathway, and modulation of Treg apoptosis pathway affects development of vascular inflammation. We found that pro-apoptotic protein Bax upregulation in Tregs is induced by IL-2 withdrawal. Treg apoptosis induced by IL-2 withdrawal is inhibited by a Bax inhibitor, suggesting that highly expressed Bax is functional. To define the role of upregulated Bax in Treg apoptosis, we established a Tregs-specific Bax transgenic mouse model. Enforced expression of Bax in Tregs promotes Treg apoptosis triggered by IL-2 withdrawal and other apoptosis stimuli, suggesting pro-apoptotic role of highly expressed Bax in wild-type Tregs. Finally, higher expression of Bax in Tregs decreases the striking threshold of vascular inflammation due to the failure of suppression of inflammatory cells resulting from Treg apoptosis. These results have demonstrated the proof of principle that the modulation of Tregs apoptosis/survival could be used as a new therapeutic approach for inflammatory cardiovascular diseases.

Keywords

Regulatory T Cells; Apoptosis; Bax; Inflammation; Vasculitis

2. INTRODUCTION

CD4+CD25^{high}Foxp3+ regulatory T cells (Tregs), comprising 5–10% of CD4+ T cells(1), exhibit potent immunosuppressive functions(2) in the regulation of autoimmunity and inflammatory atherosclerosis (3,4). Naturally occurring Treg cells (thymus-generated, nTreg cells), as an independent subset, are engaged in the maintenance of immunological self-tolerance and inhibition of various immune responses (5) and inflammatory atherogenesis. nTregs (Tregs in the rest of paper) appear to have specific apoptosis pathways since Treg cells have higher susceptibility to apoptosis (6), especially to IL-2 withdrawal-induced apoptosis. Tregs are poor IL-2 producers(7), implying that insufficient paracrine IL-2 supply to Tregs in pathological conditions could be responsible for higher susceptibility of Tregs to apoptosis. However, intracellular regulation of IL-2 insufficiency-triggered Treg apoptosis remains poorly defined. The Bcl-2/Bcl-xL protein family members play a central role in the

regulation of apoptosis(8). Recently, we identified a novel Bcl-xL-interacting anti-apoptotic protein, translationally controlled tumor protein (TCTP)(9). Although TCTP is not a Bcl-2/Bcl-xL family member, down-regulation of endogenous TCTP expression by antisense results in the increase of T cell apoptosis(9). The role of TCTP in promoting Treg survival is under intensive investigation. Our recent report showed that expression of a prototypic pro-apoptotic protein, Bax, in Bcl-2 family is higher in Tregs than in CD4+CD25⁻ T cells(10), suggesting an important role of Bax protein in regulating Treg apoptosis. A recent report suggested that Bax does not have to adopt its final conformation to drive T cell death, which supports a functional significance of higher expression of Bax in inducing Treg apoptosis(11).

Tregs play an important role in controlling the pathogenesis of inflammatory atherosclerosis(3,4). In addition, IL-2 knock-out mice have a deficiency in Tregs, and spontaneously develop autoimmune diseases including vasculitis(12). However, it remains to be determined if a deficiency in Treg generation in IL-2 knock-out mice, or higher susceptibility of Tregs to apoptosis induced by IL-2 deficiency, contributes to accelerated vasculitis. Since Tregs with high affinity IL-2 receptor have higher susceptibility to apoptosis in comparison to CD4+CD25⁻ T cells, characterization of IL-2 withdrawal-Bax pathway in Tregs would be important in generating a substantial quantity of well-survived Tregs for Treg-based immune therapeutics(13). Therefore, three important questions remained to be answered toward this goal. First, whether high expression of Bax in Tregs is under regulation by an IL-2 receptor-initiated signaling; second, whether highly expressed Bax in Tregs is functional in driving Treg apoptosis; and third, whether higher expression of Bax in Tregs accelerates the development of vascular inflammation. To examine these questions, we established a mouse CD25 promoter(14)-directed Bax transgenic mouse model (Bax Tg). We found that Bax Tg resulted in enhanced apoptosis of Tregs. Interestingly, we also found that Treg apoptosis led to accelerated development of vasculitis presumably due to promotion of Treg apoptosis and failure in inhibiting inflammatory cells. These results have demonstrated the proof of principle that novel therapeutics can be developed for treating vasculitis and atherosclerosis via inhibiting Bax expression in Tregs and promoting survival of Tregs(15).

3. MATERIALS AND METHODS

3.1. Construction of CD25⁺ T cell-targeting mouse IL-2R α promoter-Bax transgenic mice

Mouse IL-2 receptor α chain (CD25) promoter -2539 to +93 (GenBank Accession Number: M16398) vector pmIL2R α -CAT1 (6.9 kb) was generously provided by P. Reichenbach and M. Nabholz(14). The construction of the CD25⁺ T cell targeting vector pCD25-Tg was described previously (10). The transgenic vector pCD25-Bax-Tg was verified by DNA sequencing by SeqWright Company (Houston, TX). The 3.818 kb transgenic DNA fragment “Nru I-CD25 promoter-C-Myc-Bax-Sex AI” was prepared by digesting the pCD25-Bax-Tg vector with three restriction enzymes, Nru I, Sex AI, and Pvu I (New England Biolabs, Ipswich, MA), gel-purified, and microinjected into zygotes from C57BL/6 mice in the Baylor College of Medicine Genetically Engineered Mouse Core. All animal experiments in this study were performed according to the US National Institutes of Health guideline and the protocols approved by Baylor College of Medicine and Temple University Institutional Animal Care and Use Committees.

3.2. PCR and RT-PCR

Transgenic mice were identified by PCR with mouse tail DNA as the templates using the special primers (Figure 2-B). The amplified PCR products were subcloned into TOPO TA plasmid (Invitrogen, Carlsbad, CA) and confirmed by DNA sequencing. Enforced

expression of Bax in CD25+ T cell-specific manner in these transgenic mice was confirmed by RT-PCR using the primers (C-Myc primer and Bax anti-sense primer) that distinguish transgenic and endogenous RNA transcripts(16). All mice used in the experiments were age- and sex-matched. Total RNA isolation and RT-PCR were performed as previously reported(17).

3.3. Flow cytometric analysis

Single cell suspensions of samples were treated with ammonium chloride to remove erythrocytes, washed, and then stained with antibodies (Abs) for various cell markers. Cells (1×10^6) were suspended in 100 μ l of PBS containing 2% BSA and stained with 1 μ g of various fluorescent Abs for 30 min at 4°C, and analyzed on FACSCalibur (BD Bioscience, San Jose, CA). Forward and side scatter gatings were used to exclude debris from the analysis. Cells were stained with FITC-, PE-, or PE-Cy7-conjugated monoclonal Abs (mAbs) against: CD4, CD8a, TCR β , CD3 ϵ , and CD25 (BD Pharmingen, San Diego, CA). PE-, PE-Cy7- and FITC-conjugated rat and hamster IgG were used as controls.

3.4. Intracellular Staining for Bax and FOXP3

Bax-FITC antibody (Ab) and Anti-Foxp3 Ab were purchased from Santa Cruz Biotech (Santa Cruz, CA) and eBioscience (San Diego, CA), respectively. Intracellular staining of freshly isolated cells for Bax and Foxp3 was performed as previously described(10).

3.5. T Cell Activation

Mouse splenic T cells were prepared as previously reported(16) and activated by either plate-bound anti-CD3 (145.2C11 clone, 1 μ g/ml, BD PharMingen) alone, or plate-bound anti-CD3 (1 μ g/ml) and anti-CD28 (37.51 clone, 1 μ g/ml, BD PharMingen) (16,17).

3.6. Cell Culture and Induced Apoptosis *In vitro*

As normal culture condition with RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS, Hyclone, Logan, UT), mouse IL-2 (Sigma-Aldrich, St. Louis, MO) was added to the splenocytes culture at 5 ng/ml. IL-2 was withdrawn for 12 hours to induce apoptosis. After 12 and 24 hours, cell viabilities were determined by flow cytometry on the FACSCalibur flow cytometer with Annexin V-FITC Apoptosis Detection Kit (BD PharMingen)(18).

3.7. Perivascular cuff-injury induced vasculitis of femoral artery

The femoral artery was looped with a ligature and a non-flow obstructing polyethylene cuff (0.3 mm inner diameter, 0.70 mm outer diameter) was placed on the femur artery to induce stenosis, and closed with a ligature. Finally, the skin incision was closed with a running suture(19).

3.8. Histology

The images of lymphoid tissues were taken with a Zeiss Axioskopz Plus microscope (Oberkochen, Germany). The tissue areas in the images were analyzed by the software Image-Pro Plus 6.0 and used as the indices of the sizes of tissues as reported(20). Femoral arteries with cuff-induced stenosis were removed, snap frozen in liquid nitrogen, and stored at -80°C. Serial cryostatic sections (5- μ m thick) were cut consecutively: hematoxylin-eosin (H&E)-stained slides were used to examine the vessel affected by induced vasculitis with a microscopy (Zeiss LSM 310; Zeiss).

4. RESULTS

4.1. Tregs have significantly higher expression of Bax and higher rates of apoptosis after IL-2 withdrawal

We first determined whether insufficient paracrine IL-2 supply to Tregs(7) could trigger upregulation of Bax expression and higher rates of Treg apoptosis. Based on CD25 expression levels, we gated splenic CD4⁺ T cells into three fractions, CD4⁺CD25[−] fraction, CD4⁺CD25^{low} fraction, and CD4⁺CD25^{high} fraction (Figure 1A). Since over 96% of CD4⁺CD25^{high} T cells(21) express Treg-specific transcription factor FOXP3(22,23), CD25^{high} was used as a cell surface marker for Tregs in this study(24). The results showed that IL-2 withdrawal for 12 hours significantly upregulated apoptosis rates of wild-type CD4⁺CD25^{high} Tregs from 11.6% (with IL-2 control) to 19.4% (Figure 1B). In comparison, the apoptosis rates of CD4⁺CD25[−] T cells were only increased from 2.5% (with IL-2 control) to 3.5%. In addition, the higher apoptosis rates of Tregs and CD4⁺CD25^{low} cells were inhibited by a Bax-specific inhibitor from 19.4% and 17.1% to 15.4% and 11.0%, respectively, but not by negative inhibitor control (not shown), suggesting that Bax is functional in mediating IL-2 withdrawal-triggered Treg apoptosis. We then examined the issue, detected by FITC-conjugated anti-Bax monoclonal antibody as we reported(10), whether IL-2 withdrawal upregulates intracellular Bax expression in Tregs. The results showed that most (88% to 98%) of wild-type CD4⁺ T cells express low levels of Bax, as shown in the upper gates in each FACS plot (Figure 1C1). Interestingly, Treg fraction with higher Bax expression (e.g. higher fluorescence intensity, higher FI) was increased from 22.7% at 0 hour to 43.1% at 12 hours after IL-2 withdrawal (Figure 1C1, right panel and Figure 1C2) whereas CD4⁺CD25[−] T cell fraction with higher FI of Bax at 0 hour (7.8%) was not significantly changed at 12 hrs after IL-2 withdrawal (Figure 1C1, left panel and Figure 1C2). The results suggest that IL-2 withdrawal upregulated Bax expression in Tregs (Figure 1C1), which was correlated with higher apoptosis rates of Tregs induced by IL-2 withdrawal (Figure 1B). Furthermore, since tumor suppression transcription factor p53 promotes apoptosis by binding to Bax promoter and upregulating Bax expression(25), we asked whether upregulated expression of Bax in Tregs induced by IL-2 is mediated by p53 function. The results showed that upregulated expression of Bax in Tregs was inhibited by p53 inhibitor (Figure 1D1, right panel and Figure 1D2) from 37.5% (vehicle control) to 23.4%, suggesting that Bax upregulation triggered by IL-2 withdrawal is mediated via a transcription mechanism(26). Of note, it is well-known that Bax transcription is mediated by a p53 dependent pathway. However, inhibition of Bax upregulation by p53 inhibitor suggests that transcriptional mechanism plays an important role in Bax upregulation. Transgenic approach becomes only appropriate if the transcription of genes plays an important regulatory role in the expression of genes. Therefore, the results suggested the feasibility of using the transgenic approach to upregulate Bax expression to define the functional significance of upregulated Bax in Treg apoptosis. In addition, the results suggested the following working hypothesis: IL-2 withdrawal triggered Treg apoptosis is mediated by upregulated functional Bax (Figure 1E).

4.2. Bax-Tg results in increased Bax expression in Tregs

We then used the transgenic approach to express Bax in Tregs under the direction of CD25 promoter (Figure 2-A), since CD25^{high} is a reliable marker for Tregs. The detailed justification for use of CD25 promoter to direct the expression of Bax transgene (Tg), rather than other promoter, is presented in the Discussion. The results of genomic DNA PCR analysis with mouse tail DNAs showed that two founders of Bax Tg were generated (Figure 2-C). The RT-PCR analysis with RNAs prepared from three lymphoid tissues including thymus, lymph nodes (LN), spleen, and two non-lymphoid control tissues, liver and kidney, of Bax Tg showed that transgenic Bax transcripts were expressed in lymphoid tissues but

not in non-lymphoid tissues of Bax Tg mice nor in all the tissues from wild-type littermate controls (Figure 2-D). To further confirm the expression of transgenic Bax protein, we used FACS analysis of Bax intracellular staining as reported for expression of other transgene(27). The results showed that Bax protein expression in CD4+CD25+ Tregs from Bax Tg mice was statistically higher than that in CD4+CD25+ Tregs from wild-type controls (Figure 2-E). The results were correlated with the cell expression patterns of CD25 gene promoter(14).

4.3. Reduced numbers of CD25+ thymocytes in Bax-Tg result in decreased positive selection of thymocytes

We determined whether reduced numbers of Tregs affect sizes and histology of lymphoid tissues in Bax Tg mice. The results showed that the sizes of Bax Tg thymus were not statistically different from that of wild-type thymus ($p>0.05$) (Figure 3-A). Similarly, the sizes of lymph nodes (LN) of Bax Tg mice were not significantly different from that in wild-type control mice ($p>0.05$) (Figure 3-B). In contrast, the sizes of Bax Tg spleens were larger than those of wild-type spleens ($p<0.05$) (Figure 3-C). These results suggest that Treg homeostasis plays a critical role in maintaining normal sizes of spleen(28).

We examined the major subsets of thymocytes and T cells in spleen and lymph nodes in Bax Tg mice in comparison to those in wild-type control mice (Figure 4-A, upper panel). The results showed that CD4+ single positive (SP) thymocytes and CD8+ SP thymocytes were significantly reduced in Bax Tg mice by 46% and 62% ((WT-Bax Tg)/WT), respectively, at 3 weeks old. The results were correlated with a reduction of CD4+ SP thymocytes (67.2% of that in wild-type control mice) and CD8+ SP thymocytes (72.3% of that in wild-type control mice) observed in T cell-specific CD2 promoter Bax transgenic mice(29). These results also suggest that CD25+ thymocytes are a group of very important thymocytes facing positive selection. Correlated with the reduction of mature CD4+ SP and CD8+ SP, CD4+CD8+ double positive (DP) thymocytes were proportionally increased in Bax Tg mice. CD3+ thymocytes and CD4+CD8-CD25^{high} thymocytes were also reduced, suggesting that overexpression of Bax in CD25^{high} thymocytes result in decreased positive selection and thymocyte maturation from the CD4+CD8+ DP stages to the CD4+ SP stages. These results also suggest that CD25^{high} Tregs share the same positive selection pathway with other non-Tregs thymocytes(30). In addition, splenic CD4+CD25^{high} Tregs were decreased by 13% in Bax Tg mice in comparison to that of wild-type controls ((WT-Bax Tg)/WT) (Figure 4-A, middle panel). Of note, no significant changes were found in the percentages of major T cell subsets and B cells in spleens and lymph nodes from Bax Tg mice in comparison to that from wild-type control mice. Correlating with the decreased CD4+CD25^{high} Tregs in Bax Tg spleen and lymph nodes (Figure 4A, lower panel), the expression of Foxp3+ in CD4+CD25^{high} fraction in Bax Tg mice was decreased about 5% in comparison to that of wild-type mice (Figure 4-B). Since Foxp3 promotes apoptosis by itself(31), our results suggest that Bax may have a synergistic effect with Foxp3 in facilitating Treg apoptosis. Tregs from Bax Tg mice have a suppressive function similar to that from wild-type control mice by adding Tregs to inhibit the proliferation of carboxyfluorescein succinimidyl ester (CFSE)-labeled wild-type CD4+CD25- T cell culture (not shown). In contrast, the percentages of splenic CD4+ T cells and CD8+ T cells were not significantly changed in Bax Tg mice from that of wild-type control mice. Our results correlate well with the previous report that the T cell decrease and thymocyte loss were obvious in 3 week-old mice, and could also be compensated in 9 week old Bax-/- deficient mice presumably via a putative mechanism of peripheral homeostatic expansion(18).

4.4. Tregs from Bax Tg mice are more susceptible to apoptosis induced by T cell activation and IL-2 withdrawal

We then used the transgenic approach to express Bax in Tregs under the direction of CD25 promoter (Figure 2-A), since CD25^{high} is a reliable marker for Tregs. We examined the issue of whether higher expression of Bax confers Tregs the higher susceptibility to apoptosis induced by T cell activation. The results showed that stimulation for 12 hours with plate-bound anti-CD3 antibodies (1 µg/ml) and anti-CD28 antibodies (1 µg/ml) induced more apoptosis (60.5%) to CD4+CD25^{high} Tregs in Bax Tg mice in comparison to that (43.4%) of wild-type Tregs in the absence of IL-2 (Figure 5A, second panel). The apoptosis rates of CD4+CD25^{high} Tregs from Bax Tg mice and wild-type control mice were even higher when stimulated with higher concentration (5 µg/ml) of anti-CD3 and anti-CD28 antibodies (Figure 5A, third panel). In addition, we determined whether higher expression of Bax confers Tregs the higher susceptibility to apoptosis induced by IL-2 withdrawal. The results showed that the apoptosis rates of CD4+CD25^{high} Tregs were increased to 28.3% (Figure 5B, central panel) from the 14.5% of wild-type control mice in the absence of IL-2 for 12 hours (Figure 5B, left panel). The apoptosis rates in CD4+CD25^{high} cells of Bax Tg mice was higher than that of CD4+CD25^{high} cells from wild-type controls, suggesting that higher expression of Bax and IL-2 withdrawal has a synergistic effect in promoting Treg apoptosis. The sufficient IL-2 supply significantly inhibited Treg apoptosis of wild-type mice and Bax Tg mice (Figure 5B, right panel), suggesting that Bax plays a critical role in IL-2 withdrawal-triggered Treg apoptosis.

4.5. Higher expression of Bax in Tregs lowers striking threshold and accelerates development of experimental vasculitis

Tregs play an important role in suppressing the development of atherosclerotic plaques in ApoE^{-/-} mice presumably via inhibition of atherogenic autoimmune inflammatory responses(3). The IL-2 expression, a key Treg survival factor, was absent in 50% of human atherosclerotic plaques(32), suggesting that insufficient paracrine IL-2 supply is an atherogenesis-associated pathological condition. However, the question remained unknown whether insufficient paracrine IL-2 supply triggered-Bax upregulation affects the development of experimental vasculitis in ApoE^{+/+} background. To examine this question, we adopted a well-characterized perivascular cuff-injury induced vasculitis(19) (Figs. 6A and B). The results showed that higher expression of Bax in Tregs significantly promoted inflammatory responses to loose placement of a non-flow obstructing cuff around the femoral artery, which led to partial closure of vasculitis-affected artery (Figure 6C). In contrast, loose placement of a non-flow obstructing cuff did not result in severe vasculitis in wild-type control mice (Figure 6C1). The statistical analysis of the ratios of vessel wall area over whole vessel section area showed that the areas in the vessel wall in Bax Tg mice, after placing the cuff, were significantly higher than those in the vessel wall in wild-type control mice under the same condition ($p < 0.05$) (Figs. 6C2 and 3), suggesting that inflamed vessel wall in Bax Tg after placing the cuff was significantly thicker than that in wild-type mice. Correlating with the thickened vessel wall, the infiltrated inflammatory cells in the vessel from Bax Tg after cuff placement were significantly higher than that in wild-type mice (Figure 6C4). Since it has been reported that CD4+CD25⁺ Tregs can exert direct suppressive effects on monocytes/macrophages in addition to suppressing effector T cells(33), therefore, our results suggest that the higher expression of Bax in Tregs reduces the striking threshold of inflammatory pathogenesis of vasculitis due to higher rates of Treg apoptosis and presumably failure in suppressing inflammatory cells (Figure 6D).

5. DISCUSSION

Previous reports have shown that Tregs are highly susceptible to apoptosis(15,34). Since Tregs generate poorly endogenous IL-2(7), Tregs require exogenous IL-2 for survival by expressing high levels of IL-2 receptor. However, IL-2-regulated Treg apoptotic pathways that underlie homeostatic mechanisms of Tregs remain poorly defined. Since homeostasis of Tregs is critical in maintaining immune tolerance and regulation of immune responses, elucidation of Treg-specific apoptotic pathways is significant(15). Our recent report showed that expression of pro-apoptotic protein Bax in Tregs is higher than that in CD4+CD25⁻ T cells in the absence of any stimuli, which may be responsible for higher apoptosis rates of Tregs(10). This report indicates that exploration of the mechanism underlying physiopathologically higher expression of Bax in Tregs is of significance. Toward this goal, our current study has demonstrated, for the first time, the following findings: (a) IL-2 withdrawal induces upregulation of Bax presumably via a p53 dependent transcription pathway; (b) enforced expression of Bax in Tregs reduces positive selection of major subsets of mature thymocytes including Tregs, CD4⁺ single positive thymocytes and CD8⁺ single positive thymocytes, suggesting that Tregs share the same positive selection pathway with other thymocytes. Bax transgene also promotes Treg apoptosis triggered by IL-2 withdrawal and other apoptosis stimuli; (c) upregulated Bax in Tregs results in lymphadenopathy-like histology in spleen, suggesting the importance of Tregs in maintaining normal splenic histological structure. Finally, our results show that higher expression of Bax in Tregs decreases the striking threshold of vascular inflammation due to promotion of Treg apoptosis and presumably failure in suppressing anti-vascular immune cell clones. Taken together, our data suggest that modulation of Tregs apoptosis/survival could be used as a therapeutic approach for inflammatory cardiovascular diseases(15).

nTregs are a special lineage of CD4⁺ T cells developed in thymus rather than a functional status, which makes a transgenic approach feasible. In addition to be a marker for Tregs, low levels but not high levels of CD25 can also be a T cell activation marker (30). However, up to 96% of CD4⁺CD25^{high} Treg cells express FOXP3(21), suggesting that CD25^{high} is an essential marker for FOXP3⁺ Tregs (31,32). Our Bax transgenic data showed that transgenic Bax promotes higher apoptosis rates of CD25^{high}FOXP3⁺ Tregs than that of CD4⁺CD25^{low}. Of note, although FOXP3 plays a critical role in Treg development and function, FOXP3 *per se* promotes Treg apoptosis(31), and Foxp3 expression in activated T cells does not necessarily lead to acquisition of suppression function(35). These results suggest that use of CD25 promoter to generate Bax transgenic mice in this study was well justified.

Inflammatory atherosclerosis and vasculitis share the inflammatory responses against vessel wall components. Tregs play an important role in suppression of the inflammatory atherogenic process since depletion of Tregs accelerates atherosclerosis (3). Similarly, IL-2 knock-out mice have a deficiency in Tregs and spontaneously develop autoimmune diseases, including vasculitis(12). The reports suggest that IL-2 pathway is essential in maintaining survival of apoptosis-prone Tregs and suppressing inflammatory responses against vascular cell components. However, several important questions remain poorly defined including what mechanism underlies higher apoptosis of Tregs in the absence of a sufficient supply of IL-2; and whether Treg apoptosis pathway is therapeutically significant in modulating the pathogenesis of vascular inflammation. Our results demonstrate that Bax plays an important role in IL-2 withdrawal-induced apoptosis pathway of Tregs. Proliferation and migration of vascular smooth muscle cells (VSMCs) during neointima formation induced by arterial injury represent a critical component of restenosis after angioplasty of human coronary arteries and an important feature of atherosclerotic lesions(36). Peri-vascular cuff placement induces neointima formation, and this model represents the early features of atherosclerosis,

such as proliferation of VSMCs but not foam cell formation. In this model, upregulated secretion of proinflammatory cytokines and increased inflammatory cell infiltration(37) may further lead to the exhausted availability of Tregs. As outlined in Figure 6D, higher expression of Bax in transgenic Tregs “amplifies” cuff-induced vascular inflammation. Future definition of the mechanisms of upregulation of Bax in Tregs may lead to development of new therapeutics for inflammatory vasculitis and atherosclerosis.

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Figure 1.

Association of IL-2 withdrawal-induced Bax upregulation and Treg apoptosis. A. Quantitation of wild-type splenic CD4+CD25^{low} T cells, CD4+CD25^{low} and CD4+CD25^{high} Tregs by flow cytometry with PE-conjugated CD25 and PE-Cy7-conjugated CD4 antibodies. In the left upper gate are shown the percentages of gated cell fractions. B. IL-2 withdrawal induced T cell apoptosis. The apoptotic rates of three CD4+ fractions, such as CD25^{low}, CD25^{low} and CD25^{high} in each of four indicated conditions (IL-2 withdrawal for 0 hours, IL-2 withdrawal for 12 hours, cultured with IL-2 for 12 hours, and IL-2 withdrawal plus incubation with Bax channel inhibitor for 12 hours) are presented as percentages of FITC-conjugated annexin V staining positive cells. The experiments were repeated four

times and the statistical data with the mean and standard deviations are presented. C1. Bax expression in three gated CD4⁺ cell fractions was measured in three conditions including IL-2 withdrawal for 0 hours, and IL-2 withdrawal for 12 hours by flow cytometry using FITC-conjugated anti-Bax monoclonal antibody. The upper gate in each FACS plot indicates the total Bax⁺ cells whereas the lower gate in each plot shows the cell fractions with higher fluorescence and elevated expression of Bax. C2. The experiments were repeated three times and the statistical data with the mean and standard deviations are presented. D1. Bax expression in three gated CD4⁺ cell fractions was measured in two conditions including IL-2 withdrawal for 12 hours in the presence of vehicle-control, and cultured without IL-2 but with p53 transcription factor inhibitor Pifithrin for 12 hours by flow cytometry using FITC-conjugated anti-Bax monoclonal antibody. D2. The experiments were repeated three times and the statistical data with the mean and standard deviations are presented. E. A working hypothesis of Bax upregulation in Tregs induced by IL-2 withdrawal-triggered signals.

Figure 2.

Generation of TCTP transgenic mouse model. A. Bax transgenic construct. A c-myc expression tag-fused Bax cDNA was placed under the direction of mouse CD25 promoter. The locations of primers used for PCR detection of transgenic Bax DNA, and RT-PCR detection of transgenic Bax transcript and endogenous Bax transcript are presented with arrows. In the lower panel, the primer sequences for detection of β -actin by RT-PCR are presented. B. The sequences of six primers used in this study are presented. C. Detection of Bax transgenic mice by PCR using mouse tail genomic DNAs as templates. No DNA template control and non-transgenic mouse (wild-type) tail DNA control are included. D. Detection of transgenic Bax transcripts by RT-PCR in lymphoid tissues of Bax transgenic

mice (upper panel). Equal amounts of RNAs prepared from non-lymphoid tissues (kidney and liver) from Bax transgenic mice and counterpart tissues from non-transgenic wild-type control mice were used in the experiment as shown by RT-PCR detection of β -actin transcripts (lower panel). E. Detection of Bax upregulation in CD4+CD25^{high} Tregs by flow cytometry using anti-Bax monoclonal antibody. Bax expression in splenic CD4+CD25⁻ T cells in six splenocyte preparations from wild-type control mice and six Bax Tg mice, which generated the background ratios of Bax expression in Bax Tg samples over Bax expression in wild-type controls. The statistical analysis of the background ratios generated a mean $\pm$ 2 $\times$ standard deviations ($0.93 \pm 2 \times 0.11$), as shown by the two dash lines. In comparison, the ratio of Bax expression in CD4+CD25⁺ Tregs from Bax Tg mice over the Bax expression in CD4+CD25⁺ Tregs from wild-type control mice was significantly higher than the upper limit of the Bax expression ratios in CD4+CD25⁻ T cells, suggesting significant upregulation of Bax expression in CD4+CD25^{high} Tregs in Bax transgenic mice.

Figure 3.

Enlarged spleens of Bax transgenic mice with abnormal histological architecture. A. The sizes of six thymuses from wild-type control mice and from Bax transgenic mice were analyzed with digital photography followed by calculation of the surface areas of tissues (cm²). The statistical analysis of the surface areas of thymus from two groups of mice is presented in the lower panel. B. The sizes of two sets of lymph nodes from wild-type control mice and from Bax transgenic mice were analyzed with digital photography followed by calculation of the surface areas of tissues (cm²). The statistical analysis of the surface areas of lymph nodes from two groups of mice is presented in the lower panel. C. The sizes of five spleens from wild-type control mice and from Bax transgenic mice were analyzed with

digital photography followed by calculation of the surface areas of tissues (cm²). The statistical analysis of the surface areas of spleens from two groups of mice is presented in the lower panel.

Thymus (%)	CD25 Bax	Wild type	#
DN	0.21	0.61	
DP	91.68	83.63	
CD4 SP	7.42	13.87	
CD8 SP	0.70	1.89	
CD4+CD8-CD25+	2.80	2.72	
CD4+CD8-CD25 High	0.96	1.23	22%
CD3+	48.54	58.11	
CD69+CD4+CD8-	73.89	77.03	

Spleen (%)	CD25 Bax	Wild type	#
CD4 T cell	10.53	11.19	
CD8 T cell	5.56	5.04	
CD19+ B cell	33.51	38.50	
B220+ B cell	54.03	65.25	
CD4+CD8-CD25+	4.07	4.41	
CD4+CD8-CD25 High	1.91	2.2	13%

LN (%)	CD25 Bax	Wild type	#
CD4 T cell	35.31	31.29	
CD8 T cell	19.93	14.09	
CD19+ B cell	9.95	17.01	
B220+ B cell	26.91	34.56	
CD4+CD8-CD25+	4.29	5.16	
CD4+CD8-CD25 High	1.96	2.47	21%

Figure 4.

The phenotypic analysis of major T cell subsets in Bax transgenic mice. A. A representative phenotypic profile of major subsets of thymocytes, T cells in spleens and lymph nodes is presented. The experiments were repeated at least four times. B. FACS analysis of Foxp3 expression in CD4+CD25^{low} T cells and CD4+CD25^{high} is presented.

A

B

Note: 1=CD4+ CD25-, 2=CD4+ CD25 Low, 3=CD4+ CD25 High

Figure 5.

Higher susceptibility of Bax transgenic Tregs to apoptosis. A. Higher susceptibility of Bax transgenic Tregs to apoptosis induced by IL-2 withdrawal. Splenocytes from wild-type control mice (lower panel) and Bax transgenic mice (upper panel) were incubated in the presence of 0 ng/ml, 1 ng/ml, 5 ng/ml and 20 ng/ml of IL-2 for 24 hours followed by FACS analysis of apoptotic CD4+CD25high Tregs with FITC-Annexin V. The experiments were repeated three times and the representative FACS analyses are presented. B. FACS analyses of higher susceptibility of Bax transgenic Tregs and wild-type control Tregs to apoptosis induced by IL-2 withdrawal for 0 hours, 12 hours and incubation for 12 hours in the

presence of IL-2 were performed with FITC-Annexin V. The experiments were repeated three times and the statistical data are presented.

Figure 6.

Decreased striking threshold of cuff-injured vasculitis in Bax transgenic mice. **A.** A schematic representation of the experimental model of non-constrictive cuff placement-induced vasculitis in a single side of the mouse femoral artery. **B.** Non-constrictive cuff placement did not block the blood supply in the femoral artery before the tissue preparation (left panel). The left leg, without cuff placement, from the same mouse served as the control (right panel). **C.** Histological analysis of the vessel wall sections of Bax transgenic mice (Bax) and wild-type control mice (WT) with (upper panel) or without cuff placement (lower panel) (C1). The right lower panel presents a schematic representation of the calculations of surface areas of vessel wall cross section and the surface areas of whole vessel wall areas

including the lumen areas (C2). The ratios of vessel wall areas over the whole vessel wall surface areas were calculated for 10 mice in each group, and the statistical analyses are presented in the right upper panel (C3). The infiltrated inflammatory cell counts over the unit area were calculated for 10 mice in each group. The mean cell counts (grey column) and standard deviation (black portion on the top of column) for each group were presented (C4). D. A working model of how upregulated Bax in Tregs lowers the striking threshold of experimental vasculitis. Our results show that upregulated Bax expression leads to higher susceptibility of Tregs to apoptosis and decreased numbers of Tregs. Decreased Tregs presumably cannot maintain immune tolerance and suppression of inflammatory cells, which lowers the striking threshold and results in accelerated cuff placement-induced vasculitis.