Thymic retention of CD4+CD25+FoxP3+ T regulatory cells is associated with their peripheral deficiency and thrombocytopenia in a murine model of immune thrombocytopenia (ITP)

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Running Title: Thymic Tregs in ITP

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Immune thrombocytopenia (ITP) is a bleeding disorder where antibodies and/or T cells lead to enhanced peripheral platelet destruction and reduced bone marrow platelet production. Several reports have observed that ITP is associated with a peripheral deficiency of tolerance-inducing CD4+CD25+FoxP3+ T regulatory cells (Tregs). Using a murine model of ITP, we analyzed Tregs in the spleen and thymus. CD61 knockout mice were immunized against wild type (CD61+) platelets and their splenocytes were transferred into severe combined immunodeficient (SCID) mice. Compared with SCID mice receiving naïve splenocytes, within 2 weeks post-transfer, the ITP SCID mice became thrombocytopenic (<200x10^9 platelets/L) and had increased serum anti-CD61 antibodies. The quantity of thymic Tregs by 2 weeks post transfer was significantly elevated whereas Tregs in the spleens were significantly reduced. Treatment of the ITP mice with 2 g/kg intravenous immunoglobulin (IVIg) raised the platelet counts, reduced antibody production and normalized the thymic and splenic Treg populations. Compared with thymocytes from ITP mice treated with IVIg, thymocytes from untreated ITP mice delayed the onset of ITP when administered prior to engraftment with immune splenocytes. These results suggest that ITP in mice is associated with a peripheral Treg deficiency due to thymic retention and therapy normalizes the Tregs.
Introduction

Immune thrombocytopenia (ITP) is an autoimmune bleeding disorder characterized by the presence of plasma antibodies that recognize platelet autoantigens which leads to premature platelet clearance by Fcγ receptor (R) mediated phagocytosis in the reticuloendothelial system (RES).\textsuperscript{1-5} It has also been suggested that the immune response against platelets can effect the bone marrow, leading to megakaryocyte destruction and/or inhibition and impaired platelet production.\textsuperscript{6,7} Of interest, the early self administration experiments of Harrington et al\textsuperscript{8} who infused healthy volunteers with the plasma from ITP patients only caused thrombocytopenia in 17 of the 26 infusions (65 percent) suggesting that a mechanism of thrombocytopenia other than antibodies may be present. In 2003, Olsson et al\textsuperscript{9} confirmed this finding by showing that in patients with no detectible anti-platelet antibodies, thrombocytopenia was in fact due to platelet destruction by CD8+ cytotoxic T cells (CTL) and this was confirmed by other laboratories.\textsuperscript{10,11} We subsequently developed a mouse model of ITP that demonstrated both antibody- and cell-mediated thrombocytopenia where the antibody-mediated form was sensitive to intravenous gammaglobulin (IVIg) treatment whereas the cell-mediated thrombocytopenia was not.\textsuperscript{11} Taken together, these results suggest that the immunopathogenesis of ITP is more complex than was originally thought, in that some patients with ITP, particularly chronic ITP, have a cell-mediated form of thrombocytopenia in addition to autoantibody-mediated platelet destruction.

Within the last 6 years, the immunopathologic complexity of ITP has been further supported by many laboratories demonstrating that patients with active ITP have a peripheral deficiency of the CD4+CD25+FoxP+ T helper subset known as T regulatory cells (Tregs).\textsuperscript{12-29} Tregs are thymic derived T cells that are critical in maintaining self tolerance, and genetic deficiencies of this T cell population leads to overwhelming autoimmunity such as the immunodysregulation
polyendocrinopathy enteropathy X-linked syndrome (IPEX) in humans and the Scurfy phenotype in mice. Many autoimmune diseases such as rheumatoid arthritis, Type I diabetes and multiple sclerosis now are associated with peripheral Treg deficiencies. The reasons for the Treg deficiency in active ITP is unknown, but several therapies that raise platelet counts do so in association with normalizing the observed Treg deficiency. Since our murine model of ITP was dependent on the presence of CD4+ T cells, we determined whether the low platelet counts were associated with Treg abnormalities. We demonstrate here that active murine ITP is associated with a peripheral (splenic) Treg deficiency, but concomitantly, the thymus of ITP mice had significant elevations in Treg percentages. IVIg treatment raised platelet counts and normalized the thymic and splenic Treg proportions. The results suggest that the peripheral Treg deficiency in active ITP may be due to a thymic sequestration of Tregs, and these retained T cells are released when platelet counts are increased by therapy.
Materials and methods

Mice. For platelet donors or splenocyte transfer recipients, 8-10 week old female BALB/c (H-2<sup>d</sup>) and CB.17 (H-2<sup>d</sup>) severe combined immunodeficient (SCID) mice respectively were obtained from Jackson Laboratories (Bar Harbor, MA Charles River). BALB/c CD61 KO mice were obtained from Dr. Heyu Ni. All mice were housed in the Li Ka Shing Knowledge Institute’s Research Vivarium and all animal studies were approved by the St. Michael's Hospital Animal Care Committee.

Platelet Preparation and Immunization of CD61 KO mice. Leukoreduced platelets were prepared as previously described.<sup>34</sup> Briefly, blood was drawn by cardiac puncture from anesthetized donor mice into phosphate buffered saline (PBS) containing citrate/phosphate, dextrose, acetate (CPDA buffer). The blood was pooled, diluted with PBS-CPDA, centrifuged at 150 x g for 15 minutes and the platelet-rich plasma (PRP) was collected. The PRP was washed three times by centrifuging at 450 x g for 18 minutes. The washed platelets were resuspended in PBS-CPDA, counted and their concentration adjusted to 1x10<sup>9</sup> cells/mL. For immunization, BALB/c CD61 KO mice were bled 24 h prior to the first transfusion and then injected with 100 ul (10<sup>8</sup> platelets) CD61+ platelets via the tail vein weekly for 3 weeks. Their sera was tested weekly for the presence of anti-CD61 antibodies by flow cytometry using wild type CD61 platelets and when the titres were found to be >1:1,600, the immune mice were sacrificed by cervical dislocation and their spleens removed, homogenized in RPMI 1640 and washed by centrifugation at 400 x g for 15 minutes. Spleen homogenates were further treated with ACK lysing solution and washed to remove RBC. The splenocytes were then adjusted in PBS to a concentration of 1.5x10<sup>5</sup> cells/mL. Non-transfused (naïve) BALB/c mice were used as source of control splenocytes.

Generation of active ITP. ITP was induced as previously described but with modifications.<sup>11</sup> Briefly, CB.17 SCID mice were first gamma irradiated (2 cGy) and NK cell depleted by an ip infusion
of 50 uL of a rabbit anti-asialo GM1 antibody (Wako Pure Chemical Industries, Ltd) on day -1 and on
day 0, 100 ul (1.5 x10^4) of the indicated splenocytes were transferred intraperitoneally. At this low
dose of transferred splenocytes, the IVIg-resistant cell mediated thrombocytopenia is eliminated so
that only the antibody-mediated form of ITP is present. Platelet counts were measured weekly using a
Beckman Coulter Counter-LH750 hematology analyzer.

**Serum Anti-CD61 antibody Production.** To detect recipient IgG anti-CD61 production in the
transferred SCID mice, platelets from BALB/c mice were prepared as described above and 1x10^6
platelets were incubated with 3uL of undiluted serum for 30 minutes at room temperature. The
platelet-serum mixtures were washed once with PBS and then labeled with a FITC conjugated goat
anti-mouse IgG (FITC-GAM, Fc specific; Caltag Laboratories, Mississauga, ON) in the dark at room
temperature for 30 minutes. The mixture was washed before being analyzed by flow cytometry.

**IVIg treatment.** The indicated groups of engrafted SCID mice were injected intraperitoneally with 2
g/kg IVIg (Gamunex 10%; Talecris Biotherapeutics, Mississauga, ON) 1 day before splenocyte
transfer and twice weekly thereafter as previously described. All mice were bled weekly for platelet
counts and serum antibody levels.

**Thymus and Spleen preparations for Treg enumeration.** Mice from the indicated groups were
sacrificed each week and their spleens and thymuses were removed, cleaned of fat and any apparent
lymph nodes, weighed and prepared into single cell suspensions by homogenization in RPMI. The
splenocyte suspension was further treated with ACK lysing buffer to remove RBC. Cells were washed
2 times by centrifugation at 400 x g for 15 minutes. Cells were adjusted in cell medium to a
concentration of 1x10^8 cells/mL and were used to enumerate the percentage of Tregs using a
commercially available Treg kit (eBiosciences, San Diego, CA) according to the manufacturer’s
instructions. Briefly, thymocytes and splenocytes were surface stained with fluorocein (FITC)
conjugated anti-mouse CD4 and phycoerythrin (PE)-conjugated anti-mouse CD25 for 45 minutes at 4°C, washed, fixed and permeabilized using the Foxp3 staining buffer and subsequently stained with anti-mouse PE-Cy5 Foxp3. Cells were analysed using a BD FACSort flow cytometer (Becton Dickinson, San Jose, CA) by gating 10,000 CD4+CD25hi+ cells and then enumerating the percentage of gated FoxP3+ cells. The proportional data were adjusted for differences in splenic and thymic mass in order to reflect the total lymphocyte content of these organs as previously described, and to indicate whether there were absolute changes in lymphocyte numbers in addition to proportional changes in Treg distribution.

**Thymus Histology.** Mice were sacrificed and thymuses were removed, cleaned of fat and any bronchial lymph nodes and placed in buffered 10% formalin (VWR), pH 6.8-7.2 at room temperature for 2-3 days, followed by auto-processing of the tissue using a Leica TP1020 auto tissue processor (Leica Microsystem, Toronto, ON), and the fixed tissue was embedded in wax (Leica EG1160) and sectioned with a manual microtome (Leica RM 2145). The sections were placed on a glass microscope slide, fixed and stained with hematoxylin-and-eosin (H&E; Leica autostainer XL). Histological sections were visualized at 40x magnification (numerical aperture, objective lens, 0.75) with an Olympus BX50 upright microscope.

**Thymic Function.** To determine the ability of thymocytes to affect ITP development, 10^5 of the indicated thymocytes were injected ip on day -5 before the ip administration of splenocytes from CD61 immune mice. Platelet counts were performed at weekly intervals.

**Statistical Analysis.** Data were analyzed using Student's t test; p<0.05 was considered significant.
Results

Platelet counts and Anti-CD61 Antibody titres.

To ensure that ITP was induced, platelet counts and serum anti-CD61 antibody levels were analyzed. The initial fall in the platelet count at 7 days (Figure 1a) results from the irradiation treatment as previously described. Compared with SCID mice engrafted with splenocytes from naïve mice, splenocytes transferred from CD61 immune mice caused significant thrombocytopenia throughout the 28 day protocol (Figure 1a). This correlated with an increase in anti-CD61 antibody titre by day 14 post splenocyte administration (Figure 1b). Treatment of the ITP mice with 2 g/kg IVIg rescued the platelet counts (Figure 1a) and significantly reduced the anti-CD61 antibody titres (Figure 1b).

CD4+CD25hi+FoxP3+ Treg distribution is significantly skewed in the lymphoid organs of ITP mice.

The percentage of Tregs was quantified within the mouse thymi and spleens by flow cytometry (Figure 2). Compared with control BALB/c mice or with SCID mice transferred with splenocytes from naïve mice, the ITP mice developed a significant splenic Treg deficiency by 14 days post splenocyte transfer (Figure 3a). In contrast, there was a concomitant significant increase in the percentage of Tregs within the thymuses of the ITP mice (Figure 3b). Treatment of the ITP mice with 2 g/kg IVIg normalized the peripheral splenic deficiency by day 28 post transfer (Figure 3a) and significantly reduced the thymic Treg proportions to near control levels (Figure 3b). The proportional data were adjusted for differences in splenic and thymic mass in order to reflect the total lymphocyte content of these organs, and therefore to indicate whether there were absolute changes in lymphocyte numbers in addition to proportional changes in Treg distribution (Figures 3c and 3d). The normalization of the Tregs in the IVIg treated mice also closely correlated with changes in thymic
architecture (Supplemental Figure 1). Compared with untreated mice, IVIg therapy transformed the thymic architecture to that resembling healthy BALB/c mice (Supplemental Figure 1).

**Thymocyte function in modulating the development of ITP.** Irradiated and NK cell depleted SCID mice were injected intraperitoneally with $10^5$ thymocytes harvested from thrombocytopenic SCID mice and five days later, the mice were engrafted with splenocytes from CD61 immune KO mice to assess the effect of the thymocytes on the development of ITP. Thymocytes from either ITP SCID mice or ITP SCID mice treated with IVIg could not, on their own, induce thrombocytopenia when transferred into SCID mice (Figure 4a). In contrast, however, thymocytes from SCID ITP mice were able to delay the onset of thrombocytopenia induced by the subsequent administration of CD61 immune KO splenocytes (Figure 4b). The platelet counts of mice receiving thymocytes were significantly better sustained at day 14, and ITP development was delayed (Figure 4b). ITP development was not affected by thymocytes from ITP SCID mice treated with IVIG (Figure 4b).
Discussion

The immunopathogenesis of ITP has been extensively investigated in the last decade and it appears that one of the major contributing factors in the development of this disorder is a peripheral deficiency of regulatory T cells (Tregs). Tregs are a T cell subset marked by CD4+CD25hi Foxp3+ and constitute approximately 5–10% of peripheral CD4+ T cells and play an important role in self-tolerance. The reasons for the Treg deficiency in ITP are unclear, and we attempted to address this question by studying our murine model of active ITP. We report here that one of the reasons for the peripheral Treg deficiency during active disease may be due to sequestration of functional Tregs within the thymus. Treatment of the ITP mice with IVIg increased platelet counts and significantly reduced the thymic accumulation of functional Tregs thereby correcting the peripheral deficiency.

Natural Tregs (nTregs) originate from the thymus as CD4+ cells expressing high levels of CD25 together with the transcription factor (and lineage marker) FoxP3. It is clear that Tregs are essential for maintaining self tolerance since genetic deficiencies of the transcriptional factor FoxP3 leads to overwhelming autoimmunity. Indeed, many autoimmune diseases in both humans and mice are associated with a peripheral blood and splenic deficiency of Tregs respectively. This observation now appears to be true for patients with chronic ITP. Of interest in patients with ITP, therapies that raise platelet counts including dexamathasone, rituxan, thrombopoietin receptor agonists and IVIg do so in association with rescuing the peripheral Treg deficiency. Our data also support this contention where active ITP had low numbers of peripheral splenic Tregs (Figures 2 and 3) and IVIg therapy, for example, normalized the peripheral deficiency (Figure 3). What these results additionally show is that the peripheral Treg deficiency is associated with a large thymic accumulation of Tregs, and IVIg therapy reduced the thymic Treg numbers by allowing them to be released into the
periphery. The mechanism of how IVIg may accomplish this unknown but there is evidence that IVIg can modulate Tregs numbers and movements.\textsuperscript{37}

IVIg has been shown to induce Treg development and it has been hypothesized that the anti-inflammatory nature of IVIg may be due to Treg induction. For example, Kessel et al\textsuperscript{38} showed that IVIg could stimulate the expansion of CD4+CD25+Foxp3+ Tregs in vitro and Chi et al\textsuperscript{39} demonstrated that the beneficial effect of IVIg treatment of Guillain-Barré Syndrome was due to its ability to rescue their low Treg numbers. Subsequently, Ephrem et al\textsuperscript{40} demonstrated that IVIg therapy could also protect mice from experimental autoimmune encephalopathy (EAE) through induction of Tregs. More recently, two reports now have shown that IVIg treatment of patients with acute Kawasaki’s disease alleviates the disorder by increasing the number and function of Tregs.\textsuperscript{41,42} Similarly, IVIg was shown to enhance allograft survival by the induction of Tregs\textsuperscript{43} and IVIg’s beneficial effect on murine diabetes is associated with Treg induction.\textsuperscript{44} Of interest, IVIg can alter Tregs as early as 24-48 hours after therapy; the time, for example, when platelet counts begin to rise in IVIg-treated patients with ITP.\textsuperscript{43,44} Collectively, these reports suggest that IVIg’s protective anti-inflammatory effects are mediated by expanding the suppressive effects of Tregs. This is supported by the data in Figure 4 that shows that development of ITP is delayed by the Treg-laden thymocytes. Perhaps the thymocyte infusions delay antibody production via direct inhibition of immunoglobulin production or inhibition of T helper-B lymphocyte interactions. We are currently studying this.

The mechanism of how Tregs accumulate within the thymus during active ITP is unknown; however, thymic epithelial cells which are the major antigen presenting cells involved in thymic selection of T cells can express the CD61 antigen associated with CD51 to form the vitronectin receptor.\textsuperscript{45-47} It is possible that the transferred anti-CD61 immune splenocytes may damage thymic epithelial cells and cause faulty thymic adhesion or selection processes that lead to Treg retention.
Related to this, compared with healthy age-matched BALB/c mice, histological staining of the thymus in SCID mice transferred with anti-CD61 immune splenocytes revealed a thymic architecture that had an increased density of epithelial and stromal cells with a relatively moderate but homogeneous scattering of lymphocytes (Supplemental Figure 1). In contrast, however, treatment of the transferred mice with IVIg completely transformed the thymic architecture more resembling the BALB/c mouse controls (Supplemental Figure 1). It is possible that the changes in thymic architecture are related to the release of Tregs into the periphery.

Thymocytes from either the untreated or IVIg-treated ITP SCID mice could not themselves induce ITP when transferred into naïve SCID mice (Figure 4), however, if thymocytes from the ITP SCID mice were administered prior to engraftment with CD61 immune splenocytes, there was a delay in the development of ITP at two weeks post engraftment (Figure 4). Of interest, the thymocytes from ITP mice treated with IVIg did not affect the development of ITP suggesting that IVIg may allow for the release of the sequestered functional Tregs to mediate suppression of the immune mechanisms producing the ITP. Nonetheless, the sequestered thymic Tregs in mice with active ITP appear to be functional in that they can at least delay the onset of ITP.

In summary, we have demonstrated that active murine ITP is associated with a peripheral splenic Treg deficiency and corresponding thymic accumulation of Tregs. Treatment with IVIg normalizes the platelet counts and significantly reduces the thymic Treg numbers, and this correlates with a rescuing of the peripheral Treg pool in the spleen. The data suggest that a novel potential mechanism of peripheral Treg deficiency in ITP, central thymic retention of Tregs, starves the peripheral tolerance mechanism and allows the anti-platelet immune response to proceed.
Acknowledgements

The authors wish to thank Dr Alan Lazarus for his helpful discussions.

The authors have no conflict of interests to declare. R.A designed research, performed and supervised all experiments, collected data, analyzed and interpreted data, performed statistical analysis, and wrote the manuscript first draft; Y.H., S.G., G.B.S., E.R.S., L.G., and M.K. performed experiments, collected, analyzed and interpreted data; H.N. and J.F. designed research, edited the manuscript, and contributed animals and J.W.S. provided financial resources, designed research, analyzed and interpreted data, and edited the manuscript.
References


Figure Legends

Figure 1.
Induction of a) thrombocytopenia and b) anti-CD61 antibody production in irradiated SCID mice transferred with 1.5x10^4 splenocytes from either naïve BALB/c mice (N=10, ○), CD61 KO mice immunized against BALB/c platelets (N=15, □) or SCID mice transferred with 1.5x10^4 splenocytes from CD61 KO mice immunized against WT BALB/c platelets and treated biweekly with 2 g/kg IVIg (N=15, △). Thrombocytopenia at day 7 in panel a occurred in all irradiated mice as previously described. Thrombocytopenia at day 7 in panel a occurred in all irradiated mice as previously described. The data in panel a are expressed as platelet counts (x10^9/L) (+/-SEM) over time (days). The solid horizontal line in panel a represents the normal mean platelet count (+/- SEM hatched lines) from 250 healthy SCID mice [mean platelet count = 720±41 (SD). Anti-CD61 specific antibody production in panel b was performed by flow cytometry, and the data are expressed as percent gated BALB/c platelets. The stars (★) in both panels refer to statistical significance (p<0.01) between the ITP mice (□) and IVIg-treated mice (△).

Figure 2.
A representative flow cytometric dot plot analysis of CD4+CD25+ T cells and the corresponding gated histograms of FoxP3+ T cells in the spleen (left panels) and thymus (right panels) of the indicated mice at 21 days post transfer. Panels a) irradiated SCID mice transferred with 1.5x10^4 splenocytes from naïve BALB/c mouse, b) irradiated SCID mice transferred with 1.5x10^4 splenocytes from CD61 KO mice immunized against BALB/c platelets and c) irradiated SCID mice transferred with 1.5x10^4 splenocytes from CD61 KO mice immunized against WT BALB/c platelets and treated biweekly with 2 g/kg IVIg. Freshly isolated splenocytes and thymocytes were stained and analyzed by flow cytometry using a lymphocyte gate based on anti-CD4 green and CD25 red fluorescence in the dot plots. The histograms of FoxP3+ cells in the CD4+CD25^hi+ fluorescent gate are shown.
Figure 3.
The percentages of CD4+CD25hi+FoxP3+ Tregs in the a) spleens and c) thymuses of irradiated SCID mice engrafted with 1.5x10^4 splenocytes from CD61 KO mice immunized against BALB/c platelets (N=10, open columns) are shown. The percentages of Tregs in organs from irradiated SCID mice engrafted with 1.5x10^4 splenocytes from CD61 KO mice immunized against BALB/c platelets and treated biweekly with 2 g/kg IVIg (N=10, black columns) are shown for comparison. Data are expressed as the percent CD4+CD25hi+ T cells (±SEM) in the fluorescence gates as shown in Figure 2. The solid horizontal line in the panels represents the normal mean percentage (+/- SEM hatched lines) of CD4+CD25hi+FoxP3+ Tregs in aged-matched BALB/c mice (N=10). The stars (★; p<0.05, ★★; p<0.01) in panels a and c refer to statistical significance between the ITP mice (open columns) and IVIg-treated mice (black columns). In Figures 3b and 3d, the proportional data were adjusted for differences in splenic and thymic mass respectively in order to reflect the total lymphocyte content of these organs.

Figure 4.
a) Platelet counts in control SCID mice transferred with 1.5x10^4 splenocytes from naïve BALB/c mice (N=5, ●) or SCID mice transferred with 10^5 thymocytes from either SCID ITP mice (N=5, □) or SCID ITP mice treated biweekly with 2 g/kg IVIg (N=15, ◊). b) Platelet counts in SCID mice transferred with 1.5x10^4 splenocytes from CD61 immune KO mice (SCID ITP, N=5, O) or SCID mice transferred with 10^5 thymocytes from SCID ITP mice 5 days before receiving splenocytes from CD61 immune KO mice (N=5, △) or SCID mice transferred with 10^5 thymocytes from SCID ITP mice treated with IVIg 5 days before receiving splenocytes from CD61 immune KO mice (N=5, ◼). Thrombocytopenia at day 7 occurred in all irradiated mice as previously described.11 The data are expressed as platelet counts (x10^9/L) (+/-SEM) over time (days). The solid horizontal lines represents the normal mean platelet count (+/- SEM hatched lines) from 250 healthy SCID mice.
[mean platelet count = 720±41 (SD). The star (★) in panel b refers to statistical significance (p<0.01) between the SCID mice administered thymocytes (△) and control ITP SCID mice (○).
Figure 1

(a) Platelet Count (x10^9/L) vs. Days Post Transfer

(b) Serum Anti-CD61 (% Gated) vs. Days Post Transfer
Figure 2
Figure 3

(a) CD4⁺CD25⁺FoxP3⁺ (%) over days post transfer.

(b) CD4⁺CD25⁺FoxP3⁺ corrected for organ wt. (mg) over days post transfer.

Days Post Transfer

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Figure 4

Platelet Count (x $10^9$/L) vs. Days Post Transfer
Thymic retention of CD4+CD25+FoxP3+ T regulatory cells is associated with their peripheral deficiency and thrombocytopenia in a murine model of immune thrombocytopenia (ITP)

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