Foxp3 Regulates Megakaryopoiesis and Platelet Function


Objective—Platelets are crucial for hemostasis and are vital regulators of inflammation. Foxp3 is a key transcription factor for T regulatory cell development. Humans with IPEX (immune dysregulation, polyendocrinopathy, enteropathy, x-linked) and the scurfy (Foxp3sf) mouse have mutations in the Foxp3 gene that lead to a host of pathologies including autoimmunity and skin diseases. Scurfy mice and some humans with IPEX are also thrombocytopenic. The purpose of this study was to determine whether the absence of functional Foxp3 leads to defects in megakaryocytes and platelets.

Methods and Results—We discovered that human and mouse megakaryocytes express Foxp3 mRNA and protein. Using shRNA and Foxp3sf mice, we demonstrated that Foxp3-deficient mouse and human megakaryocyte progenitors exhibited proliferation defects. Striking platelet abnormalities were observed in both an IPEX patient and Foxp3sf mice. Impaired platelet spreading and release of TGF-β and CD40 ligand (CD40L), and abnormal levels of plasma CD40L were observed in a case of IPEX syndrome. Foxp3sf mice were thrombocytopenic and had increased platelet volume and altered serum levels of CD40L, TXB2, and TGF-β.

Conclusion—These findings provide compelling new evidence that Foxp3 is needed for proper megakaryopoiesis and plays a role in regulating platelet function including spreading and release. (Arterioscler Thromb Vasc Biol. 2009;29:1874-1882.)

Key Words: megakaryocytes ▪ platelets ▪ Foxp3 ▪ IPEX

Platelets play vital roles in the normal hemostatic response to injury and are key cellular elements in diseases such as stroke and myocardial infarction. They are also now recognized as contributing to chronic diseases such as type-2 diabetes. Platelets contain mRNAs, pre-mRNAs, and splicing machinery to synthesize proteins. In addition, they release proinflammatory eicosanoids and proteins such as CD40 ligand (CD40L; formally called CD154), a potent cytokine that activates immune cells and structural cells such as endothelial cells. These biological processes make platelets important regulators of the immune system and the inflammatory response. In addition to unwanted platelet activation, loss of platelet function can also lead to morbidity and mortality. Reduced platelet number through autoimmunity, cancer chemotherapy, or ionizing radiation exposure can be fatal.

Normal megakaryopoiesis is necessary for optimal platelet production and function. Megakaryopoiesis is a complex process that involves the differentiation of bipotential erythroid/megakaryocyte progenitors to megakaryocyte progenitors followed by their differentiation to megakaryocyte precursor cells. While megakaryocyte progenitors proliferate, megakaryocyte precursors lack proliferative potential, but instead replicate DNA and increase cellular content to form mature polyploid megakaryocytes that shed platelets.

Forkhead box protein 3 (Foxp3) is a key transcription factor believed to be restricted to a subset of regulatory T (Treg) cells and is required for their development. Genetic mutations in Foxp3 lead to an X-linked often fatal autoimmune disease known as IPEX (immune dysregulation, polyendocrinopathy, enteropathy, x-linked) syndrome in humans. A similar disease arising from a Foxp3 mutation occurs in the spontaneous mouse mutant scurfy (Foxp3sf) in which a frameshift mutation in Foxp3 results in a dysfunctional product lacking the forkhead domain. The defects observed in IPEX and scurfy have been ascribed to dysfunctional Treg.

Based on the low platelet numbers and gastrointestinal bleeding in the presence of mutant Foxp3 in Foxp3sf mice and IPEX patients, we hypothesized that Foxp3 plays an intrinsic role in megakaryocyte maturation and thrombopoiesis. In this article, we report the expression of Foxp3 in human and mouse megakaryocytes, demonstrate its importance in megakaryopoiesis, and describe the platelet phenotype in Foxp3sf mice and in a case of IPEX syndrome.

Materials and Methods

Cell Culture and Treatment Conditions

Meg-01 cells were originally established from the bone marrow of a patient with Philadelphia chromosome–positive chronic myelogenous leukemia, and M07e cells, human leukemic cells with megakaryoblastic features, were purchased from the American Type Culture Collection (Rockville, Md). These and Dami cells, established from the blood of a patient with megakaryoblastic...
leukemia, were cultured as previously described. The primary human lung fibroblast strain, L828, were cultured as previously described.

**Megakaryocyte Differentiation From Human Cord Blood-Derived CD34+ Cells**

Human CD34+ cord blood cells were obtained from AllCells (Emeryville, Calif). Cells were plated at 2.5x10^6 cells per well in a 12-well plate and cultured in serum-free medium as previously described and supplemented with 100 ng/mL of recombinant human thrombopoietin (rThPO; R&D Systems). After 14 days in culture, primary human megakaryocytes were identified by staining with a CD61-FITC antibody and analyzed on a BD Biosciences FACScalibur flow cytometer. Cells were greater than 95% CD61 positive. Data were analyzed using FlowJo software (Treestar).

**Quantitative Real-Time PCR Analysis**

Sequences for PCR primer pairs were as follows: human Foxp3, forward 5'-GAAACAGACATCCGAGGTTC-3' and reverse 5'-AGTGGCCAGATGTTTCTG-3'; human 7S, forward 5'-ACCACAGGTTGCTTAAGGA-3' and reverse 5'-CACGGGAGTTTGACTGCT-3'. Another set of human Foxp3 primer pairs was used to confirm results from primers listed above: human Foxp3, forward 5'-CGGACCATCTTCTGGATGAG-3' and reverse 5'-TGTGCGGATCATCCACAG-3'.

**Western Blot Analysis**

Whole cell lysates were prepared using ELLB buffer plus protease and phosphatase inhibitors as previously described. One hundred micrograms of protein was electrophoresed and membranes probed with hFOXY antibody (1:5000) (eBioscience). Human Foxp3 transfected cell lysate (BioLegend) was used as a positive control.

**Flow Cytometric Analysis**

1x10^6 cells were fixed and permeabilized with Foxp3 staining buffer (eBioscience) and stained for Foxp3 using the clone PCH101 (eBioscience) or clone 236A/E7 (eBioscience) according to the manufacturer's instructions. Cells were incubated with isotype control antibodies (eBioscience) and fluorescence was analyzed as described. For DNA content analysis, cells were fixed overnight in 95% ethanol at 4°C. Cells were washed and incubated with 3 μmol/L of DAPI for 20 minutes at 37°C.

**Immunofluorescence**

Cell cytospins were mounted on slides and stained for Foxp3 intracellularly as described above. Cells were visualized using an Olympus BX51 light microscope (Olympus), photographed with a SPOT camera, and analyzed with SPOT RT software.

**Animals**

Blood was obtained from the orbital sinus of male B6.Cg-Foxp3sf/J (Foxp3gl) and background strain C57BL/6J mice (Jackson Laboratories) at age 24 days. C57BL/6J, age-matched, male mice were used as a comparative control because Foxp3+/- females were backcrossed to C57BL/6 males to maintain Foxp3gl mice. Platelet counts were also performed on healthy C57BL/6J male siblings of the Foxp3gl mice. Platelets were counted, and mean platelet volume (MPV) was determined using the Heska CBC-Diff Veterinary Analyzer. Serum was prepared by allowing whole blood clot for 30 minutes at 37°C and placing the blood on ice for 2 hours. Bone marrow was harvested from the femora of Foxp3gl and C57BL/6J mice. 2x10^6 megakaryocytes were cytopsin on glass slides and stained with a Diff-Quik stain set (Dade Behring).

**Analysis of Megakaryocytes in Foxp3gl Mice**

Megakaryocyte number was assessed by counting the number of megakaryocytes in 5 low power (10×) fields. Megakaryocyte progenitor (Meg-CPCs) assay was performed as previously published. Meg-CFCs were defined by the expression of GPlbα (Emfret Analytics) and by their ability to generate colonies containing at least 3 megakaryocytes. Foxp3 expression was analyzed in cells expressing CD41 (BD Biosciences) by flow cytometry. Megakaryocytes were stained for Foxp3 using the 150D/EF clone (eBiosciences) according to the manufacturer's instructions.

**siRNA to Knock-Down Foxp3**

Foxp3 shRNA individual clone was obtained from Open Biosystems. 1x10^6 Meg-01 cells were nucleofected with 2 μg of pLKO.1 HIV-based lentiviral vector expressing human shRNA Foxp3 plasmid or an empty vector plasmid using Amaxa Biosystem’s Cell Line Nucleofector Kit C, program V-001. Meg-01 cells were analyzed for Foxp3 expression 24, 48, and 72 hours posttransfection by flow cytometry. Four hours after nucleofection, cells were washed and proliferation was analyzed using CellTrace CFSE (carboxyfluorescein diacetate, succinimidyl ester) according to the manufacturer’s instructions (Invitrogen). Mitomycin C (Sigma), which crosslinks DNA and inhibits cell division, was used as a proliferation negative control and Meg-01 cells that were not nucleofected were used as a positive control.

**Measuring TXB2, CD40L, TGF-β, 12(S)-HETE, and Platelet Factor 4 (PF4)**

Mouse blood was collected and anticoagulated with acid-citrate-dextrose (ACD) or activated with either ADP (10 μmol/L) or thrombin (10 U/mL) for 30 minutes. Plasma was obtained by centrifuging anticoagulated blood at 3000 g for 10 minutes. Serum was obtained by centrifuging coagulated blood at 3000 g for 10 minutes. TXB2 was measured using a competitive enzyme immunoassay (ELIA) kit (Cayman Chemical), CD40L, TGF-β, and platelet factor 4 (PF4) were measured using sandwich enzyme-linked immunosorbent assays (ELISAs; R&D Systems), 12-hydroperoxyeicosatetraenoic acid (12(S)-HETE) was measured using an ELIA (Assay Designs).

**Human Serum Collection and Blood Platelet Isolation**

Whole blood was obtained under University of Rochester IRB approval from male donors and an IPEX donor using Vacutainer tubes containing buffered sodium citrate or no anticoagulant for serum collection (BD Biosciences). The IPEX patient was previously revealed to have a G to A transition at 1150G>A in exon 11, resulting in a substitution of Ala to Thr at residue 384, within the DNA binding domain of Foxp3. Platelets were isolated as described. Complete blood counts were performed on an Abbott Cell-Dyn 1700. Platelet purity was typically greater than 99%. Plasma and serum were analyzed for human TGF-β and TXB2 (Cayman Chemical). Human CD40L was analyzed by ELISA as described.

**Human Platelet Activation**

Washed platelets were spread on fibrinogen-coated (150 μg/mL) slides as described. Platelet spreading is critical for platelet-surface contact during wound healing and is used as a measure of platelet function. In addition, 3x10^7 platelet/300 μL were incubated (37°C) with platelet activators: Thrombin 0.8 U/mL (Sigma), collagen 10 μg/mL (Chrono-log Corporation), and adenosine diphosphate (ADP) 10 μmol/L (Chrono-log Corporation). After treatment, platelets were centrifuged (120g/1 minute) and supernatants were analyzed for human TGF-β, TXB2, platelet factor 4 (PF4), and CD40L.

**Statistical Analysis**

Results are expressed as the mean±SD. Statistical analysis was performed using a paired 2-tailed Student t test with P<0.05 deemed as statistically significant. All experiments were repeated at least 3 times unless otherwise stated.

**Results**

Megakaryocytes and Megakaryoblastic Cell Lines Express Foxp3 mRNA and Protein

To determine whether Foxp3 mRNA is expressed in megakaryocytes, real-time PCR analysis was used to evaluate 3 different
megakaryocytic cell lines (Meg-01, M07e, and Dami) and primary human megakaryocytes. L828 RNA was used as a negative control as human fibroblasts were found to be negative for Foxp3 mRNA.24 Human CD4⁺CD25⁺ T cells were isolated from peripheral blood and activated with anti-CD3 and anti-CD8 beads.25 The RNA was isolated and used as a positive control. As shown in Figure 1, all 3 human megakaryocyte cell lines expressed Foxp3 mRNA, as did primary human megakaryocytes. However, Foxp3 transcript levels were lower compared to activated T cells. Expression of Foxp3 mRNA was confirmed using 2 different primer sets for Foxp3.

To determine whether the expression of Foxp3 mRNA resulted in the production of Foxp3 protein, human megakaryocytes were examined by flow cytometry using intracellular staining and by Western blotting using antibodies with different specificities for Foxp3. Flow cytometric analysis using the PCH101 antibody clone, which binds the C terminus of the transcription factor, showed that all 3
megakaryoblastic cell lines and primary human megakaryocytes expressed intracellular Foxp3 protein (Figure 1B). Foxp3 was not detected in human blood platelets. These results were confirmed using the 236A/E7 antibody clone, which binds to an internal portion of Foxp3 (data not shown). These data reveal that megakaryoblastic cell lines have about 2 to 4 times as much Foxp3 protein as primary human megakaryocytes.

To support the flow cytometric data, Western blotting was performed using the hFox3 antibody clone. Human Foxp3 transfected cell lysate and human PBMC lysate were used as positive controls. Figure 1B demonstrates that megakaryocytes express Foxp3 protein, yet platelets themselves lack detectable Foxp3 protein. Western blotting also confirmed that 2 to 4 times more Foxp3 protein was detected in Meg-01, M07e, and Dami cells compared to primary human megakaryocytes (Figure 1C).

Next, immunofluorescent staining using the PCH101 antibody was used to determine the subcellular localization of Foxp3. Figure 1D shows the presence of Foxp3 protein in 3 different megakaryoblastic cell lines and in primary human megakaryocytes, where it was predominantly detected in the nuclei (Figure 1D).

**Foxp3 Protein Expression Is Enhanced With Phorbol Ester Myristate Acetate–Induced Megakaryocyte Maturation**

Meg-01 cells and primary human megakaryocytes were treated with the megakaryocyte differentiation agent, phorbol ester myristate acetate (PMA; 50 nmol/L to 1 μmol/L), for 24 hours. Figure 2 demonstrates that PMA dose-dependently increased Foxp3 expression in Meg-01 cells. The optimal dose for Meg-01 cells was 500 nmol/L PMA, increasing the mean fluorescence intensity from 13 to 19 (Figure 2A). PMA (50 nmol/L) induced Foxp3 expression in primary human megakaryocytes (Figure 2B) and increased megakaryocyte DNA content (Figure 2C). Cells with a DNA content of 4N had a higher expression of Foxp3 protein compared with cells with a DNA content of 2N (Figure 2D), which correlates with the data describing nuclear localization of Foxp3 in megakaryocytes. However, the PMA-treated 2N primary human megakaryocytes had higher Foxp3 expression compared with vehicle-treated 2N megakaryocytes (MFI PMA [50 nmol/L]: 83 versus MFI vehicle: 60). These results show that Foxp3 is induced during megakaryocyte maturation.

**Characterization of Megakaryocytes in Foxp3sf Mice**

In support of our human megakaryocyte data, mouse bone marrow megakaryocytes were also found to express Foxp3 protein (Figure 3). To determine the functional significance of megakaryocytic Foxp3, Foxp3sf mice were studied. To assess the influence of mutant Foxp3 on platelet levels, platelet counts were performed on 4 individual Foxp3sf and C57BL/6J mice. Wild-type, C57BL/6J, healthy siblings of the Foxp3sf mice had platelet counts similar to the C57BL/6J mice (data not shown). Foxp3sf mice have up to 53% fewer platelets compared to C57BL/6J mice (Figure 3B) and we newly report that Foxp3sf mice have ~4-fold less mature bone marrow megakaryocytes than normal mice (Figure 3C and 3D).
Characterization of Megakaryocyte Progenitors in Foxp3sf Mice

To determine whether this reduction in megakaryocyte number was attributable to a reduction in megakaryocyte progenitors, we next performed a Meg-CFC colony assay. Meg-CFC-derived colonies were identified by labeling with anti-GPIb/H9252 antibodies. Figure 4 demonstrates that Foxp3sf mice have an ~50% reduction in megakaryocyte progenitors compared to the C57BL/6J mice. In addition, mean colony size was lower in Foxp3sf mice, exhibiting significantly fewer colonies in the 3 to 50 cell count range, compared to the C57BL/6J mice (Figure 4B).

Meg-01 Cells Lacking Foxp3 Protein Have Reduced Proliferation

Figure 4C demonstrates that nucleofecting Meg-01 cells with a pLKO.1 lentiviral vector expressing human shRNA Foxp3 plasmid reduced Foxp3 protein to undetectable levels by 24 hours. This knockdown persisted through 72 hours (data not shown). Four hours after nucleofection, cells were washed and labeled with CFSE to measure the proliferative response of the Foxp3 knockdown. CFSE passively diffuses into cells and forms fluorescent conjugates.26 Labeled cells are retained during meiosis, and the label is inherited by the daughter cells during cell division.27 Fluorescence was analyzed after 72 hours.

Figure 4. Lack of Foxp3 influences megakaryocyte proliferation. A, Bone marrow was harvested and cultured in the presence of rhTPO, IL-3, IL-6, and IL-11 for 7 days. Bar graph demonstrates that Foxp3sf bone marrow had significantly fewer Meg-CFCs than C57BL/6J bone marrow. Meg-CFCs were defined by their ability to generate colonies containing at least 3 megakaryocytes. Results are presented as mean±SD (*P<0.04). B, Bar graph demonstrates that Foxp3sf colonies had a lower mean colony size compared to C57BL/6J colonies (mean is 12 colonies for C57BL/6J mice and 9 colonies for Foxp3sf). Mean colony size is indicated by #. C, Meg-01 cells were nucleofected with a Foxp3 shRNA plasmid. After 24 hours Foxp3 expression was analyzed by flow cytometry. Histogram shows the percentage of Foxp3-positive cells. D, CFSE dye was added to Meg-01 cells 4 hours post-nucleofection, and cell proliferation was analyzed by flow cytometry after 72 hours. Histogram shows cell division in Meg-01 cells treated with mytomycin C or expressing the empty vector plasmid or the Foxp3 shRNA plasmid. Foxp3 knock down Meg-01 cells failed to divide.
hours by flow cytometry. Figure 4D demonstrates that the Meg-01 cells expressing the Foxp3 shRNA plasmid failed to proliferate within the 72-hour period. Histogram peaks of the Foxp3 knockdown cells and the mitomycin C–treated cells were identical. In contrast, Meg-01 cells expressing the empty vector plasmid underwent 1 or 2 cell divisions by 72 hours.

Characterization of Platelets in Foxp3sf Mice
Impaired megakaryopoiesis can lead to platelets abnormal in size and function, so we hypothesized that Foxp3sf mice would have platelet dysfunction in addition to thrombocytopenia. Platelet size may reflect altered function, and we found no differences in plasma levels of TGF-β, CD40 ligand (CD40L), thromboxane B_2 (TXB_2), and 12(S)-hydroperoxyeicosatetraenoic acid 12(S)-HETE. Whereas only 5% of IPEX platelets fully spread, whereas 60% of normal unstimulated platelets. These levels were approximately doubled with activation in both IPEX and normal platelet supernatants (Figure 6B). No differences in serum TGF-β and CD40L levels were observed between the IPEX patient and an age-matched control, suggesting that multiple platelet agonists in conjunction could promote the release of TGF-β and CD40L (data not shown). However, the IPEX patient had plasma CD40L levels 3 times higher than the control (Figure 6C).

Discussion
The specificity and restriction of Foxp3 expression to a subset of T lymphocytes has provoked controversy in the scientific literature. However, more recent studies indicate that Foxp3 is expressed in some epithelial cells and some tumor cells. The abnormal platelet levels observed and the hemorrhaging that occurs in IPEX syndrome and in Foxp3sf mice prompted us to examine Foxp3 expression in the megakaryocyte lineage and determine whether it plays a role in megakaryopoiesis and platelet production. Herein, we demonstrate that primary human megakaryocytes and megakaryoblastic cell lines express Foxp3 mRNA and protein, although to a lesser extent than in T_reg cells. Consistent with its putative role as a transcription factor, Foxp3 protein expression in megakaryocytes was predominantly nuclear, which may account for the failure to detect Foxp3 in human platelets (Figure 1C). Nuclear localization of Foxp3 may also explain increased levels of Foxp3 protein expression after PMA treatment (Figure 2), as PMA increases cellular DNA content in megakaryocytes by inducing endomitosis. We also demonstrated that Foxp3 protein expression was greater in megakaryoblastic cell lines compared to primary human megakaryocytes (Figure 1B and 1C). However, the reason for and the consequences of this increased expression are unknown. It has been speculated that Foxp3 plays an intrinsic role in malignant transformation and tumor survival. Interestingly, the Meg-01 cell line and the Dami cell line were derived from patients with megakaryoblastic leukemias that presented elevated bone marrow blast cells and thrombocytosis. Future studies will examine the importance of Foxp3 expression in megakaryoblastic cell lines.

Our findings indicate that Foxp3 plays an important role in megakaryopoiesis. Foxp3sf mice, which lack the full-length functional Foxp3 protein, had 4-fold fewer bone marrow megakaryocytes compared to C57BL/6J mice (Figure 3D). Further, megakaryocyte colony number was approximately 6 times more platelet factor 4 (PF4) in the supernatant compared with normal unstimulated platelets. These levels were approximately doubled with activation in both IPEX and normal platelet supernatants (Figure 6B). No differences in serum TGF-β and CD40L levels were observed between the IPEX patient and an age-matched control, suggesting that multiple platelet agonists in conjunction could promote the release of TGF-β and CD40L (data not shown). However, the IPEX patient had plasma CD40L levels 3 times higher than the control (Figure 6C).

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addition, megakaryocyte mean colony size was lower in Foxp3sf mice compared with that in C57BL/6J suggesting that a reduction in progenitor proliferation contributes to reduced progenitor number (Figure 4B). These new findings demonstrate that the reduced colony number in Foxp3sf mice is attributable to a defect in megakaryocyte progenitor proliferation and could also be attributable to a potential role of Foxp3 in the generation of megakaryocyte progenitors from upstream bipotential or multipotential progenitors. We also observed that platelet count does not necessarily correlate with megakaryocyte number or megakaryocyte progenitor number. The platelet counts from individual mice exhibited more variability, which may indicate differences in the ability of mature megakaryocytes to produce platelets. These data, however, do not rule out the possibility that the reduced megakaryocyte number is a consequence of profound defects in T regulatory cells. Megakaryopoiesis may be impaired in vivo because of the wide range of autoimmune-associated symptoms which characterize the Foxp3sf mouse. However, Foxp3 knockdown human Meg-01 cells demonstrate a greatly reduced proliferative response, suggesting a direct role for Foxp3 in megakaryopoiesis (Figure 4D).

The precise mechanism by which Foxp3 regulates megakaryopoiesis remains unknown. Foxp3 functions in T lymphocytes, in part, as a transcriptional repressor by recruiting both histone acetyl transferases and histone deacetylases. Foxp3 also functions as a passive transcriptional repressor by physically interacting with proteins such as nuclear factor-kappa B (NF-κB) and acute myeloid leukemia 1 (Aml1)/runt-related transcription factor 1 (Runx1). Foxp3 may be playing a similar role in megakaryocytes by suppressing or activating transcription factors.

Genetic lesions in megakaryocytes that cause thrombocytopenia often cause abnormal platelet function. Foxp3sf mice have reduced platelet counts and our new findings demonstrate that they exhibit striking activation abnormalities. TGF-β serum levels were significantly lower in Foxp3sf mice. Because platelet-derived TGF-β is a cytokine mainly involved in wound healing and tissue repair, we speculate that reduced TGF-β could potentiate the dermatitis and the skin lesions which characterize the IPEX disease and the Foxp3sf mice. In addition, TGF-β can suppress T cell responses. TGF-β reduces T cell proliferation by inhibiting IL-2 production and upregulating cell cycle inhibitors and inhibits the differentiation of Th1 to Th2 by downregulating T-bet and GATA-3. TGF-β also inhibits the activation of macrophages and reduces the ability of dendritic cells to present antigens to T cells. Interestingly, we found that Foxp3sf serum had elevated levels of CD40L and 2 arachidonic acid metabolites, TXB2 and 12(S)-HETE, despite having fewer platelets. TXB2 is a more stable metabolite of thromboxane A2 (TXA2), a cyclooxygenase-derived product generated by platelets which induces irreversible platelet aggregation and vascular smooth muscle contraction. 12(S)-HETE is a 12-lipoxygenase-derived product that is produced abundantly in platelets during activation. These data suggest that the Foxp3sf platelets produce more arachidonic acid metabolites during activation. As described in the Introduction, CD40L activates immune and structural cells, as well as platelets. The majority of the circulating soluble CD40L originates from...
platelets and CD40L levels are elevated during inflammatory disease states.\textsuperscript{44–46} Collectively, these new data suggest that the platelet phenotype in \emph{Foxp3}{\textsuperscript{3/0}} mice contributes to the inflammation observed in the ‘scuffy’ disease.

\emph{Foxp3}{\textsuperscript{3/0}} mice also had increased mean platelet volumes (Figure 5A). There are many intrinsic and reactive reasons why platelet volume is elevated in disease states. For example, Gata-1 knockout mice have deficiencies in megakaryocyte maturation, and as a result their platelets have elevated volumes.\textsuperscript{47} The peripheral platelet destruction from circulating antiplatelet antibodies increases platelet volume in immune thrombocytopenic purpura (ITP) patients because a higher percentage of platelets are younger.\textsuperscript{48,49} Therefore, the increased platelet size observed in \emph{Foxp3}{\textsuperscript{3/0}} mice may indicate both impaired platelet production and peripheral platelet destruction or in contrast that the elevated mean platelet volume may be compensating for the decrease in platelet number.

To determine whether a similar platelet phenotype was observed in IPEX, the human correlate of scuffy, we examined the platelets of an IPEX patient. IPEX is rare disease involving various \emph{Foxp3} mutations and can result in death at an early age.\textsuperscript{29} About 50\% of IPEX patients are reported to be thrombocytopenic, and hemorrhage is one of the most common causes of death in untreated patients.\textsuperscript{9} Gastrointestinal bleeding has occurred in a case with normal platelet counts, suggesting inadequate platelet function.\textsuperscript{50} The IPEX donor evaluated herein was aged 17 years and had a G to A transition (1150G\textrightarrow{}A) in exon 11, resulting in a substitution of Ala to Thr at residue 384, within the DNA-binding domain of \emph{Foxp3}.\textsuperscript{50} His platelets demonstrated striking abnormalities in spreading (Figure 6A) and a reduced ability to release CD40L and TGF-\emph{B} in response to potent platelet activators (Figure 6B). Our IPEX donor also demonstrated a profound elevation in plasma levels of CD40L (Figure 6C). These data indicate that the IPEX patient did not respond normally to platelet activators and possibly that his platelets already released internal stores of CD40L in vivo.

We also demonstrated that the IPEX patient had 6 times more platelet factor 4 (PF4) in the supernatants of both unactivated and activated platelets compared with the normal donor. This suggests that the IPEX platelets contain higher levels of PF4 or that the release of PF4 from alpha granules is enhanced. Because IPEX platelets release less TGF-\emph{B} and CD40L than the normal platelets, we speculate that IPEX platelets contain higher levels of PF4. The increased release of PF4 may implicate platelets in the symptomology of IPEX. IPEX disease is characterized by severe atopic dermatitis, and recently plasma levels of PF4 were shown to be elevated in patients with atopic dermatitis and in a mouse model of atopic dermatitis.\textsuperscript{51–53} In addition, PF4 is a negative regulator of megakaryopoiesis, suggesting that elevated PF4 may be a mechanism for inhibiting megakaryocyte proliferation.\textsuperscript{54} Collectively, these new findings demonstrate that the defect in \emph{Foxp3} observed in IPEX influences platelet function.

Our study adds considerable new information to the ongoing discussion of the presence of \emph{Foxp3} in cell types other than T\textsubscript{reg}. Overall, we have shown that \emph{Foxp3} deficiency results in a lesion of megakaryocyte proliferation that is associated with platelet dysfunction. These new findings support the concept that genetic disorders that cause thrombocytopenia also cause abnormal platelet function such as occurs in myelodysplasias. Therefore, we have elucidated an underlying mechanism of megakaryopoiesis that contributes to the pathophysiology of IPEX syndrome and the ‘scuffy’ disease.

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Disclosures

None.

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