HHS Public Access

Author manuscript

Immunol Res. Author manuscript; available in PMC 2017 December 01.

Published in final edited form as:

Immunol Res. 2017 October ; 65(5): 1089–1094. doi:10.1007/s12026-017-8951-x.

Platelet Factor 4 Increases Bone Marrow B cell Development and Differentiation

David J. Field[1], **Angela A. Aggrey-Amable**[1], **Sara K. Blick**[2], **Sara K. Ture**[1], **Andrew Johanson**[1], **Scott J. Cameron**[1], **Sukanya Roy**[1], and **Craig N. Morrell**[1,*]

[1]Aab Cardiovascular Research Institute, University of Rochester Medical Center, Rochester, New York, USA

[2]Rochester Institute of Technology, Bridges to the Doctorate for Deaf and Hard of Hearing Students, Rochester, New York, USA

Abstract

Platelet factor 4 (PF4) is a megakaryocyte/platelet derived chemokine with diverse functions as a regulator of vascular and immune biology. PF4 has a central role in vessel injury responses, innate immune cell responses, and T-helper cell differentiation. We have now discovered that PF4 has a direct role in B cell differentiation in the bone marrow. Mice lacking PF4 ($PF4^{-/-}$ mice) had fewer developing B cells in the bone marrow beginning after the Pre Pro-B cell stage of differentiation. *In vitro*, PF4 increased the differentiation of hematopoietic progenitors to B cell lineage cells, indicating that PF4 has a direct effect on B cell differentiation. STAT5 activation is essential in early B cell development and PF4 increased the phosphorylation of STAT5. Taken together, these data demonstrate that PF4 has an important role in increasing B cell differentiation in the bone marrow environment.

Introduction

B cell differentiation and development are initiated in complex bone marrow microenvironments. Hematopoietic stem cells (HSC) differentiate into committed lymphoid progenitors (CLP) that further differentiate into either T cell or B cell lineages. B cell differentiation occurs under the influence of largely bone marrow stromal cell derived cytokines and chemokines including CXCL12 (SDF-1), FMS-like tyrosine kinase 3 ligand (FLT3L), interleukin 7 (IL-7), and stem cell factor (SCF) that each exert their greatest effects at specific stages of B cell development (1, 2). For example, CXCL12 and FLT3L have their greatest effects on the CLP to Pre Pro-B cell stages of B cell development, whereas IL-7 and SCF exert their greatest influence once B cell commitment and Pro-B cell differentiation has occurred. These factors activate signaling pathways to drive gene expression programs that regulate B cell lineage differentiation. Once B cell maturation progresses to the immature B

*Corresponding Author: Craig N. Morrell, Box CVRI, 601 Elmwood Ave, Rochester NY, 14642. Phone: (585) 276-7693. Craig_Morrell@URMC.Rochester.edu.

Author Contributions: DJF, AAA, SKT, AJ, SKB, SR, and SJC performed experiments and analyzed data; CNM performed experiments, analyzed data, and wrote manuscript.

Competing Interests: We have no competing interests.

cell stage, immature B cells leave the bone marrow and enter the periphery to become antibody producing, antigen presenting, and cytokine secreting members of adaptive immune responses (2).

Platelet factor 4 (PF4 or CXCL4) is typically described as a megakaryocyte and platelet derived chemokine. PF4 is a highly abundant platelet alpha granule constituent and found in the plasma at ng/mL concentrations, but mice lacking PF4 do not have an overt bleeding defect (3). Consistent with its chemokine structure, the greatest influences of PF4 are on vascular inflammation, immune responses, and immune development. PF4 exacerbates vessel wall injury (4) and is essential for maintaining normal T helper cell differentiation (5). PF4 has also been implicated in maintaining hematopoietic stem cell (HSC) quiescence in a proposed megakaryocyte-HSC niche (6). Receptors for PF4 include CXCR3 on trafficking leukocytes, αv integrins on endothelial cells, and PF4 binds and signals through Low Density Lipoprotein Receptor-Related Protein 1 (LRP1) to megakaryocytes, endothelial cells, vascular smooth muscle cells, and multiple myeloma cells (7–10). There are indications that PF4 may interact with B cell lineage cells as PF4 has been noted to be genetically silenced in multiple myeloma cells (11) and PF4 inhibited growth and induced apoptosis in myeloma cells by negatively regulating STAT3 (12).

We have now discovered that mice lacking PF4 ($PF4^{-/-}$ mice) have fewer maturing B cells furthering our understanding of the central role for PF4 in acquired immune response homeostasis.

Materials and Methods

Mice and cell isolations

All mice were on a C57Bl/6J background as described in our prior studies (4, 5). Complete blood counts were performed using an Abaxis Vetscan HM5. Bone marrow was isolated by flushing the mouse femurs with a PBS, 2% FBS and 1 mM EDTA solution. All bone marrow cell counts represent the number of cells from the 2 femurs of an individual mouse.

All antibodies for flow cytometry and the B cell depleting anti-CD20 clone AISB12 were purchased from eBioscience. Anti-IL-17 antibody and control IgG were purchased from BioXcell. For LPS treatment mice were given 0.1 μg/g LPS via intraperitoneal injection and the bone marrow harvested the next day.

Immunoblot

Mouse pre-B cell HB-34 cells were purchased from ATCC and grown with RPMI media and 20% FBS. Immunoblots for p-STAT5 (Santa Cruz Biotech, Dallas, Tx) and total STAT5 (Cell Signaling Technology, Danvers, MA) were performed using standard conditions with a primary antibody titer of 1:1000 in 3% BSA/TBS-T with gentle agitation. Secondary antibody (GE Healthcare, Buckinghamshire, UK) was used in a 1:2000 titer in 5% milk/TBS-T for 1 hour at room temperature. Final autoradiographic films (Bioblot BXR, Laboratory Product Sales, Rochester NY) were quantified by densitometry using ImageJ software (NIH).

Statistical Analysis

For experiments involving repeated measurements, Friedman's chi-square test, a non-parametric test, was computed. Additionally, pairwise comparisons between the baseline and various concentration or time point values were made using the paired t-test. When independent samples were compared, the standard Student's t-test was used as noted. A *p*-value of less than 0.05 was considered statistically significant.

Results

Our prior studies demonstrated that PF4 limited Th17 type of T-helper cell differentiation (5). To determine whether a lack of PF4 alters the development of immune cells in the bone marrow, we isolated the femurs of WT and $PF4^{-/-}$ mice and the total number of marrow leukocytes in both femurs of each mouse was determined. $PF4^{-/-}$ mice had a trend towards more total marrow cells compared to WT mice (Fig 1A). The number of specific cell types was also determined. $PF4^{-/-}$ mice had more bone marrow neutrophils, but similar numbers of monocytes as WT mice (Fig 1B). In contrast, both the percent and total number of bone marrow B cells were significantly reduced in $PF4^{-/-}$ mice (Fig 1C). Circulating $PF4^{-/-}$ B cells were also significantly reduced, but not to the same extent as marrow B cells (Fig 1D). These data indicate that PF4 is needed for normal B cell differentiation and development in the bone marrow environment.

To define what step in B cell differentiation PF4 exerts its positive effects we first determined the percent and number of $cKit^{+}$ (hematopoietic progenitor) marrow cells and found no significant difference in the per cent between WT and $PF4^{-/-}$ mice, but there was a strong trend to increased total $cKit^{+}$ cells in $PF4^{-/-}$ mice (Fig 2A). CLP cells are the stage of development just before B cell commitment that is followed by the Pre Pro-B cell stage. The number of CLPs and Pre Pro-B cells were very similar between WT and $PF4^{-/-}$ mice (Fig 2B–C). However, the number of Pro (Fig 2D), Pre (Fig 2E), and Immature (Fig 2F) B cells in the bone marrow were all significantly reduced in $PF4^{-/-}$ compared to WT mice.

$PF4^{-/-}$ mice have increased numbers of neutrophils likely due to their elevated IL-17. Increased neutrophil counts can reduce bone marrow B cell production (13). To determine whether reduced marrow B cells in $PF4^{-/-}$ mice is secondary to increased IL-17 and increased neutrophils, we treated $PF4^{-/-}$ mice with either control IgG or anti-IL-17 antibody every 4 days over 3 weeks. Anti-IL-17 antibody reduced the neutrophil count in $PF4^{-/-}$ mice to WT levels demonstrating a direct IL-17 effect on neutrophils (Fig 3A). However, $PF4^{-/-}$ mice with normal neutrophil numbers still had significantly fewer bone marrow B cells compared to WT mice (Fig 3A), indicating that increased neutrophils does not account for the suppression of B cell production in $PF4^{-/-}$ mice.

To determine whether PF4 has a role in B cell differentiation not only in basal conditions, but also in response to increased B cell production demands, we treated WT and $PF4^{-/-}$ mice with a low dose anti-CD20 antibody (50 μg/mouse) to reduce the number of B cells. $CD19^{+}$ cells in the circulation as a percent of $CD45^{+}$ cells were then serially determined and normalized for fewer beginning B cells in $PF4^{-/-}$ mice (expressed as change from baseline). Twenty-four hrs after anti-CD20 antibody treatment WT and $PF4^{-/-}$ mice had a similar

reduction in the number of circulating B cells (Fig 3B). On days 3–4 post depletion WT mice had a return of circulating B cell numbers that greatly exceeded that of $PF4^{-/-}$ mice (Fig 3B). To further demonstrate a role for PF4 in response to increased B cell production demands, WT and $PF4^{-/-}$ mice were treated with a sub-lethal dose of LPS (0.1 μg/g) or control buffer and 20 hrs later bone marrow was harvested to evaluate the number of B cells as compared to mice of same genotype not LPS treated. WT mice had an about 50% increase in post-LPS total bone marrow B cells, pro-B cells, and immature B cells compared to buffer treated WT mice (Fig 3C). However, $PF4^{-/-}$ responses varied from no change to a decrease in the number of marrow B cells as compared to control $PF4^{-/-}$ mice (Fig 3C). These data indicate that in addition to maintaining basal B cell production PF4 increases B cell differentiation in response to an acute increase in production demands.

To demonstrate a direct effect of PF4 on B cell differentiation we isolated hematopoietic progenitor cells from WT mice and cultured the progenitors on OP9 stromal cells with control media or B cell differentiation induced by the addition of IL-7 and Flt3L (2 ng/mL each). Recombinant PF4 (100 ng/mL) was added either at the same time as IL-7/Flt3L (D0) or 24 hrs later (D1). Four days after the induction of B cell differentiation the number of $CD19^{+}$ cells were determined. The addition of PF4 both at D0 and D1 increased the number of $CD19^{+}$ cells (Fig 4A), although the PF4 effect was greatest when added on D0. These data demonstrate that PF4 has a direct effect on increasing the differentiation of hematopoietic progenitors to B cells.

B cells leave the marrow compartment at the immature stage of development and mature into antibody producing plasma cells in the periphery. In contrast to developing bone marrow B cells, WT and $PF4^{-/-}$ mice had similar plasma cell numbers both in the bone marrow and the spleen, and similar plasma IgG concentrations (Fig 4B). STAT5 is necessary for B cell development in the bone marrow, but STAT5 is not needed once B cells leave the marrow compartment (14, 15). Because $PF4^{-/-}$ mice had fewer developing B cells, but similar numbers of plasma cells, we determined whether PF4 increased the phosphorylation of STAT5 (P-STAT5). HB-34 cells, a pre-B cell line, were incubated with increasing concentrations of PF4 overnight and total STAT5 and P-STAT5 determined by immunoblot. PF4 increased P-STAT5 in a dose dependent manner (Fig 4C–D). To determine whether PF4 has a similar effect *in vivo*, P-STAT5 was measured in bone marrow derived B cells by intracellular flow cytometry. Compared to $PF4^{-/-}$ mice, WT mice had increased total $CD19^{+}$ and Pro-B cell P-STAT5 (Fig 4E). These data demonstrate that PF4 may drive B cell differentiation by increasing STAT5 phosphorylation.

Discussion

These data demonstrate that PF4 has an important role in B cell differentiation in the bone marrow environment and helps to further the concept that megakaryocytes and platelets have a central role in the regulation of immune responses and immune development both in the periphery, and in the bone marrow microenvironment. Others have demonstrated that megakaryocytes contribute to maintaining a quiescent HSC niche, in part through PF4 and/or TGF-β mediated mechanisms (6, 16). We have now found that in the bone marrow

environment PF4 also supports B cell differentiation, perhaps indicating a more general role for megakaryocytes in hematopoietic development.

Megakaryocytes constitutively release PF4 in the bone marrow (6). Inflammatory stimuli, such as non-lethal doses of LPS, induces B cell production in the bone marrow and non-lethal doses of LPS also induce megakaryocytopoiesis and platelet production (17). This perhaps indicates a link between megakaryocytes and B cell development in the bone marrow environment, particularly in inflammatory states. We have implicated megakaryocytes as a PF4 source to support B cell differentiation, but our results do not rule out other non-megakaryocyte sources. PF4 basally is largely megakaryocyte/platelet restricted, but we have found that activated T cells are a source of PF4 (5). We have isolated mesenchymal stromal cells from the bone marrow and by qRT-PCR they do not express *Pf4* (data not shown), but this does not rule out other unknown PF4 sources.

We demonstrate that PF4 has a major role in driving B cell differentiation, in part by increasing P-STAT5. This has a direct impact on our understanding of B cell differentiation and immune responses with the potential to impact the development of means to modulate B cell responses. More importantly, it also indicates that in addition to being the source of circulating platelets, megakaryocytes impact the development of potentially many cell types in the bone marrow.

Acknowledgments

Funding: This study was supported by NIH training grants 5T32HL066988 to SJC, 5T32HL007937 to AAAA and GM107739 to SKB. CNM was supported by American Heart Association 13EIA14250023 and National Institutes of Health R01HL124018 grants.

References

1. Herzog S, Reth M, Jumaa H. Regulation of B-cell proliferation and differentiation by pre-B-cell receptor signalling. Nature reviews Immunology. 2009; 9:195–205.
2. Nagasawa T. Microenvironmental niches in the bone marrow required for B-cell development. Nature reviews Immunology. 2006; 6:107–116.
3. Eslin DE, Zhang C, Samuels KJ, Rauova L, Zhai L, Niewiarowski S, Cines DB, Poncz M, Kowalska MA. Transgenic mice studies demonstrate a role for platelet factor 4 in thrombosis: dissociation between anticoagulant and antithrombotic effect of heparin. Blood. 2004; 104:3173–3180. [PubMed: 14764524]
4. Shi G, Field DJ, Long X, Mickelsen D, Ko KA, Ture S, Korshunov VA, Miano JM, Morrell CN. Platelet factor 4 mediates vascular smooth muscle cell injury responses. Blood. 2013; 121:4417–4427. [PubMed: 23568488]
5. Shi G, Field DJ, Ko KA, Ture S, Srivastava K, Levy S, Kowalska MA, Poncz M, Fowell DJ, Morrell CN. Platelet factor 4 limits Th17 differentiation and cardiac allograft rejection. J Clin Invest. 2014; 124:543–552. [PubMed: 24463452]
6. Bruns I, Lucas D, Pinho S, Ahmed J, Lambert MP, Kunisaki Y, Scheiermann C, Schiff L, Poncz M, Bergman A, Frenette PS. Megakaryocytes regulate hematopoietic stem cell quiescence through CXCL4 secretion. Nat Med. 2014; 20:1315–1320. [PubMed: 25326802]
7. Liang P, Cheng SH, Cheng CK, Lau KM, Lin SY, Chow E, Chan N, Ip R, Wong R, Ng M. Platelet factor 4 induces cell apoptosis by inhibition of STAT3 via upregulation of SOCS3 expression in multiple myeloma. Haematologica. 2012

8. Lambert MP, Xiao L, Nguyen Y, Kowalska MA, Poncz M. The role of platelet factor 4 in radiation-induced thrombocytopenia. Int J Radiat Oncol Biol Phys. 2011; 80:1533–1540. [PubMed: 21740995]
9. Aidoudi S, Bikfalvi A. Interaction of PF4 (CXCL4) with the vasculature: a role in atherosclerosis and angiogenesis. Thromb Haemost. 2010; 104:941–948. [PubMed: 20806113]
10. Lambert MP, Wang Y, Bdeir KH, Nguyen Y, Kowalska MA, Poncz M. Platelet factor 4 regulates megakaryopoiesis through low-density lipoprotein receptor-related protein 1 (LRP1) on megakaryocytes. Blood. 2009; 114:2290–2298. [PubMed: 19605848]
11. Cheng SH, Ng MH, Lau KM, Liu HS, Chan JC, Hui AB, Lo KW, Jiang H, Hou J, Chu RW, Wong WS, Chan NP, Ng HK. 4q loss is potentially an important genetic event in MM tumorigenesis: identification of a tumor suppressor gene regulated by promoter methylation at 4q13.3, platelet factor 4. Blood. 2007; 109:2089–2099. [PubMed: 17077331]
12. Liang P, Cheng SH, Cheng CK, Lau KM, Lin SY, Chow EY, Chan NP, Ip RK, Wong RS, Ng MH. Platelet factor 4 induces cell apoptosis by inhibition of STAT3 via up-regulation of SOCS3 expression in multiple myeloma. Haematologica. 2013; 98:288–295. [PubMed: 22929979]
13. Forlow SB, Schurr JR, Kolls JK, Bagby GJ, Schwarzenberger PO, Ley K. Increased granulopoiesis through interleukin-17 and granulocyte colony-stimulating factor in leukocyte adhesion molecule-deficient mice. Blood. 2001; 98:3309–3314. [PubMed: 11719368]
14. Dai X, Chen Y, Di L, Podd A, Li G, Bunting KD, Hennighausen L, Wen R, Wang D. Stat5 is essential for early B cell development but not for B cell maturation and function. J Immunol. 2007; 179:1068–1079. [PubMed: 17617599]
15. Malin S, McManus S, Cobaleda C, Novatchkova M, Delogu A, Bouillet P, Strasser A, Busslinger M. Role of STAT5 in controlling cell survival and immunoglobulin gene recombination during pro-B cell development. Nat Immunol. 2010; 11:171–179. [PubMed: 19946273]
16. Zhao M, Perry JM, Marshall H, Venkatraman A, Qian P, He XC, Ahamed J, Li L. Megakaryocytes maintain homeostatic quiescence and promote post-injury regeneration of hematopoietic stem cells. Nat Med. 2014; 20:1321–1326. [PubMed: 25326798]
17. Wu D, Xie J, Wang X, Zou B, Yu Y, Jing T, Zhang S, Zhang Q. Micro-concentration Lipopolysaccharide as a Novel Stimulator of Megakaryocytopoiesis that Synergizes with IL-6 for Platelet Production. Sci Rep. 2015; 5:13748. [PubMed: 26330186]

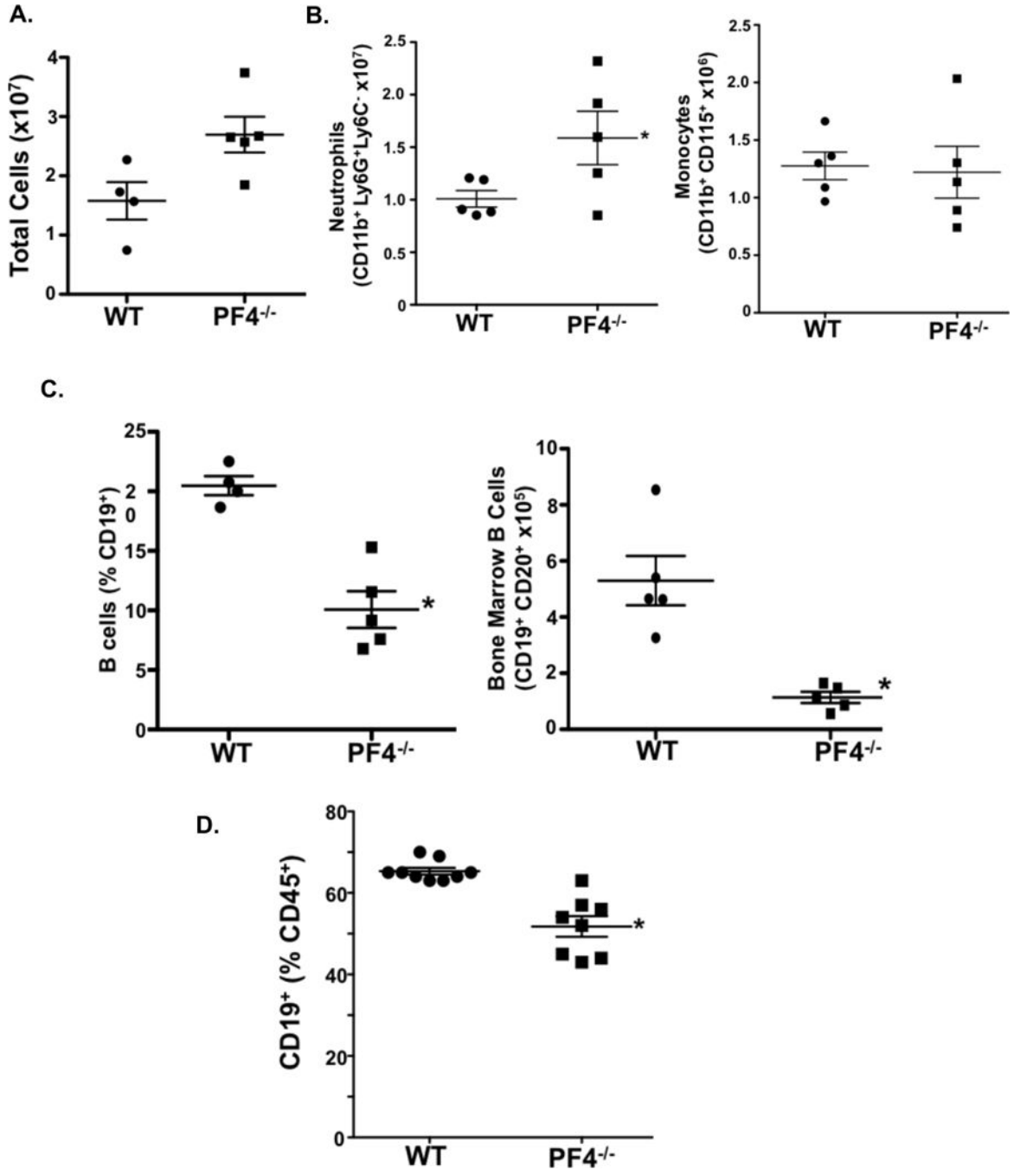


Figure 1.

PF4$^{-/-}$ mice have fewer bone marrow B cells. A) WT and PF4$^{-/-}$ mice have similar numbers of total bone marrow cells. Each data point is representative of the combined number of cells from both femurs of an individual mouse. B) PF4$^{-/-}$ mice have more bone marrow neutrophils and similar numbers of monocytes as WT mice (*P<0.05 vs WT). C) PF4$^{-/-}$ mice have fewer cells of B cell lineage cells in bone marrow compared to WT mice (*P<0.01 vs WT). D) PF4$^{-/-}$ mice have fewer B cells the peripheral circulation compared to WT mice (*P<0.01 vs WT).

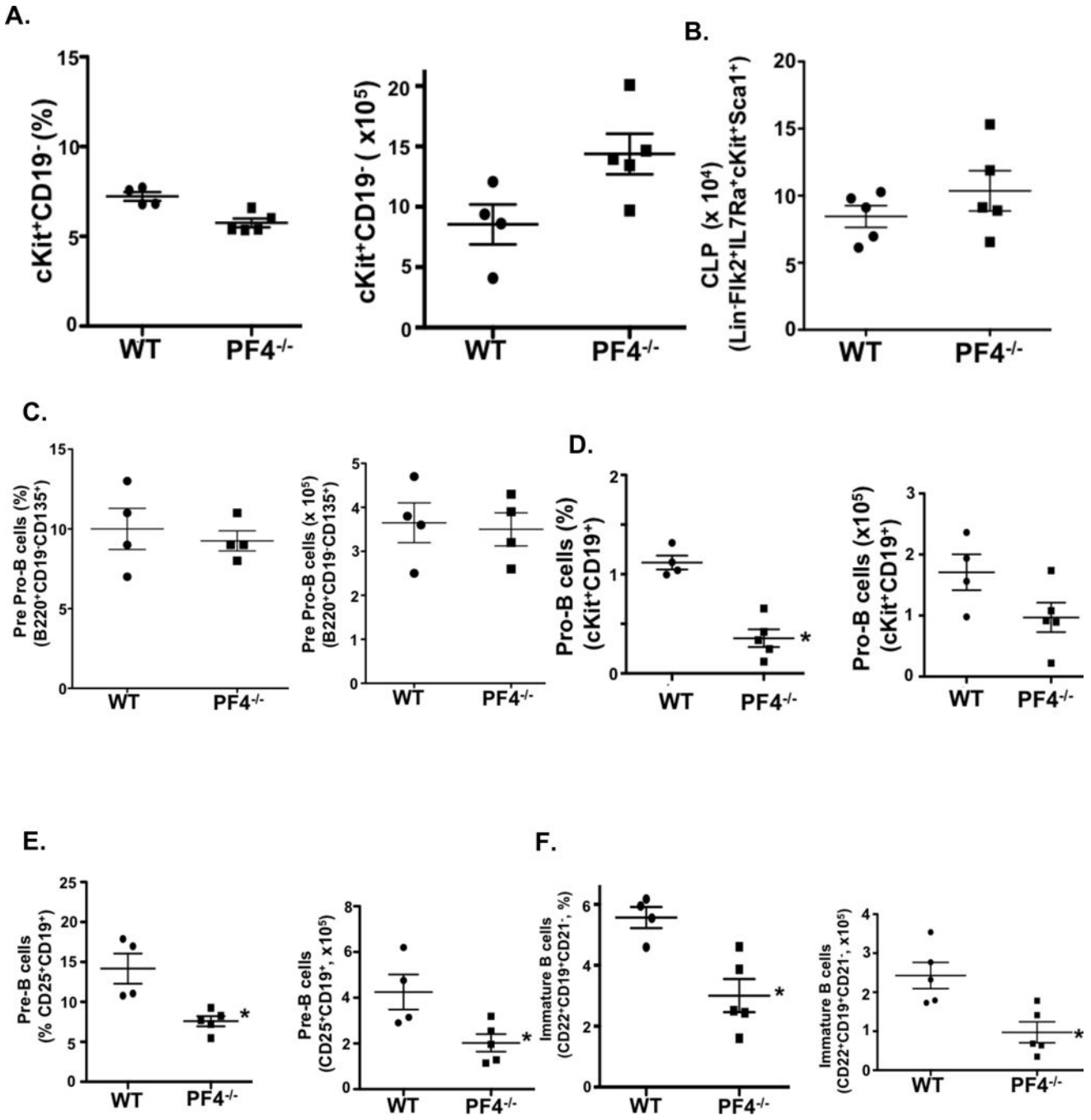
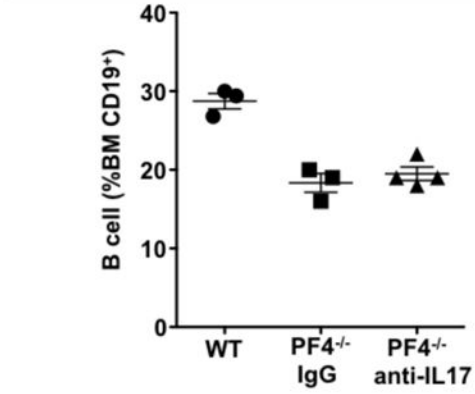


Figure 2.

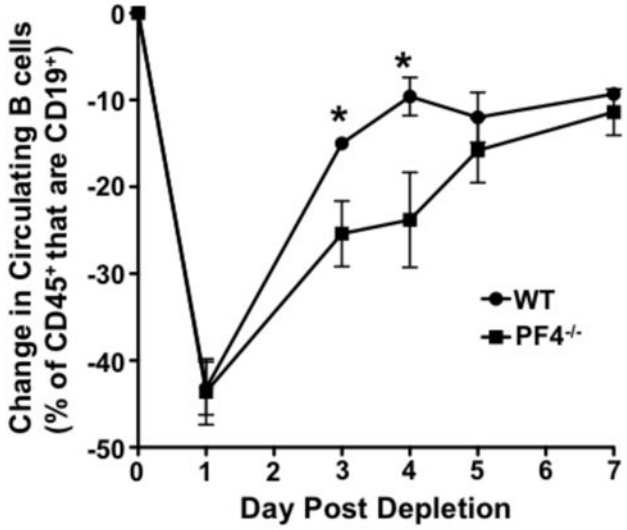
PF4$^{-/-}$ mice have a decline in B cell lineage cells at the Pro-B cell stage of development. A) PF4$^{-/-}$ and WT mice have similar hematopoietic progenitor cells. B) The number of bone marrow CLP cells are similar in WT and PF4$^{-/-}$ mice. C) The number of bone marrow Pre Pro-B cells are similar in WT and PF4$^{-/-}$ mice. D-F) PF4$^{-/-}$ mice have fewer Pro-B cells, Pre-B cells, and Immature B cells compared to WT mice (*P<0.01 vs WT).

A.

	WBC	Lymph	Mono	PMN	RBC	Platelet
WT	9.2 ± 0.5	8.43 ± 0.5	0.17 ± 0.05	0.56 ± 0.2	10.39 ± 0.3	606 ± 55
PF4$^{-/-}$ + IgG	12.3 ± 1.4	9.97 ± 0.7	0.49 ± 0.1	1.74 ± 0.6*	9.73 ± 0.1	791 ± 35
PF4$^{-/-}$ + Anti-IL17	10.2 ± 1.6	8.47 ± 0.4	0.24 ± 0.1	0.65 ± 0.2	9.25 ± 0.6	718 ± 97



B.



C.

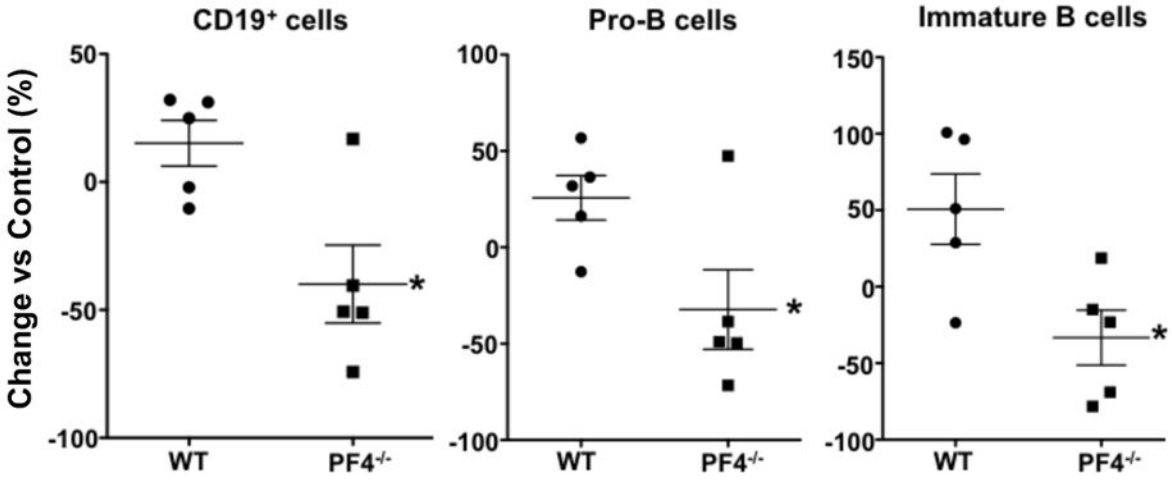


Figure 3.

PF4 mediates B cell production with increased bone marrow demands. A) Reduced bone marrow B cells in PF4$^{-/-}$ mice is not neutrophil dependent. Mice were treated with IL-17 blocking antibody or control IgG (300 μg first dose then 100 μg every 4 days for 3 weeks). CBC were performed and bone marrow CD19^{+} cells were quantified. IL-17 blocking returned PF4$^{-/-}$ neutrophil numbers. B) PF4$^{-/-}$ mice have delayed B cell recovery post-depletion. WT and PF4$^{-/-}$ mice were treated with anti-CD20 antibody (50 μg) and circulating B cell numbers determined by flow cytometry and reported as a change from

baseline (N=5, *P<0.05 vs WT). C) $PF4^{-/-}$ mice have reduced stimulus induced B cell differentiation responses. WT and $PF4^{-/-}$ mice were given a sub-lethal dose of LPS (0.1 μg/g) and 20 hrs later bone marrow B cells were quantified and expressed as % change from the average of 3 control mice of each genotype (*P<0.03 vs WT).

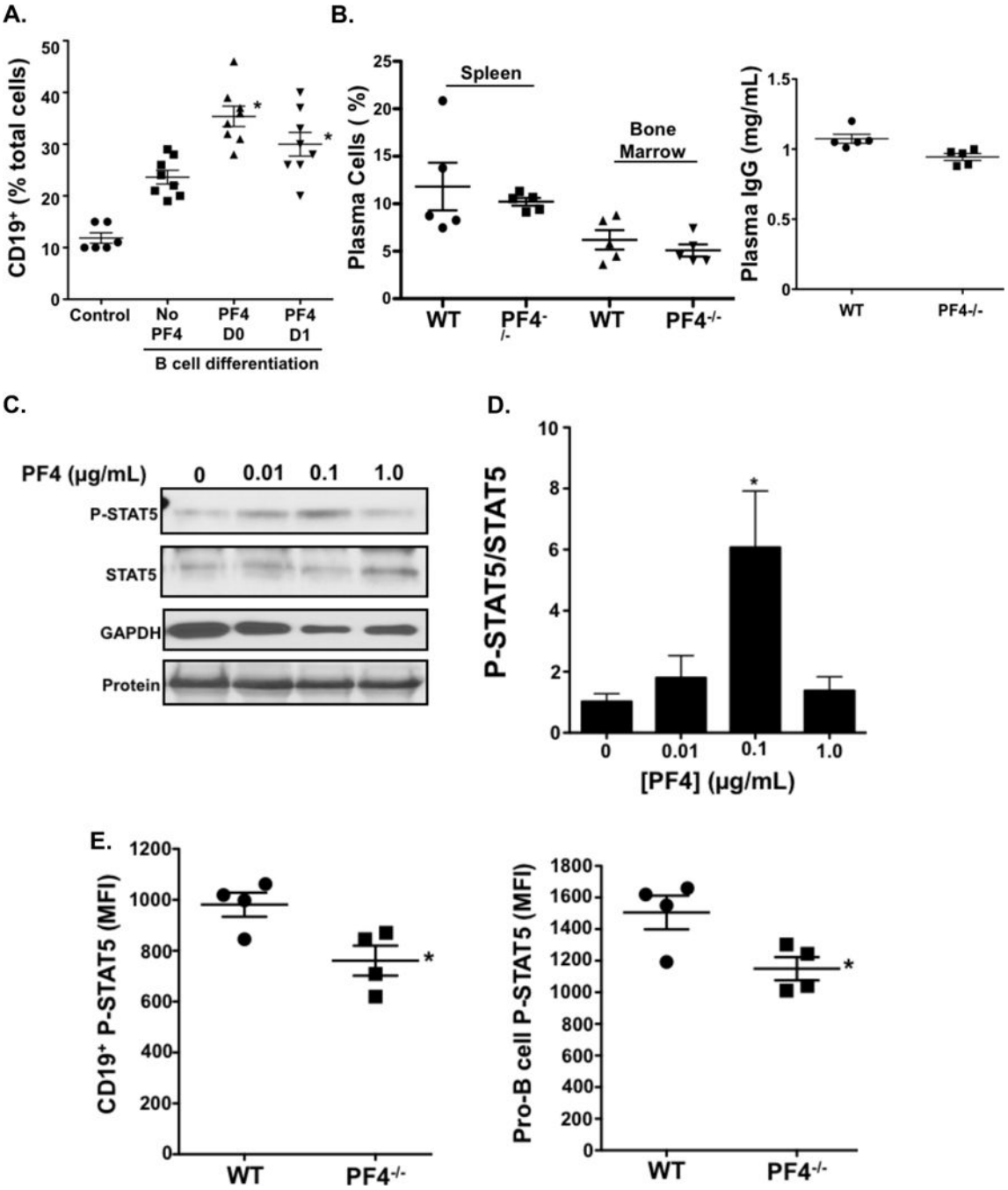


Figure 4.

PF4 increases B cell progenitor STAT5 activation. A) PF4 increases B cell differentiation *in vitro*. WT mouse hematopoietic progenitor cells were isolated and differentiated to B cells *in vitro* (cultured with OP9 cells and 2 ng/mL IL-7, 2 ng/mL Flt3L, ± 100 ng/mL PF4 added at day 0 or day 1). PF4 increased the number of $CD19^+$ cells (*$P<0.05$ vs No PF4). B) $PF4^{-/-}$ mice have normal plasma cell numbers and plasma IgG. C) PF4 induced P-STAT5. Cells were treated with PF4 overnight and P-STAT5 determined by immunoblot. D) P-STAT5/

STAT5 quantification (N=4, *P<0.05 vs 0). E) P-STAT5 is reduced in $PF4^{-/-}$ bone marrow B cells. Intra-cellular flow cytometry (*P<0.04 vs WT).