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Bone Marrow Remodelling Supports Hematopoiesis in Response to Immune Thrombocytopenia Progression

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Abstract:

Immune thrombocytopenia (ITP) is an acquired autoimmune condition characterized by both reduced platelet production and the destruction of functionally normal platelets by sustained attack from the immune system. However, the effect of prolonged ITP on the more immature hematopoietic progenitors remains an open area of investigation. Using a murine *in vivo* model of extended ITP, we reveal that ITP progression drives considerable progenitor expansion and bone marrow (BM) remodelling. Single cell assays using Lin⁻Scal⁺c-Kit⁺CD48⁻CD150⁺ long-term hematopoietic stem cells (LT-HSCs) revealed elevated LT-HSC activation and proliferation *in vitro*. However, the increased activation did not come at the expense of LT-HSC functionality as measured by *in vivo* serial transplantations. ITP progression was associated with considerable BM vasodilation and angiogenesis, as well as a 2-fold increase in local production of CXCL12; a cytokine essential for LT-HSC function and BM homing expressed at high levels by LepR⁺ BM stromal cells. This was associated with a 1.5-fold increase in LepR⁺ BM stromal cells and a 5.5-fold improvement in progenitor homing to the BM. Whereas the increase in stromal cells was transient and reverted back to baseline after platelet count returned to normal, vasculature changes in the BM persisted. Together, these studies demonstrate that LT-HSCs expand in response to ITP, and that LT-HSC functionality during sustained hematopoietic stress is maintained through an adapting BM microenvironment.

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Bone Marrow Remodelling Supports Hematopoiesis in Response to Immune Thrombocytopenia Progression in Mice

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Key Points

- Sustained ITP activates and increases the number of functional LT-HSCs
- The remodelled ITP bone marrow enhances hematopoiesis

Abstract

Immune thrombocytopenia (ITP) is an acquired autoimmune condition characterized by both reduced platelet production and the destruction of functionally normal platelets by sustained attack from the immune system. However, the effect of prolonged ITP on the more immature hematopoietic progenitors remains an open area of investigation. Using a murine *in vivo* model of extended ITP, we reveal that ITP progression drives considerable progenitor expansion and bone marrow (BM) remodelling. Single cell assays using Lin⁻Sca1⁺c-Kit⁺CD48⁻CD150⁺ long-term hematopoietic stem cells (LT-HSCs) revealed elevated LT-HSC activation and proliferation *in vitro*. However, the increased activation did not come at the expense of LT-HSC functionality as measured by *in vivo* serial transplantations. ITP progression was associated with considerable BM vasodilation and angiogenesis, as well as a 2-fold increase in local production of CXCL12; a cytokine essential for LT-HSC function and BM homing expressed at high levels by LepR⁺ BM stromal cells. This was associated with a 1.5-fold increase in LepR⁺ BM stromal cells and a 5.5-fold improvement in progenitor homing to the BM. Whereas the increase in stromal cells was transient and reverted back to baseline after platelet count returned to normal, vasculature changes in the BM persisted. Together, these studies demonstrate that LT-HSCs expand in response to ITP, and that LT-HSC functionality during sustained hematopoietic stress is maintained through an adapting BM microenvironment.

Introduction

Increased destruction and decreased production of functionally normal platelets is a hallmark of Immune Thrombocytopenia (ITP), which manifests as a bleeding tendency¹⁻³. The majority of ITP patients develop serum platelet specific autoantibodies that target platelets for destruction and are primarily against platelet glycoprotein (GP) IIb/IIIa (CD41/CD61), GPIb/IX (CD42c/CD42a), GPV (CD42d) and GPIa/IIa (CD49b)⁴⁻⁷. Of these, autoantibodies against CD41/CD61 are the most common^{6,7}. The spleen is the major site of platelet clearance, which is primarily mediated through phagocytosis by splenic macrophages or dendritic cells^{8,9}. ITP patients can be classified as 'newly diagnosed' (< 3 months) or 'persistent' (< 12 months) which is more common in children, as well as 'chronic' (> 12 months) which is more common in adults^{10,11}.

During conditions of chronic hematopoietic stress, such as infection and inflammation, hematopoietic stem cells (HSCs) are activated to meet the increased demand in blood cell production^{12,13}. Exit from quiescence has been previously associated with a loss in regenerative capacity and may eventually cause pancytopenia¹⁴⁻¹⁶. During ITP, the requirement for HSC differentiation is twofold. Firstly, the increased platelet demand caused by autoimmune-mediated platelet destruction drives megakaryopoiesis¹⁷⁻²⁰. Secondly, sustained immune system activation driving platelet destruction requires replenishment from progenitors^{12,13,21}. Despite this, there have been no reports of HSC exhaustion or pancytopenia in chronic ITP patients, suggesting that HSCs may receive additional support.

Recent evidence shows that the bone marrow (BM) microenvironment provides supportive, rather than instructive input for HSC differentiation²². Furthermore, the microenvironment is pliable, and can contribute to disease progression by driving the expression of proliferative/pro-inflammatory factors or by downregulating factors which traditionally support HSC function. Such changes may be induced by the presence of infection^{23,24}, the development of malignancy^{25,26} or the administration of drugs²⁷. Whether the BM microenvironment actively remodels itself in response to various chemical or cellular insults to resolve and/or minimise hematopoietic stress remains unclear.

Antibody mediated platelet destruction can be recreated *in vivo* by the administration of anti-platelet antibodies to cause thrombocytopenia²⁸. This is known as the passive transfer model of ITP, and its historical use has been in the form of a single injection^{29,30}, or multiple injections of anti-platelet

antibodies spanning 1-2 weeks³¹⁻³⁵. To better model sustained ITP, we extend this model to 4 weeks, showing that the increased demand on hematopoiesis is met by an increase in the number of functional HSCs. We hypothesise that this is achieved through an interactive and iterative relationship between differentiating HSCs and an adapting supportive BM microenvironment in an effort to maintain homeostasis.

Methods

Mice

Mice used in this study were female wild type (WT) C57Bl/6J (B6)-CD45.1 or CD45.2 mice unless otherwise stated. *Cxcl12*^{DsRed/+} knock-in mice were previously bred onto a B6 genetic background and were generated by recombining *DsRed-Express2* into the endogenous *Cxcl12* locus as previously described³⁶. Both WT and *Cxcl12*^{DsRed/+} mice were used between 8-12 weeks of age. Female B6-*W⁴¹/W⁴¹*-CD45.1 mice aged between 7-9 months of age were used as recipients for low dose Lin⁻ Sca1⁺c-Kit⁺CD48⁺CD150⁺ long-term hematopoietic stem cell (LT-HSC) transplantation experiments. All mice were bred and housed under specified pathogen-free conditions. All animal procedures were approved by the University of York Animal Welfare and Ethical Review Board and carried out under UK Home Office licence (PPL P24552FC1). Animals were killed by CO₂ asphyxia and cervical dislocation, or overdose of anaesthesia and exsanguination. Mice were randomly allocated to the IgG or anti-CD41 group and downstream sample analysis was performed blinded. Primers for genotyping of the *Cxcl12*^{DsRed} allele: 5'-AAGAAGCCCGTGAAGCTGC-3' and 5'-TCCTCGTTGTGGGAGGTGAT-3'.

Passive ITP Model

Mice were administered anti-mouse CD41 antibody (clone MWReg30; BD Pharmingen) to selectively deplete platelets (or IgG1 to act as a control) via intraperitoneal injection every 48 h for up to 4 weeks (duration dependent on the experiment). Concentration of anti-CD41/IgG1 ranged between 0.2-0.4 mg/kg as indicated in Figure 1. As described previously, antibody dose escalation is necessary to maintain thrombocytopenia over time³⁴. In our study, 0.2 mg/kg anti-CD41 was sufficient to maintain thrombocytopenia for the first 2 weeks, followed by an increase of 0.1 mg/kg per week thereafter.

Competitive Transplantation Assay

WT (CD45.2) donor mice were injected with anti-mouse CD41 antibody, whilst WT (CD45.1) donor mice were injected with IgG1 for 4 weeks (as described above). CD45.1 recipient mice were administered total body gamma radiation with two doses of 5.5 Gy, 24 h apart (lethal irradiation). For primary transplants, flushed BM from donor mice was prepared by mixing CD45.1 and CD45.2 BM cells in a 3:1, 1:1, and 1:3 ratio and resuspended in sterile PBS. A total of 5×10^6 cells in a final volume of 200 μ L was injected into the tail vein of each of the recipient mice. For secondary transplants, 2×10^7 BM cells from primary transplant donors (pooled within each group) were further transplanted into CD45.1 recipient mice. All recipient mice were administered oral antibiotic Baytril (Bayer, Leverkusen, Germany) in drinking water for 14 days post-transplant as a prophylactic treatment against bacterial infection. Recipient mice were bled at 4, 8, 12, 16 and 20 weeks to monitor donor chimerism and relative mature cell production by flow cytometry. Full details of antibodies used and flow cytometry methodology are available in the Supplementary Methods.

Cell Sorting and Transplantation Experiments

Lin⁻ donor cells were immunomagnetically enriched prior to cell sorting based upon the negative expression of lineage markers via the EasySep Mouse Hematopoietic Progenitor Cell Isolation Kit (19856, StemCell Technologies) according to the manufacturer's instructions. Following enrichment, cells were stained for progenitor markers as described in the Supplementary Methods.

For homing assays, approximately 15,000 Lin⁻ Sca1⁺c-Kit⁺ (LSK) cells from naïve CD45.1 mice were administered in 100 μ L sterile PBS per recipient mouse via tail vein injection. Recipient mice were lethally irradiated CD45.2 mice, previously administered anti-CD41 or IgG for 4 weeks. 36 h later, BM was harvested and stained for donor cells followed by flow cytometry analysis as described in the Supplementary Methods.

For low dose HSC transplantation experiments, 100 LT-HSCs from either 4 week anti-CD41 or IgG injected CD45.2 mice were bulk sorted into 1 mL of PBS, and diluted so that each sub-lethally irradiated (one dose of 3.6 Gy) B6-*W⁴¹/W⁴¹*-CD45.1 mouse received either 10 or 3 LT-HSCs. Recipient B6-*W⁴¹/W⁴¹*-CD45.1 mice were bled 4, 8 or 11 weeks post transplantation and peripheral blood analysed to determine levels of chimerism as described in the Supplementary Methods. The experiment was terminated at 11 weeks due to COVID-19 restrictions, therefore it was not possible to

confirm whether mice with < 1% chimerism (trace chimerism) would progress to develop > 1% chimerism with time. Mice with trace chimerism are highlighted in the results.

For *in vitro* LT-HSC assays, 1 LT-HSC was sorted per well of a round bottom 96-well plate, with each well containing 50 μ L of LT-HSC media as described in the Supplementary Methods.

Cells were sorted using a four laser Beckman Coulter Astrios Eq sorter.

Statistics

Statistical analyses were performed using GraphPad Prism software (Version 8.4.3). In statistical graphs, data points indicate individual samples and bars represent mean values $\pm$ SD. $P < 0.05$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$, whilst non-significant values ($P > 0.05$) are not shown. P values are reported in the figure legend.

Results

ITP Progression Drives Hematopoietic Progenitor Expansion

To determine the long-term impact of ITP on hematopoiesis, a model for sustained ITP was established by repeated (every 48 h) intraperitoneal injection of mice with a monoclonal rat anti-mouse CD41 antibody or rat IgG1 isotype control for a period of 4 weeks (Figure 1). From 2 weeks, increased doses of rat anti-CD41 antibody were necessary to maintain thrombocytopenia, presumably to counteract compensatory thrombopoiesis³⁴ and/or antibody generation against the injected rat IgG1 antibody. Hereafter, mice treated with anti-CD41 are referred to as the 'ITP' group; mice treated with IgG1 are referred to as the 'control' group.

This passive transfer model maintains severe thrombocytopenia (defined here as a mean platelet count below $200 \times 10^9/L$), from a mean resting platelet count of $865 \times 10^9/L$. Mice in the ITP group had a platelet count range of $57-327 \times 10^9/L$, whilst controls had a platelet count range of $584-1402 \times 10^9/L$. Additionally, mice with ITP had an increase in mean platelet volume indicating a higher platelet turnover (Figure 1Ai). Plasma thrombopoietin levels were not affected by ITP, although an increase in the number of BM megakaryocytes was observed at later time points (Figure 1B-C). As inflammatory factors are known to drive emergency megakaryopoiesis independently of thrombopoietin³⁷, the plasma and BM supernatant were analysed. Biological processes involved in the inflammatory response and positive regulation of MAPK signalling (such as CCL21) were upregulated in both the

plasma and the BM (Figure 1D and Figure S1). Upregulation of these biological processes were higher in the BM than the plasma which was of interest as inflammation and MAPK signalling have previously been shown to affect LT-HSC quiescence and function^{37,38}. Additionally, some biological processes and reactome pathways also shown to affect LT-HSC quiescence and function such as IL-1 signalling and smooth muscle cell proliferation (such as VEGF, MMP2, WISP1 and IGFBP-3) were uniquely upregulated in the BM (Figure 1D and Figure S1)³⁹⁻⁴³.

To test whether sustained ITP affects hematopoietic progenitors, mice in the ITP group and controls were compared by flow cytometry. Whilst ITP had no effect on total BM cellularity (Figure 2A), ITP caused a 1.3-fold expansion in the percentage of BM-derived Lin⁻Sca1⁻c-Kit⁺ (LK cells, enriched for myeloid progenitors) and 1.7-fold expansion in LSK cells, which are enriched for HSCs (Figure 2Bi and ii). Progenitor expansion in the spleen was also observed (Figure S2C-E), with a 2 and 2.9-fold expansion in the LK and LSK populations, respectively, suggesting the presence of extramedullary hematopoiesis in ITP. The LSK population was subdivided further using SLAM markers⁴⁴, with the largest expansion in the LT-HSC fraction (2.8-fold in the BM and 9.7-fold in the spleen) (Figure 2Bv). Gating strategies of analysed BM and splenic populations are shown in Figure S2A-B.

To determine the functional effect of ITP on LT-HSCs, single LT-HSCs from mice in the ITP group or controls were isolated and cultured in conditions which maintain LT-HSC activity (Figure 2B) and analysed for changes in cell division, colony size and cell phenotype^{45,46}. LT-HSCs from mice with ITP proliferated faster, with less time taken to complete first, second and third division (Figure 2C). This faster division formed larger colonies at day 10 of culture (Figure 2D), which were driven by an increase in LSK progenitors (Figure 2Ei). However, despite the overall increase in number of cells per clone, ITP did not alter the proportion of differentiated and stem/progenitor cells within clones, suggesting that progenitor expansion was not accompanied by differentiation or self-renewal defects (Figure 2Eii-iii). Our results indicate that sustained ITP induces the cycling of phenotypic LT-HSCs, causing an expansion of hematopoietic progenitors without compromising LT-HSC function.

Sustained ITP Increases the Number of Functional LT-HSCs

In order to assess the *in vivo* functional capacity of the expanded progenitor compartment, competitive transplantation assays were performed using 5x10⁶ whole BM cells from ITP and control donors in 3:1, 1:1 and 1:3 ratios (Figure 3A). This was followed by secondary transplantations after 20

weeks using 2×10^7 pooled whole BM cells from mice within each ratio group. In the 1:1 group (Figure 3C), the contribution of ITP donors to peripheral chimerism increased to $63 \pm 6\%$ (mean values $\pm$ SD), accompanied by an expanded contribution to the BM LT-HSC phenotype at $85 \pm 10\%$. Secondary transplantation data similarly showed an increased contribution by donor cells derived from the ITP group to mature cell production. Together, these transplantation data demonstrate the existence of an expanded, self-renewing pool of functional LT-HSCs in the BM of mice with sustained ITP.

In order to test LT-HSC functional capacity more directly, we next transplanted limiting numbers of highly purified LT-HSCs isolated using the $\text{Lin}^- \text{Sca1}^+ \text{c-Kit}^+ \text{CD48}^- \text{CD150}^+$ phenotype. Either 3 or 10 LT-HSCs from the ITP or control groups were isolated and transplanted into sub-lethally irradiated B6- W^{41}/W^{41} -CD45.1 mice (Figure 3E). Whereas 20% of mice receiving 3 LT-HSCs and 40% of mice receiving 10 LT-HSCs from the control group were positive, none of the mice receiving LT-HSCs from mice with ITP were repopulated (Figure 3F), suggesting that the LT-HSC phenotype in the ITP group does not contain as high a frequency of functional LT-HSCs.

ITP Progression Drives Physical and Biochemical Changes Within the BM HSC Microenvironment

LT-HSC function is maintained through cytokine-cytokine receptor interactions and direct cell-cell contact between the LT-HSC and components of the perivascular BM microenvironment, including endothelial cells and LepR^+ stromal cells^{36,47,48}. As sustained ITP causes expansion and functional changes in LT-HSCs, we next assessed whether this was accompanied by alterations in the BM microenvironment. Immunofluorescence microscopy identified significant changes in non-hematopoietic components of the HSC BM microenvironment, which were associated with ITP progression (Figure 4). Mice with 4 week ITP had a 2-fold increase in sum vessel area, which was attributed to an increase in average vessel area rather than any changes in vessel number. Effects were also observed at an earlier (2 week) time point, with a 1.5-fold increase in sum vessel area with no change in vessel number (Figure S3). Furthermore, at 4 weeks there was a significant LepR^+ stromal cell expansion (1.5-fold increase), whilst there were no changes in stromal cell number at 2 weeks.

To explore whether this remodelling of the BM microenvironment was reversible following recovery from thrombocytopenia, mice were allowed to recover for 4 weeks ('ITP Recovered' group) prior to BM analysis (Figure 4). During this period of recovery, the platelet count reverted back to baseline by

day 18 after the final anti-CD41 antibody injection (Figure S4). Despite the amelioration of thrombocytopenia, the substantial structural differences observed in the BM vasculature persisted with a further 1.5-fold increase in sum vessel area relative to the initial ITP group. In these mice, a significant increase in vessel number was observed as opposed to a further increase in average vessel area (Figure 4C). Conversely, numbers of LepR⁺ perivascular stromal cells reverted back to control levels, suggesting that stromal cell expansion was transient (Figure 4B).

Since perivascular LepR⁺ stromal cells are a major source of factors influencing LT-HSC function, including key microenvironment factors CXCL12, SCF and Angiopoietin-1^{36,47,49,50}, and share the same microenvironment as LT-HSCs^{47,51,52}, we hypothesised that a transient increase in LepR⁺ stromal cells support LT-HSCs in response to sustained ITP. We measured the expression of *Cxcl12*, *Kitl* and *Angpt1* in the BM and found that *Cxcl12* expression transiently increased to 2-fold levels in the ITP group (Figure 5A), but there were no significant changes in *Kitl* or *Angpt1* (Figure S5B). Confocal imaging of the BM microenvironment from *Cxcl12*^{DsRed/+} reporter mice from an ITP group relative to a control group of mice revealed an increase in perivascular *Cxcl12* expression in mice from the ITP group (Figure 5B), and that this was driven by the expansion of LepR⁺ stromal cells (Figure S5A). CXCL12 levels were increased 1.8-fold in the BM supernatant of mice with ITP, but not in circulation or spleen, confirming a BM specific elevation in CXCL12 (Figure 5C). As CXCL12 has an essential role in progenitor homing to the BM⁵³⁻⁵⁶, we hypothesised that mice with ITP would show an increased potential for progenitor homing (Figure 5D). Flow cytometry analysis of recipient BM revealed that naïve LSKs homed preferentially (5.5-fold increase) to the BM of irradiated mice with ITP relative to controls. Together, the data shows that the BM actively remodels in response to ITP progression to create a site preferential for hematopoiesis. Whilst the expansion in LepR⁺ stromal cells and associated CXCL12 expression was transient, vasculature changes persisted, the consequences of which are unknown.

Discussion

Whilst the mechanisms behind the development and maintenance of thrombocytopenia have been the subject of intensive research, the wider long-term effects of ITP progression are unclear. Here, we establish a model for investigating the effect of ITP progression on hematopoiesis and identify a significant expansion in phenotypic hematopoietic progenitors accompanied by a remodelling of the

BM microenvironment. Functional assays demonstrated an increased proliferation and self-renewal potential of LT-HSCs, but also indicated a shift in LT-HSC frequency within the LT-HSC phenotype. Assessment of the BM microenvironment identified alterations in key mediators of LT-HSC function, including an increase in total blood vessel area and a specific increase in LepR⁺ stromal cells. Together, these changes in LT-HSC activation and BM remodelling combine to counteract the stress of sustained ITP to ensure homeostasis within the hematopoietic system.

The murine ITP model we present shares a number of features with the clinical progression of chronic ITP such as increased megakaryopoiesis whilst maintaining normal circulating thrombopoietin levels^{17,19,20,57}, giving us confidence that our model was an appropriate choice to study the effect of sustained ITP on hematopoiesis. Although increased megakaryopoiesis has been previously reported to occur as soon as 24 h after anti-CD41 injection⁵⁸, we did not observe increased megakaryopoiesis until 4 weeks of injections. Cytokine analysis of BM and plasma samples at 4 weeks revealed an upregulation of proteins involved in the inflammatory response, which has been previously shown to drive megakaryopoiesis³⁷. As the cytokine signature in the ITP BM appeared to be more divergent from controls than the periphery, and that several upregulated biological processes and pathways have been previously shown to affect LT-HSC function³⁸⁻⁴⁰, we asked if sustained ITP may affect hematopoiesis more broadly.

The expansion in stem and progenitor cell numbers in ITP in combination with the increase in proliferation observed in single LT-HSC *in vitro* assays suggests that LT-HSCs might be activated in response to ITP. Competitive transplantation assays revealed that ITP drives an expansion of LT-HSCs with durable self-renewal, suggesting that this activation did not compromise LT-HSC activity. However, caution is needed when interpreting the relative numbers and activation status of LT-HSCs in diseased states. Whereas whole BM competitive transplants allow LT-HSC activity to be assessed in an unbiased fashion and indicate that numbers of functional LT-HSC are increased, experiments assume that the expression of cell surface markers are not altered as a result of the experimental conditions. Indeed, it has been reported that during immune stimulation, BM cells have increased expression of Sca1 and CD150, causing non-HSC populations to appear in gates expected to be enriched in LT-HSC populations⁵⁹. This can lead to dilution of experimental LT-HSCs vs control LT-HSCs when performing downstream experiments. Our low dose LT-HSC transplantation experiments suggest that this may also occur in sustained ITP, where sub-lethally irradiated mice receiving LT-

HSCs from ITP CD45.2 mice failed to repopulate compared to control CD45.2 mice. Together these data indicate that LT-HSCs might reside outside the traditional phenotype and that the LT-HSC phenotype is contaminated with a larger proportion of non-HSCs in an ITP setting.

It has recently been shown that progenitors activate and proliferate in response to acute antibody mediated thrombocytopenia³³. However, when ITP is extended to 2 weeks, LT-HSC functionality was impaired during serial transplants. This discrepancy with our results is potentially due to differences in experimental setup. Ramasz et al³³ performed a primary competitive transplant using 300 LT-HSCs from mice with sustained ITP and 5×10^5 BM competitor cells from controls. After 16 weeks, they transplanted 300 ITP LT-HSCs from primary recipients into secondary recipients alongside 5×10^5 fresh BM competitor cells. The decreased contribution to peripheral blood reconstitution after each round of competitive transplant can be explained by our observation that the traditional LT-HSC phenotype is contaminated with a larger proportion of non-HSCs in ITP. However, when analysing the LT-HSC pool as a whole (e.g. using unbiased whole BM transplants), LT-HSC functionality is increased and preserves hematopoiesis long-term.

The ability of LT-HSCs to maintain functionality in conditions of hematological stress is essential for the preservation of hematopoiesis long term. When LT-HSCs are unable to meet the increased demand such as in chronic infection^{23,24} or the development of malignancy^{25,26}, LT-HSCs exit quiescence and pancytopenia may arise. The increase in the functional LT-HSC pool in ITP therefore presents an intriguing dichotomy and we studied the HSC BM microenvironment to determine whether beneficial changes in cell extrinsic factors may occur. ITP progression was associated with vasodilation and angiogenesis as well as LepR⁺ stromal cell expansion. The stromal cells maintained their classically defined perivascular location and therefore close/adjacent proximity to LT-HSCs^{36,49,60} and their expansion was associated with an increase in CXCL12, which has a crucial role in maintaining LT-HSC function, including retention in the BM^{36,54,55}, repopulating activity⁶¹ and quiescence^{56,62}. Furthermore, this increase in CXCL12 was BM specific and was expected to be primarily derived from LepR⁺ stromal cells^{36,60}, which we confirmed by confocal microscopy.

Intriguingly, LepR⁺ stromal cell number reverted back to levels seen in control mice after recovery from ITP, suggesting that thrombocytopenia may indirectly feedback to increase LT-HSC support during ITP progression.

The BM is the primary and preferential site for steady state hematopoiesis in healthy adults, which is maintained by complex and multifactorial interactions from many different niche components⁶³. Extramedullary hematopoiesis during infection or malignancy is often associated with loss of BM CXCL12 signalling^{23,26,64-66}. Our observation that ITP progression coincides with a transient increase in BM CXCL12 expression and associated increase in LT-HSC BM homing may be an important mechanism to maintain LT-HSC functionality in conditions of elevated differentiation pressure, which is essential for maintenance of homeostasis long-term. As BM sinusoids are the preferred sites of progenitor homing due to low blood flow velocities and low wall shear rates⁶⁷, it is possible that the increase in average vessel area in ITP would further reduce blood flow velocity and aid progenitor homing. As discussed, it has been shown that progenitors activate and proliferate in response to acute antibody mediated thrombocytopenia, driven by the relocalisation of SCF from the cytoplasm to the cell membrane of megakaryocytes³³. Interestingly, this was not accompanied by an increase in total BM *Kitl* expression, suggesting that the proliferative effect on progenitors was post-transcriptionally regulated. In addition to its proliferative effects, SCF is essential for LT-HSC function^{47,48}. We have not assessed membrane bound SCF expression in our model of sustained ITP, which may present a further mechanism acting to preserve LT-HSC functionality during sustained ITP.

Our research therefore points to a concerted effort from various components of the BM microenvironment to create a nurturing environment maximising megakaryopoiesis whilst minimising LT-HSC exhaustion. BM examination is not routinely performed for chronic ITP patients¹⁰; it is therefore possible that significant changes in BM architecture can occur which requires further investigation. As we have only tested a single anti-CD41 antibody, it is important to investigate whether the effects on hematopoiesis and the BM microenvironment are comparable with other antiplatelet antibodies, such as anti-CD61 which are also common in ITP patients^{6,7}. Future studies should also address the effect of T cell mediated autoimmunity on hematopoiesis and the BM microenvironment, as our model of sustained ITP is T cell independent. This is of relevance as a minority of patients do not display autoantibodies, and in these patients ITP is thought to be driven through cell-mediated autoimmunity²¹.

Data Sharing Statement

For original data, please contact ian.hitchcock@york.ac.uk.

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Authorship

Contribution: O.J.H wrote the manuscript, all authors interpreted data and read and edited the final manuscript. O.J.H, I.S.H and D.G.K designed experiments. O.J.H performed all experiments with assistance from G.F.R, J.P.H, K.H, A.P.S, D.G.K and I.S.H.

Conflict-of-interest disclosure

The authors declare no competing financial interests.

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Figure Legends

Figure 1. Repeated intraperitoneal injection of mice is a model for sustained ITP. A) A single intraperitoneal injection of anti-CD41 antibody (delineated by an arrow) every 48 h induces severe thrombocytopenia associated with an increase in platelet mean volume (MPV). For routine monitoring of ITP induction, mice were bled 24 h after injection. N = 5-10, *P* values calculated by a Two-way ANOVA with Sidak's multiple comparison test. **B)** Circulating thrombopoietin (TPO) levels during ITP progression (control vs 2 week ITP *P* > 0.99, control vs 4 week ITP *P* > 0.99, 2 week ITP vs 4 week ITP *P* > 0.99). N = 4-5 (2 independent experiments). *P* values calculated by a Kruskal-Wallis test with Dunn's multiple comparisons test. **Ci)** BM megakaryocyte (Mk) numbers during ITP progression (control vs 2 week ITP *P* > 0.99, control vs 4 week ITP ***P* = 0.0069, 2 week ITP vs 4 week ITP *P* = 0.10). N = 5-8 (2 independent experiments). *P* values calculated by a Kruskal-Wallis test with Dunn's multiple comparisons test. **ii)** Representative hematoxylin and eosin stained diaphysis BM sections from WT femurs with Mks indicated by yellow stars. Images were obtained using the Zeiss AxioScan.Z1 slide scanner. **D)** Gene Ontology terms within the biological process domain and reactome pathways identified by STRING analysis of upregulated cytokines in the **i)** BM and **ii)** plasma of mice with 4 week ITP relative to control. Cytokine analysis was performed using the Proteome Profiler Mouse XL Cytokine Array Kit (R&D systems). All data shows mean values ± SD.

Figure 2. Sustained ITP drives progenitor expansion and LT-HSC proliferation. **A)** Total cell counts from flushed BM $P = 0.46$ (Mann-Whitney test). $N = 7$, (2 independent experiments). **B)** Flow cytometry analysis of hematopoietic progenitor populations: **i)** Lin⁻c-Kit⁺ (LK); $*P = 0.034$, **ii)** Lin⁻Sca⁻1⁺c-Kit⁺ (LSK); $**P = 0.0055$, **iii)** LSK CD48⁺CD150⁻ (Multipotent Progenitors, MPP); $P = 0.068$, **iv)** LSK CD48⁻CD150⁻ (Short Term HSC, ST-HSC); $*P = 0.019$, **v)** LSK CD48⁻CD150⁺ (Long Term HSC, LT-HSC); $***P = 0.0005$. $N = 13$ (3 independent experiments), P values were calculated by Mann-Whitney tests. **B)** Experimental outline for C-E. **C)** Cumulative time taken for a single LT-HSC to complete first (**i**), second (**ii**), and third (**iii**) division. $N = 4-5$ (2 independent experiments), P values calculated by a Two-way ANOVA with Sidak's multiple comparison test. **D)** Colony size after 10 days in culture ($*P = 0.020$). $N = 3-4$ (representative of 2 independent experiments). P values calculated by a Two-way ANOVA. Colonies were categorised as very small (< 50), small (51-500), medium (501-10,000) or large ($> 10,000$). **E)** After 10 days in culture, colonies were analysed by flow cytometry for the expression of Lineage and LSK markers. LSK numbers (**i**, $**P = 0.006$) and frequency (**ii**, $P = 0.92$). Frequency of Lin⁺ cells (**iii**, $P = 0.55$). P values were calculated by Mann-Whitney tests. $n = 143$ and $n = 208$ clones from controls and mice with sustained ITP, respectively. $N = 3$ (representative of 2 independent experiments). All data shows mean values $\pm$ SD with the exception of E, which shows mean values $\pm$ SEM.

Figure 3. Sustained ITP increases the frequency of functional LT-HSCs *in vivo*. **A)** Experimental outline for B-D. **Bi, Ci and Di)** peripheral blood from primary recipients 4-20 weeks after transplantation. **Bii, Cii and Dii)** analysis of BM from primary recipients 20 weeks after transplantation. **Biii, Ciii and Diii)** analysis of peripheral blood from secondary recipients 4-20 weeks after transplantation. **Biv, Civ and Div)** analysis of BM from secondary recipients 20 weeks after transplantation. The dotted line shows the expected ratio if ITP had no effect on chimerism; for primary recipients this was calculated based upon the transplanted ratio of donor cells, for secondary recipients this was calculated based upon the end point of the primary recipients. $N = 5$ per recipient group. **E)** Experimental outline for F. **F)** After 11 weeks, 2/9 recipients receiving 3 control LT-HSCs and 2/5 recipients receiving 10 LT-HSCs experienced chimerism, whilst 0 recipients receiving ITP LT-HSCs experienced chimerism, $P = 0.013$. P value was calculated by a Chi-squared test. A successful transplantation was defined as $> 1\%$ CD45.2 chimerism. After 11 weeks, 1 mouse transplanted with

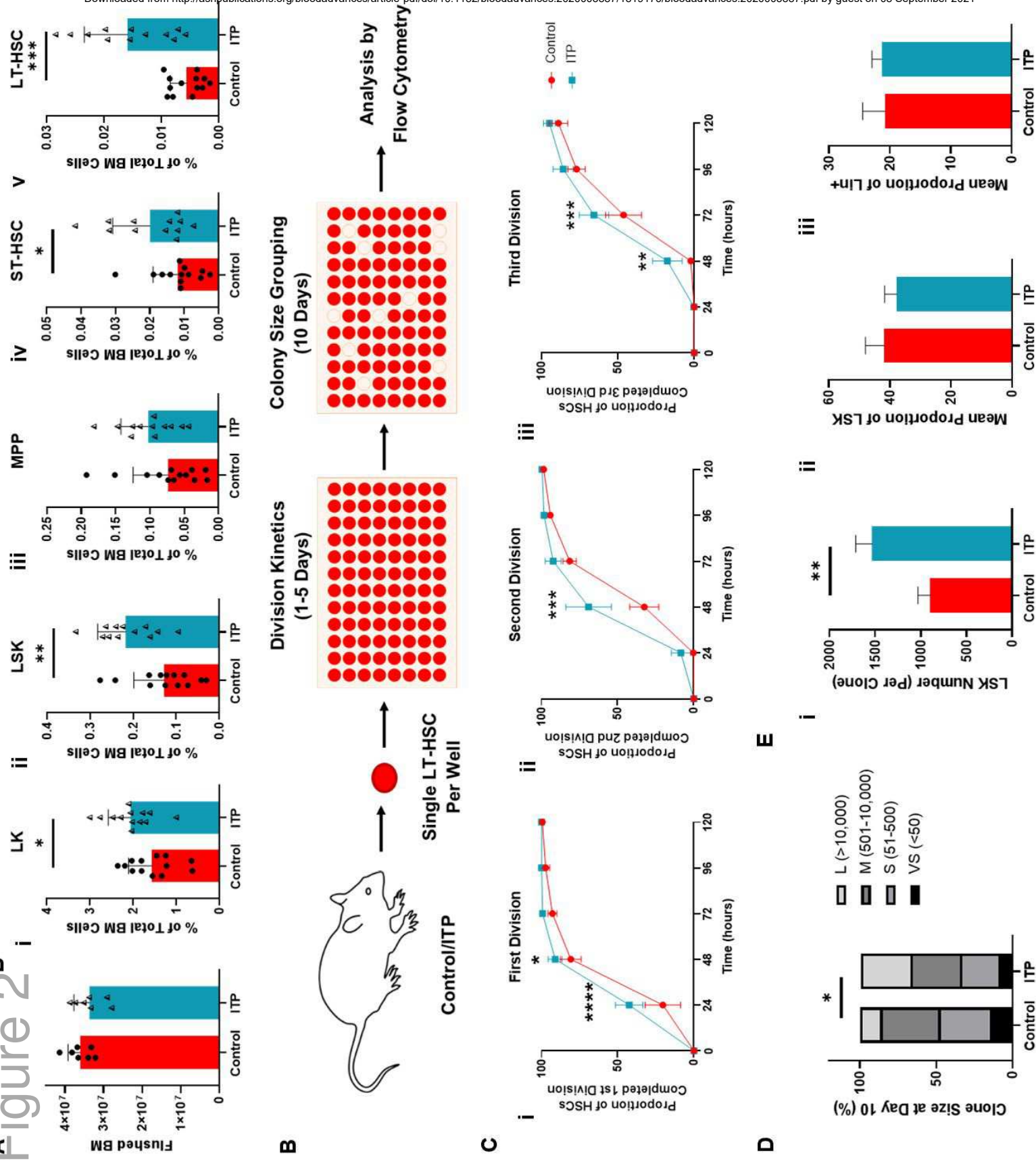
10 control LT-HSCs showed > 1% CD45.2 chimerism, whilst 1 mouse showed trace amounts of chimerism (< 1%) as marked by an asterisk (*). All data shows mean values $\pm$ SD.

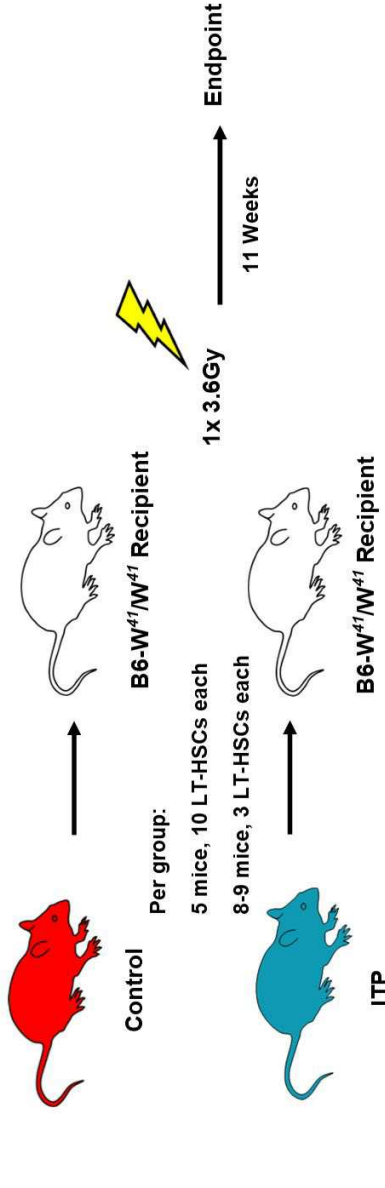
Figure 4. Sustained ITP remodels the BM microenvironment. ITP progression was associated with blood vessel structural changes and reversible changes in stromal cell number. Mice were treated with IgG (control) or anti-CD41 (ITP) for 4 weeks. A further group of ITP mice were allowed to recover for a further 4 weeks after the last injection of anti-CD41, where platelet count returned to normal by day 18 ('ITP Recovered' group). All data shown from controls and mice with ITP are representative of 3 independent experiments, whilst the data from ITP Recovered mice is representative of 1 independent experiment. **A)** Representative confocal images of control, ITP and ITP Recovered diaphysis BM from WT femurs. **B)** Relative numbers of LepR⁺ stromal cells were inferred by comparing total LepR staining between groups. Staining was quantified by exporting the LepR channel images as TIFFs and quantifying total staining relative to controls using ImageJ software. Control vs ITP * P = 0.018, control vs ITP Recovered P = 0.91, ITP vs ITP Recovered ** P = 0.0021. P values were calculated by a Kruskal-Wallis test with Dunn's multiple comparison test. N = 4-9. Values show mean values $\pm$ SD, with an average of 10 images analysed per mouse. **C)** Vessel analysis showing: **i)** Sum vessel area (control vs ITP **** P < 0.0001, control vs ITP Recovered **** P < 0.0001, ITP vs ITP Recovered *** P = 0.0001), **ii)** Average vessel area (control vs ITP **** P < 0.0001, control vs ITP Recovered **** P < 0.0001, ITP vs ITP Recovered P = 0.066), **iii)** Vessel number (control vs ITP P > 0.99, control vs ITP Recovered *** P = 0.0008, ITP vs ITP Recovered *** P = 0.0007). Vessel information was quantified using StrataQuest analysis software (TissueGnostics). P values were calculated by a Kruskal-Wallis test with Dunn's multiple comparison test, n = 44-55. Violin plots show median values and upper and lower quartiles, with an average of 10 images analysed per mouse. All data shows mean values $\pm$ SD.

Figure 5. ITP progression increases BM CXCL12 expression and is associated with increased progenitor homing. **Ai)** BM *Cxcl12* expression (control vs 2 week ITP P > 0.99, control vs 4 week ITP * P = 0.021, control vs 4 week ITP Recovered P > 0.99, 2 week ITP vs 4 week ITP P = 0.14, 4 week ITP vs 4 week ITP Recovered P = 0.43). N = 4-6 (1-2 independent experiments), P values were calculated by a Kruskal-Wallis test with Dunn's multiple comparison test. **Aii)** Spleen *Cxcl12* expression (P = 0.82). N = 6 (2 independent experiments). P value was calculated by a Mann-Whitney test. **B)** Representative confocal images of diaphysis BM from femurs of *Cxcl12*^{Dsred/+} mice

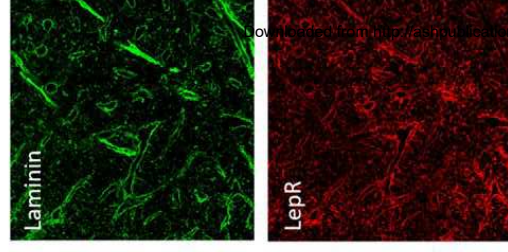
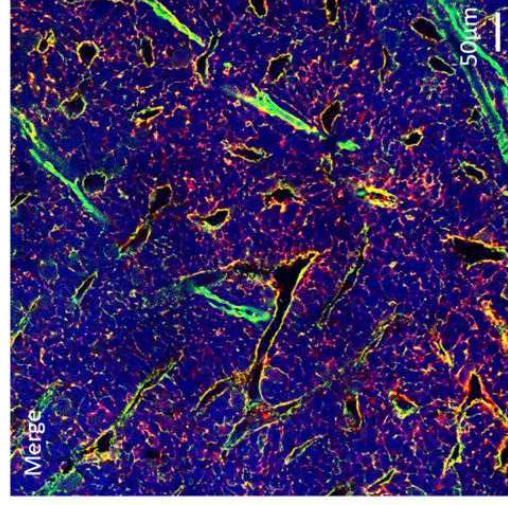
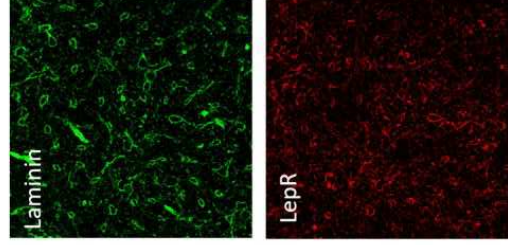
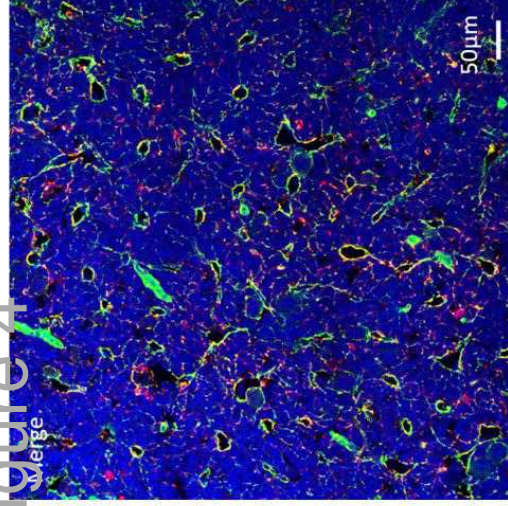
with sustained ITP vs controls. **C)** CXCL12 ELISA using: **i)** BM supernatant ($**P = 0.0023$, $N = 7$), **ii)** spleen homogenate ($P = 0.38$, $N = 7$), **iii)** and plasma ($P = 0.23$, $N = 3-4$). 2 independent experiments, P values were calculated by Mann-Whitney tests. **D)** Experimental outline for E. **E)** Homing of naïve progenitors to the BM of mice with 4 week ITP vs controls ($P = 0.004$, $N = 8-9$). 2 independent experiments, P value was calculated by a Mann-Whitney test. All data shows mean values $\pm$ SD.

Figure 2

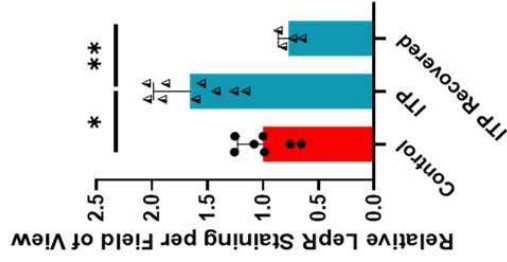




HSC Dose	Positive	
	Control	ITP
3	2/9	0/8
10	2*/5	0/5



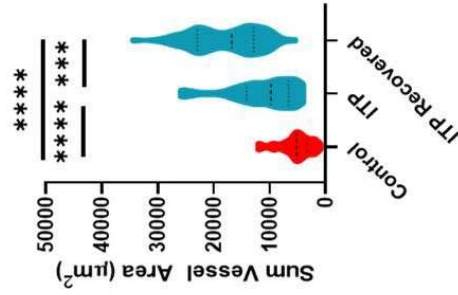
ITP Recovered



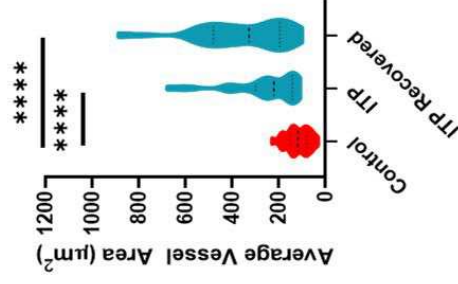
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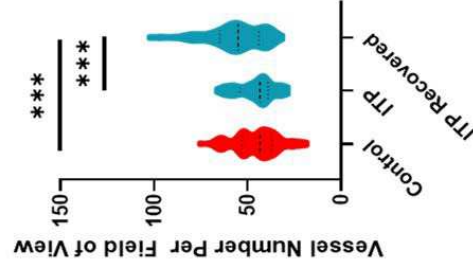
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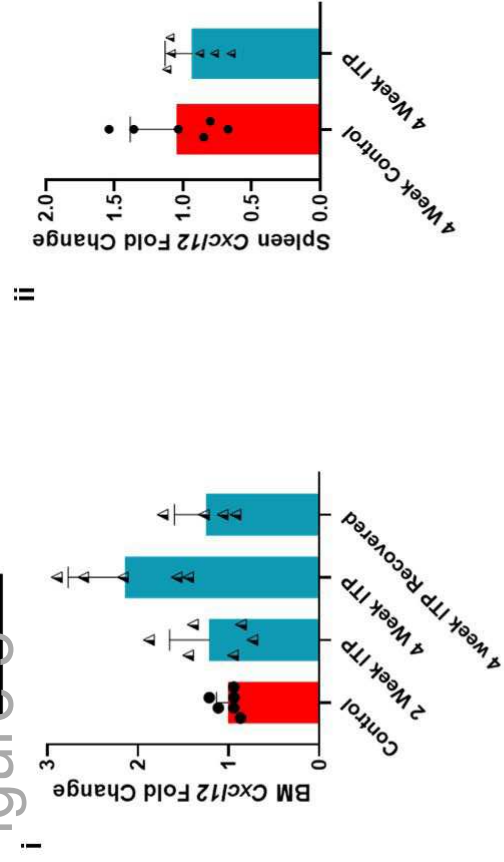


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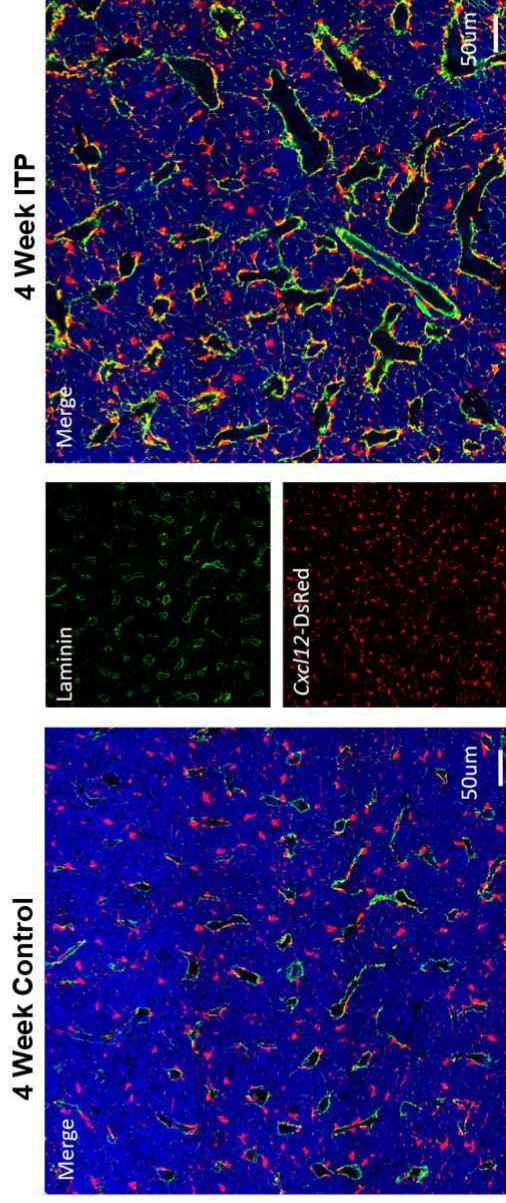


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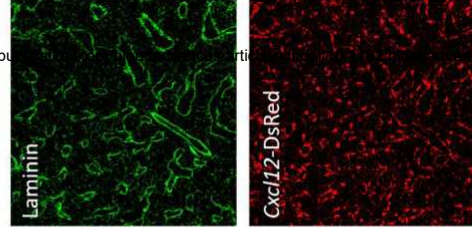




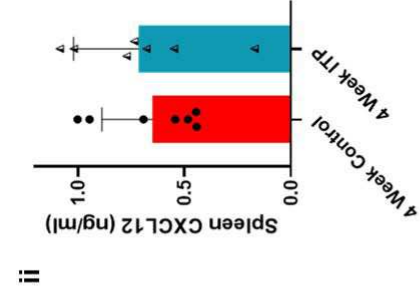
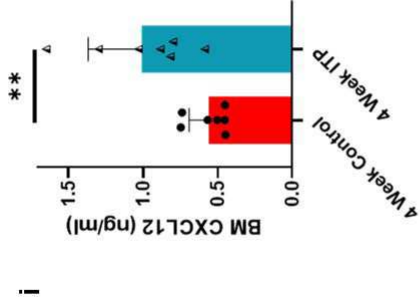
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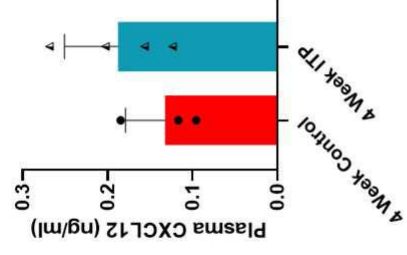
4 Week ITP



C



iii



D

