Improved regulatory T cell activity in patients with chronic immune thrombocytopenia treated with thrombopoietic agents

Weili Bao,1 James B. Bussel, 2 Susanne Heck, 3 Wu He, 3 Marissa Karpoff, 2 Nayla Boulad, 2 and Karina Yazdanbakhsh1.

1Laboratory of Complement Biology, New York Blood Center, New York, NY; 2Platelet Disorders Center, Division of Pediatric Hematology-Oncology, Weill Cornell Medical College, New York, NY; 3Flow Cytometry Laboratory, New York Blood Center, New York, NY

Corresponding author:

Karina Yazdanbakhsh, PhD

Laboratory of Complement Biology,

New York Blood Center,

310 E67th Street

New York, NY 10065.

Tel: 212-570-3383

Email: kyazdanbakhsh@nybloodcenter.org

Running title: Improved Treg function with TPO-R agonists
Abstract

Immune thrombocytopenia (ITP) is an autoantibody-mediated bleeding disorder with both accelerated platelet destruction and impaired platelet production. We and others have described impaired regulatory CD4⁺CD25^{hi} T cells (Treg) numbers and/or suppressive function in ITP patients. Clinical trials using thrombopoietic agents to stimulate platelet production have shown favorable outcomes in ITP patients, but information on the immunologic responses of treated patients are lacking. We studied the immunological profile of chronic ITP patients before (n=10) and during treatment with thrombopoietin receptor (TPO-R) agonists (n=9). Treg activity, as measured by suppression of proliferation of autologous CD4⁺ CD25⁻ cells, was improved in patients on treatment (p<0.05) and the improvement correlated with reduction in IL-2-producing CD4⁺ cells, consistent with dampening of immune responses. There was a concomitant increase in total circulating TGF-β1 levels (p=0.002) in patients on treatment and the levels of TGF-β1 correlated with the degree of improvement in platelet counts (r=0.8, p=0.0002). This suggests that platelets in patients on TPO-R treatment may play a role in improving Treg function, either directly or indirectly by enhanced release of TGF-β1 as a result of greater platelet turnover. In conclusion, our findings suggest that thrombopoietic agents in patients with ITP have profound effects to restore immune tolerance.
Introduction

Immune thrombocytopenia (ITP) is a bleeding disorder due to low platelet counts with an incidence of 2 and 12 per 100,000 adults and children, respectively per year and a mortality rate of 1-3% per year in severely affected cases. Autoreactive antibodies to platelet antigens, mainly the platelet glycoprotein IIb/IIIa complex, are considered responsible for accelerated destruction of platelets by the reticuloendothelial system and also reduced platelet production. While healthy individuals harbor platelet-specific autoreactive T cells that are tolerized in the periphery, patients with ITP possess activated platelet-autoreactive T cells with increasing cytokine imbalance toward IL-2 and IFN-γ especially in patients with chronic ITP with some also reporting higher levels of circulating pro-inflammatory cytokines tumour-necrosis factor (TNF-α) and soluble CD40 ligand (sCD40L). These data are consistent with loss of peripheral tolerance and an inflammatory phenotype in chronic ITP patients.

CD4+ regulatory T cells (Tregs) play a critical role in maintenance of peripheral tolerance by both directly and indirectly suppressing the activation and proliferation of many cell types, including T cells, B cells, dendritic cells, natural killer (NK) cells, and NKT cells in vivo and/or in vitro. Because of their ability to control homeostasis and immunopathology, the level of Tregs and their function are among the most informative criteria of a patient’s immune status. Tregs are characterized by high expression of the CD25 molecule (the IL-2 receptor α-chain) and expression of the transcription factor Foxp3, and make up 5–10% of the normal peripheral CD4+ T cell population. As with a number of other autoimmune diseases, recent studies in patients with ITP have shown reduced levels of Foxp3 mRNA and protein in circulating mononuclear cells and abnormal Treg function in spleen biopsies. We recently showed that circulating Treg suppressive activity was reduced in patients with chronic ITP and that the defect was intrinsic to Tregs rather than a result of effector T cells resisting suppression. These studies indicate that deficiency in generation and/or defective functions of Tregs may contribute to the loss of immunologic self-tolerance and pathogenesis in patients with ITP. In particular, failure to maintain immune suppression by Tregs may explain the reported platelet autoantigen-specific T cell proliferative responses and the proinflammatory phenotype in ITP patients. Interestingly, chronic ITP patients treated with rituximab whose platelet counts improve show restored numbers of Tregs as well as restored regulatory activity as determined by in vitro cell
proliferation assays.\textsuperscript{20} Similarly, improvement in Treg frequency and activity have been reported following treatment with high dose dexamethasone in patients with ITP,\textsuperscript{17} and in vitro studies indicate a positive effect of IVIG on Treg function.\textsuperscript{21} Altogether, the data are consistent with the immunomodulatory nature of such treatment modalities in the setting of chronic ITP.

More recently, a number of thrombopoietic agents have been developed and shown to be highly effective in the treatment of ITP.\textsuperscript{22} These agents include a thrombopoietic mimetic containing the thrombopoietin receptor (TPO-R)-activating peptides attached to the Fc portion of IgG (Nplate or Romiplostim or AMG 531) given as weekly subcutaneous injections, and small molecule TPO-R agonists administered as daily oral tablets (eltrombopag or Promacta; AKR-501; and LGD4665). They increase thrombopoiesis by activating TPO receptors (TPO-R), thereby increasing the production of mature megakaryocytes and platelets.\textsuperscript{23} Romiplostim and eltrombopag have completed phase I-III clinical trials and over 70\% of patients had achieved “safe” levels of platelet counts that prevent bleeding ($\geq 50 \times 10^9$/L) with favorable outcomes with respect to safety and tolerability with both drugs.\textsuperscript{24-28} AKR-501 has completed a phase I trial, demonstrating an increase of more than 50\% over the baseline platelet count in 5 out of 6 healthy volunteers tested\textsuperscript{29} and additional clinical trials in patients with chronic ITP are underway. No information is currently available on the immune responses of patients treated with any of these drugs as it relates to prognosis and response to treatment.

In this study, we analyzed the immunological profiles of a cohort of patients before treatment and in patients who had been on treatment with thrombopoietic agents. Surprisingly, we found an improvement in the Treg activity of patients on treatment and a decrease in their pro-inflammatory sCD40L with a concomitant increase in their circulatory TGF-$\beta$1 that correlated with increase in platelet counts. These data suggest that thrombopoietic agents possess immunomodulatory activity as indicated by the improved peripheral Treg function in patients on treatment and an accompanying decrease in inflammatory state. In addition, the data raises the interesting possibility that the increase in platelet counts may play a role in mediating the increase in Treg function in patients on treatment.
**Materials and Methods**

**Patient population**

All the studies were approved by the institutional Review Boards of the Weill Medical College of Cornell University and of the New York Blood Center. Peripheral blood was obtained from 17 patients (all Caucasian) with chronic ITP study (Table 1) and 9 healthy normal healthy volunteers as controls upon informed consent in accordance with the Declaration of Helsinki. In two cases (patient #9 and patient #10), we were able to sample blood from the same patient before they started treatment with thrombopoietic agents (“pre-treatment”) and after 4 months on treatment (Table 1). The “pre-treatment” group (total n=10, including patients #9 and #10) consisted of 8 females and 2 males with median age 58.5 years (range 17-70) who had completed their previous treatment (non-thrombopoietic agents) at least 2 weeks prior their study visit (Table 1). The on-treatment group (total n=9, including patients #9 and #10) consisted of 8 females and 1 male with median age 54 years (range 46-80) who had been on treatment with thrombopoietic agents (with romiplostim, n=4, eltrombopag, n=1 or an investigational thrombopoietic agent AKR-501, n=4) for at least 4 months at the time of the visit (Table 1).

**Proliferation/Treg cell suppression assay**

To purify Tregs, CD4+ cells were first enriched by positive selection (Miltenyi Biotech, Auburn, CA), followed by separation of CD4+CD25hi and CD4+CD25- T cells (94% purity) on a MoFLo cell sorter (Beckman-Coulter Inc, Hialeah, FL). For the proliferation assay, CD4+CD25- effector cells (5 x 10⁴) were cultured alone or in combination with CD4+CD25hi Tregs in either duplicates or triplicates at various effector to Treg cell ratios in wells containing irradiated T cell depleted allogeneic PBMC. On day 5 of culture, 1 μCi of ³H thymidine (Perkin Elmer, Shelton CT) was added to each well and incubated for an additional 16 h and the uptake of labeled thymidine was measured by liquid scintillation (PerkinElmer, Waltham, MA). Percentage inhibition which is referred to as Treg suppressive activity was determined as 1 – (cpm incorporated in the coculture)/cpm of responder cells alone) x 100.

**Surface and Foxp3 staining**

To determine the frequency of Tregs, freshly obtained whole blood (150μl/tube) was first incubated with CD4-PerCP and CD25-APC (BD Biosciences, San Jose, CA) prior to staining for
intracellular Foxp3 (clone PCH101 and an isotype control) following manufacturer’s instructions. Cells were analyzed by BD FACSCanto using Diva software (BD, Franklin Lakes, NJ).

**Cytokine detection**

For detection of circulatory TGF-β1 and sCD40L levels, platelet-poor plasma was first prepared following a modified protocol by Lee et al.30 Briefly, blood samples were collected in acid citrate dextrose solution A (BD Vacutainer, Franklin Lakes, NJ) and first centrifuged at 200g for 15 minutes to remove RBCs and WBCs, followed by centrifugation at 750g for 20 minutes to remove platelets and a final centrifugation step at 16,000g for 20 minutes to remove debris. Samples were placed at 37 °C for 15 minutes following each centrifugation step and prostacyclin (5ng/ml, Sigma Aldrich, St Louis, MO) was added before each centrifugation to maintain platelet quiescence. The samples were then defibrinated (1U/mL thrombin, Sigma Aldrich, St Louis, MO) and stored at 80 °C. Circulating TGF-β1 levels were determined by the human TGF-β1 Quantikine ELISA kit (R&D Systems Inc. Minneapolis, MN) following the manufacturer’s instructions. To measure total TGF-β1, including the biologically inactive latent TGF-β1 form,31 samples were first acid-activated (with 1N HCl) before assaying. sCD40L was determined by the human ELISA kit by Bender Medsystems (eBioscience Inc., San Diego, CA).

Intracellular cytokine staining for IL-2 was done on CD4+CD25– sorted T cells stimulated for 5 h with PMA (50ng/ml) and ionomycin (1μM) in the presence of brefeldin A (1μg/ml, BD, San Jose, CA). Cells were fixed and made permeable with Cytofix/Cytoperm according to the manufacturer’s instructions (BD Biosciences, San Jose, CA). Cells were stained with FITC-labeled anti- IL-2 and were analyzed on a FACSCanto (BD, Franklin Lakes, NJ). Flow cytometric data were analyzed with BD FACS Diva software (Franklin Lakes, NJ).

**Statistical analysis**

Statistical analyses were performed using Mann-Whitney *U* test (platelet counts, Treg frequency, cytokine measurements), non-parametric Spearman correlation (all correlation studies), and 2-way ANOVA for repeated measurements (Treg assay). Differences were considered significant at *p* < 0.05.
Results

Treg activity improves in patients on treatment with thrombopoietic agents

To determine whether Treg frequency/activity is affected following treatment with thrombopoietic agents, we compared the peripheral Treg compartment of a group of chronic ITP patients with platelet counts of below 40 x10^9/L before they went on treatment (n=10) with that of a second group (n=9) who had been on treatment with thrombopoietic agents for more than 4 months (Table 1). All but one patient (#10) in the “on treatment” group was being treated exclusively for ITP with thrombopoietic agents. The “on treatment” group showed an improved platelet counts (Fig. 1, p=0.002). We found no statistically significant differences in the Treg frequencies (Foxp3+CD25^hi in the CD4^+ population) of patients before treatment and those on treatment (2.7% ± 0.4% vs 3.1% ±0.6 % all p values >0.1, Fig.2) and similarly no differences in the levels of Foxp3 expression in the CD4^+CD25^hi population (data not shown).

We next compared the functional activity of CD4^+CD25^hi subset in patients before and on treatment by purifying and testing them ex vivo using a mixed lymphocyte proliferation assay. CD4^+CD25^- T cells from patients before and on treatment had similar proliferation capacity (30,825± 6518 cpm, vs. 29,248 ± 4865 cpm, p= 0.8) and were not significantly different from that of healthy controls (40,387 ± 8054 cpm). Similarly, the sorted CD4^+CD25^hi from patients in the pre-treatment and on treatment groups were equally anergic (4885± 899 cpm vs. 3436 ± 577 cpm, p=0.3), a characteristic feature of in vitro stimulated Tregs. However, Treg suppressive activity as measured by suppression of proliferation of autologous CD4^+CD25^- cells from patients on treatment was significantly higher than the Treg activity of patients in the pre-treatment group (Fig. 3, at 1:1 ratio, suppressive activity for patients on treatment 63% ±4% vs. 53% ±7% for pre-treatment group and at 1:4, 51% ±6% vs. 35% ±7% respectively, overall p=0.045), but was lower than those of healthy volunteer controls (at 1:1 ratio, 70% ±2% and at 1:4 ratio, 50% ±5%). To determine whether the improved Treg activity correlated with altered helper T cell cytokine secretion pattern, we analyzed the intracellular cytokine expression in stimulated sorted CD4^+ population. It has been reported that PBMCs from a majority of patients with chronic ITP when stimulated secrete higher levels of IL-2, possibly as a result of continuously activated autoimmune responses. We found a significant negative correlation between levels of IL-2 expression in stimulated, sorted CD4^+ T cells and Treg suppressive
activity (Fig 4, r=0.47, p=0.04), suggesting that improved Treg activity is linked to dampening of immune responses, in particular related to IL-2.

**Circulating plasma levels of TGF-β1 and sCD40L**

TGF-β1 is primarily considered an anti-inflammatory cytokine, promoting Treg development and function, and inhibiting Th1 and Th2 development as well as B cell proliferation and antibody production. A number of studies have indicated that ITP patients have low circulating TGF-β1 levels which increase as platelet counts improve following various treatments. We therefore measured total circulating TGF-β1 in some of our patient cohort using the platelet poor fraction of the plasma to avoid potential in vitro platelet activation/degranulation. We found that circulating TGF-β1 levels strongly correlated with platelet counts in our patient cohort (Fig. 5, r=0.8, p=0.0002) with higher levels of TGF-β1 in the patient group on treatment compared to the pre-treatment group (Fig. 6, 2211pg/ml ± 345 pg/ml vs 1083 pg/ml ± 127 pg/ml, p=0.02). Concurrently, we also tested levels of circulating sCD40L, a proinflammatory cytokine in the platelet poor plasma fraction in our patient cohort and found a decrease in the levels in patients on treatment (0.88±0.13 ng/ml vs 0.53±0.05 ng/ml, p=0.02) as has previously been reported in treated patients with ITP.
Discussion

We have found in a small cross-sectional study improved in vitro Treg function in chronic ITP patients who have been on thrombopoietic agents, suggesting for the first time that these agents may possess immunomodulatory activity in addition to their profound effects on platelet counts. The increase in Treg suppressive activity correlated with a reduction in effector T helper functions as manifested by a negative association between Treg activity and the frequency of IL-2 expressing CD4⁺ T cells. Moreover, patients on treatment had increased circulating TGF-β1 levels but decreased sCD40L levels, both suggesting a reduction in the overall inflammatory state. Whether the increase in Treg activity in patients on treatment is a cause or consequence of decrease in the inflammatory state remains to be determined by future longitudinal studies.

Because TPO receptor expression is largely restricted to the megakaryocytic compartment, it is unlikely that the thrombopoietic agents directly interact with the Tregs to improve their activity. Instead, our hypothesis is that the effect of thrombopoietic agents on Tregs is mediated indirectly by TGF-β1 released by platelets and/or megakaryocytes. In support of this latter possibility, we found a strong association between platelet counts and circulating TGF-β1 levels. Platelets are a rich source of TGF-β1, containing 40-100 times more than other cell types and they release TGF-β1 following activation/degranulation. The cause of elevated TGF-β1 levels in patients on thrombopoietic agents is not known, but an artifactual platelet activation during venipuncture in only in one patient group, but not healthy controls who have equal or higher platelet counts seems unlikely. Moreover, we find decreased levels of sCD40L, known to be abundant in platelets and released following platelet activation in the same plasma samples in patients on TPO-R agonists, further arguing against an artifactual platelet activation during venipuncture. These data further support a previous report that platelets are not activated in patients on thrombopoietic agents. The reason why circulatory sCD40L, which are mostly platelet-derived, are higher in patients in our pre-treatment group as well as in patients with low platelet counts is not known, but it may be that sCD40L is derived from a non-platelet source (eg. T cells) in patients who have low platelet counts. In addition, the mechanisms that cause the release of sCD40L, but not TGF-β1 in patients with low platelet counts remain to be fully determined, but it is possible that these cytokines may belong to different platelet granule sorting/release pathways.
TGF-β1 may also be released by (increased numbers of) megakayocytes.\textsuperscript{43} It is possible that with increased platelet turnover in patients on thrombopoietic agents, there is increased platelet clearance with concomitant release of TGF-β1. Interestingly, ITP patients with improved platelet counts following treatment with immunosuppressive drugs, high dose dexamethasone, IVIG or splenectomy were also shown to have increased TGF-β1 levels,\textsuperscript{34-36} although direct correlation studies between TGF-β1 levels and platelet counts were not reported. These latter treatments when effective are thought to prevent or minimize platelet destruction which is in contrast to the effect of thrombopoietic agents that increase platelet turnover. Future studies are needed to determine the mechanisms by which TGF-β1 levels are increased in patients on thrombopoietic agents and how these mechanisms may differ, if at all, in patients, who are on treatments that interfere with platelet destruction. Alternatively, it may also be that as platelet counts improve, there is altered processing/presentation of platelet antigens by the antigen presenting cells, resulting in Treg normalization.

TGF-β1 can induce Foxp3-negative T cells and convert them to Foxp3\textsuperscript{+} Tregs.\textsuperscript{44} Although we did not find any correlation between TGF-β1 levels and the frequency of Foxp3\textsuperscript{+} cells in the CD4\textsuperscript{+}CD25\textsuperscript{hi} population, we did find a positive correlation between TGF-β1 levels and the frequency of total CD4\textsuperscript{+}Foxp3\textsuperscript{+} cells (r=0.6, p=0.02, Supplementary Figure 1). These latter cells consisted of CD4\textsuperscript{+}CD25\textsuperscript{hi} Treg population with known regulatory function as well as the CD4\textsuperscript{+}CD25\textsuperscript{med} and CD4\textsuperscript{+}CD25\textsuperscript{neg} populations, whose regulatory role remains questionable.\textsuperscript{45,46} Longitudinal studies are needed to determine whether TGF-β1 levels correlate with induction of Foxp3\textsuperscript{+} T cells in various CD4\textsuperscript{+}CD25\textsuperscript{hi/med/neg} populations and more importantly if these Foxp3-expressing CD4\textsuperscript{+}CD25\textsuperscript{med} and CD4\textsuperscript{+}CD25\textsuperscript{neg} subpopulations possess regulatory activity in ITP patients on treatment.

TGF-β1 has also been shown to be essential for maintenance of Treg functions.\textsuperscript{44} Specifically, CD4\textsuperscript{+}CD25\textsuperscript{+} Treg from transgenic mice overexpressing a truncated version of the TGF-β type II receptor that acts as a dominant negative mutant receptor had decreased in vivo suppressive capacity, indicating that TGF-β1 signaling is essential to maintain Treg suppressive function.\textsuperscript{47} We were unable to find a direct correlation between Treg suppressive activity and TGF-β1 levels. However, in 2 cases (patients #9 and #10, Table 1) we were able to perform analysis before and on treatment on the same patients. With patient #9 whose platelet count was 26 x10\textsuperscript{9}/L before
treatment and increased to 66 x10⁹/L on the drug, we found a 20% increase in peripheral TGF-β1 levels and a concomitant 20% improvement in Treg activity while in patient #10 with platelet counts of 36 x10⁹/L prior to treatment to 290 x10⁹/L on treatment, TGF-β1 levels and Treg activity both increased by 30%. As our study had a cross sectional design, longer term longitudinal studies are required to validate the clinical significance of the elevation of Treg activity observed in patients on treatment with thrombopoietic agents and to further explore the role of platelets and its association with Tregs in ITP. The recent development of a mouse model of ITP⁴⁸ is likely to be helpful in addressing some of these questions.

It is our prediction that antigen-specific Treg responses are increased in patients on treatment in parallel to polyclonal Treg activity, but these studies have not yet been performed. In the study by Kuter et al.,²⁵ only about 10% of the ITP patients treated with a thrombopoietic agent for 6 months did not relapse in the 12 weeks of follow up. What fraction of patients will relapse when longer ie years instead of months of therapy is discontinued remains unexplored and needs further study. Of note, 5/9 patients in the on treatment group had been taking the thrombopoietic agents for ≥1 year (Table 1). It is possible that because functional Tregs are already induced in these patients, treatment regimens optimized to fully restore platelet counts will gradually lead to establishment of stable Treg populations to achieve long term immune tolerance.

In summary, we have found an improved in vitro Treg activity with a concomitant decrease in effector T helper functions in patients on treatment with TPO-R agonists. Furthermore, patients on treatment had increased circulating TGF-β1 levels but decreased sCD40L levels, indicating that a reduction in the overall inflammatory state. While the data needs to be validated in longitudinal study, our current study suggests that thrombopoietic agents which have already been shown to be highly effective in the treatment of ITP, may also be pro-tolerogenic too.
ACKNOWLEDGEMENTS

We are grateful to Dr. Zeeshan Hafeez, and Jared Levan, Jessica Cruz and Greg Lallos (Weill Cornell Medical College) for help with coordinating some of the patient recruitment and clinical data analysis. We thank Dr. Beau Mitchell (NYBC) for helpful discussions. This work was supported in part by National Heart, Lung, and Blood Institute grant HL096497-01 (K.Y.).

Contributions: W.B., S.H. and W.H. performed research, analyzed and interpreted data; M.K. and N.B. recruited patients, and analyzed data; J.B.B. designed the research, selected and recruited patients and wrote the paper; K.Y. designed, directed and performed research and wrote the paper.

Conflict of Interest Disclosure: J.B.B. receives clinical research support from the following companies: Amgen, Camgene, GlaxoSmithKline, Genzyme, Immunomedics, Ligand, Eisai, Inc., Shionogi, and Sysmex. He also participates for France Foundation in their speaker's bureau program. His family owns stock in Amgen and GlaxoSmithKline. He has participated in Advisory Boards for Amgen, GlaxoSmithKline, Ligand, Shionogi, and Eisai.

References


### Table 1. Demographic and clinical characteristics of chronic ITP patients before and during treatment with thrombopoietic agents.

All patients “on treatment” were considered responsive to the thrombopoietic agents, where responsiveness is defined as increasing the platelet count from >30 x10^9/L before starting treatment to a count usually over 50 x10^9/L while on treatment. The platelet counts indicated in the Table correspond to the patients’ counts on the day that their blood was analyzed for this study. Thus, although patient #12 had a platelet count of 31 x10^9/L when we collected blood for our analysis, the common count for this patient while on treatment was over 50 x10^9/L. Out of a total of 17 patients, 3 patients were receiving daily low (less than 3mg) dose immunosuppressive prednisone regimen and one on higher (60mg) dose at the time of their visit and all except one (patient #10) was in the pre-treatment group.

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<th>Gender</th>
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<th>Plt (x10^9/L)</th>
<th>Thrombopoietic agent (time on treatment) at time of blood sampling/collection</th>
<th>Previous/ongoing non-TPO agonist treatment type at time of blood sampling/collection</th>
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<td>Gender</td>
<td>Treatment</td>
<td>Days</td>
<td>Drug</td>
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**On treatment**

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<th>Days</th>
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<tr>
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<td>NO</td>
<td>290</td>
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Figure Legends

Fig. 1. Platelet counts in patients before and on treatment with thrombopoietic agents.
Platelet counts are shown for the same day that blood was sampled for immunological analysis. Platelet counts of patients on treatment (open squares) were statistically higher (p=0.002) compared to before treatment (filled circles). Median pre-treatment count was 22 x10^9/L, IQR 11-30 x10^9/L versus 105 x10^9/L, IQR 44-243 x10^9/L on treatment group.

Fig. 2. Frequency of CD25^{hi} Foxp3^{+} in the patient cohort. (A) Whole blood was stained with anti-CD4 PerCP and anti-CD25 APC, followed by intracellular staining using PE conjugated anti-Foxp3. The cells were first gated on the CD4+ lymphoid population in PBMCs, and the frequency of CD25^{hi}Foxp3^{+} cells was measured based on the shown gating strategy. (B) Percentage of CD25^{hi}Foxp3^{+} in the CD4^{+} population in patients before (filled circles) and on treatment with thrombopoietic agents (open squares) as well as the normal healthy controls (filled diamonds) is shown. No statistical differences amongst the groups were seen.

Fig. 3. Circulating Treg suppressive activity in patients before and on treatment with thrombopoietic agents. Suppression of proliferation by CD4^{+}CD25^{hi} Treg was analyzed in patients before treatment (n=10) and in patient on treatment with thrombopoietic agents (n=9) as well as normal healthy controls (n=9). Sorted populations of CD4^{+}CD25^{−}T cells were stimulated with plate-bound 0.1 µg/ml anti-CD3 antibodies and allogeneic accessory cells, alone or cocultured at varying ratios (1:1 shown in solid lines and 1:4 shown as dotted lines) with autologous sorted CD4^{+}CD25^{hi} cells and mean inhibition was calculated as described in Methods and Materials. Although this is a cross-sectional study with different patients in the pre-treatment and on treatment groups, we have joined the pre-treatment and on treatment data points by a line (solid or dotted) to highlight the difference in the suppressive activity between the 2 groups. Suppression measured at 1:1 ratio of CD4^{+}CD25^{−} to CD4^{+}CD25^{hi} in patients before treatment was 53% ±7%, but was higher in patients on treatment (63% ±4%). Similarly, at 1:4, the pre-treatment group had a lower suppressive activity (35% ±7%) compared to on treatment group of 51% ±6% (overall p=0.045). For comparison, the healthy volunteer controls suppressive activity at 1:1 ratio (70% ±2%) and at 1:4 ratio (50% ±5%) is also indicated. The raw proliferation data is shown as a supplementary figure 2.
**Fig. 4.** Correlative analysis of Treg functional activity and frequency of IL-2 expressing sorted CD4⁺ T cells. Sorted CD4⁺CD25⁻ T cells from the patient cohort were stimulated with PMA and ionomycin for 5 hours in presence of protein transport inhibitor and intracellular expression of IL-2 was measured by flow cytometry. The frequency of IL-2 expressing CD4⁺ cells negatively correlated with the patients’ Treg suppressive activity at 1:1 ratio of CD4⁺CD25⁻ and CD4⁺CD25high (r=-0.47, p=0.04).

**Fig. 5.** Correlative study of platelet counts and TGF-β1 levels. Circulating total TGF-β1 levels of the patient cohort from Table 1 were measured by ELISA using platelet poor plasma. Correlative analysis indicated a strong positive association between the patients platelet counts and TGF-β1 levels (r=0.8, p=0.0002).

**Fig. 6.** Circulating TGF-β1 levels in patients before and on treatment with thrombopoietic agents. Plasma levels of TGF-β1 were significantly increased in patients on treatment (filled squares) compared to before treatment (filled circles, p=0.002, using the rank order Mann-Whitney test). As comparison, levels of TGF-β1 in normal healthy controls are shown (filled diamonds).

**Fig. 7.** sCD40L levels in platelet poor plasma of patient cohort. Circulating levels of sCD40L in platelet poor plasma were significantly lower in patients on treatment (filled squares) compared to pre-treatment (filled circles, p=0.02). As comparison, levels of sCD40L in normal healthy controls are shown (filled diamonds).
Figure 1

Plt count (x\(10^9\)/L)

Pre-Treatment
\(n=10\)

On treatment
\(n=9\)

\(p=0.002\)
Figure 3

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Figure 5

$r = 0.8$
$p = 0.0002$

TGF-β1 (pg/ml) vs. Plt count ($x10^9$/L)
Figure 6

p=0.002

TGFβ1 (pg/ml)

Pre-treatment

On treatment

Healthy controls

[Graph showing comparison of TGFβ1 levels among different groups with statistical significance]
Figure 7

The figure shows a scatter plot comparing the levels of sCD40L (ng/ml) in different groups:

- **Pre-treatment**
- **On treatment**
- **Healthy controls**

There is a significant difference between the groups, indicated by the p-value of 0.02. The y-axis represents the concentration of sCD40L, with the x-axis indicating the different treatment phases.
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