Deletion of Vhl in Dmp1-Expressing Cells Causes Microenvironmental Impairment of B Cell Lymphopoiesis

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The contributions of skeletal cells to the processes of B cell development in the bone marrow (BM) have not been completely described. The von-Hippel Lindau protein (VHL) plays a key role in cellular responses to hypoxia. Previous work showed that Dmp1-Cre; Vhl conditional knockout mice (Vhl cKO), which deletes Vhl in subsets of mesenchymal stem cells, late osteoblasts and osteocytes, display dysregulated bone growth and reduction in B cells. Here, we investigated the mechanisms underlying the B cell defects using flow cytometry and high-resolution imaging. In the Vhl cKO BM, B cell progenitors were increased in frequency and number, whereas Hardy Fractions B-F were decreased. Vhl cKO Fractions B-C cells showed increased apoptosis and quiescence. Reciprocal BM chimeras confirmed a B cell-extrinsic source of the Vhl cKO B cell defects. In support of this, Vhl cKO BM supernatant contained reduced CXCL12 and elevated EPO levels. Intravital and ex vivo imaging revealed Vhl cKO BM blood vessels with increased diameter, volume, and a diminished blood-BM barrier. Staining of Vhl cKO B cells with an intracellular hypoxic marker indicated the natural existence of distinct B cell microenvironments that differ in local oxygen tensions and that the B cell developmental defects in Vhl cKO BM are not initiated by hypoxia. Our studies identify novel mechanisms linking altered bone homeostasis with drastic BM microenvironmental changes that dysregulate B cell development.

Keywords: B lymphocytes, osteoimmunology, hypoxia, microenvironment, bone marrow niches

INTRODUCTION

The mechanisms by which changes in bone homeostasis affect immune development in the bone marrow (BM) are not fully understood (1–4). A detailed understanding of how bone microenvironments affect immune cell development and function could provide strategies towards novel therapeutic approaches to immune deficiencies. B cells produce antibodies (Abs), which are crucial for a robust adaptive immune response. B cells are generated from hematopoietic stem cells (HSCs) in the liver during fetal life, and in the BM in the adult (5). B cell development in the BM occurs in a series of defined stages that rely on growth factors that are produced by several
non-hematopoietic stromal cells, including mesenchymal stem cells (MSCs) and osteoblasts (OBs) (1).

The von-Hippel Lindau protein (VHL) regulates hypoxia-inducible factor (HIF) degradation, which is involved in cellular adaptation to low oxygen environments (6). When HIF1α accumulates in normoxic conditions, it travels to the nucleus to activate over 100 hypoxia-inducible target genes (7). VHL is expressed ubiquitously in many cell types, and global deletion of the Vhl gene results in embryonic lethality, so conditional knockout approaches are necessary to investigate the cell-specific roles of VHL in specific microenvironments. Conditional deletion of Vhl in OBs and in hematopoietic progenitors have demonstrated a role for VHL in these cell types (8, 9). The role of HIF and its regulation on the immune system has been extensively reviewed (10), but the mechanisms by which cell-intrinsic and cell-extrinsic VHL regulate specific immune cell lineages has not fully been addressed.

The BM microenvironment manifests hypoxic heterogeneities in a spatio-temporal manner (11–13), however the implications of these oxygen tension (pO₂) differences on hematopoiesis are not well characterized. Hypoxia slows the processes of angiogenesis and osteogenesis during fracture healing and bone formation, but also promotes OB differentiation into OCYs (14), and can stimulate osteoclast formation (15). Studies have shown HIF stabilization as a therapeutic option for treating bone fractures (16, 17) and osteoporosis (18–20), but the underlying molecular mechanism remains poorly understood. Vhl plays an important role regulating HIF expression, and disruption of Vhl in bone cells leads to improper bone homeostasis (7, 8, 21, 22). Vhl depletion in osteochondral progenitor cells and osteocalcin-positive OBs leads to an increase in bone mass through an increase in OB number (7, 22). Furthermore, disrupting VHL in OBs induces expression of β-catenin, revealing the mechanism by which VHL/HIF pathway promotes bone formation through the Wnt pathway (7, 23, 24). Altogether, these studies of Vhl deletion in osteolineage cells have not examined the cell-extrinsic effects of these changes on the immune cells residing in the BM.

The BM contains specialized microenvironments that maintain blood cells and supply factors required for their development and maintenance. Perivascular stromal cells, osteoprogenitor cells, endothelial cells (ECs), MSCs, OBs and OCYs are critical B cell “niches” and are all cells that support B cell development (1, 4, 25, 26), in part through production of cytokines. Essential cytokines for B cell development include CXC-chemokine ligand 12 (CXCL12) (27–29), FLT3 ligand (FLT3L) (30), IL-7 (30–33), stem-cell factor (SCF) (31, 32) and receptor activator of nuclear factor-κB ligand (RANKL) (34). The BM contains a dense vascular network and vascular sinususes creating the perivascular region, which provides a niche where B cells are known to develop and reside (35). A model of B cell developmental niches based on CXCL12 and IL7 levels has been proposed (4) in which B cells start at the pre-pro-B cell (Fraction A) stage where they are located in the perisinusoidal niche, especially near CXCL12+ reticular cells. As B cells continue to mature to the pro-B cell stage (Fractions B–C), they also interact with IL-7 expressing cells, and then pre-B cells migrate away from the sinusoids toward galectin-1+ stromal cells that do not express IL7 (36). This model has been updated given recent reports of the four new MSC subsets, their ability to support B lymphopoiesis, and their locations within the BM (37). During aging, vascular density decreases in many tissues due to impaired angiogenesis caused by EC dysfunction (38, 39). Vascular “hyperpermeability” also increases with age, via changes in ECs lining the blood vessel wall, disrupting the blood-BM barrier (40–42). The role of the vasculature and regulation of vessel permeability in hematopoiesis, especially in B cell development, remains unknown.

To understand how changes in bone homeostasis may affect immune cell development, we previously utilized Dmp1-Cre;Vhl conditional knockout mice (VhlcKO), in which Vhl is deleted primarily in OCYs, but also in some MSC subsets and late OBs (43). In the VhlcKO bones, the number of hematopoietic cells is severely reduced, and B cell development is stunted (21). Here, we provide evidence for molecular, cellular and structural changes in the VhlcKO BM niche that adversely affect B cell development in a cell-extrinsic manner, such as decreased production of B cell supporting cytokines and structural changes in the BM vasculature. We also observed an age-dependent change in hypoxia that could further contribute to the B cell defects. These studies reveal novel molecular mechanisms by which Vhl deletion in Dmp1-expressing cells affect B cell niches.

MATERIALS AND METHODS

Study Design

A G*Power statistical (44) power analysis (α=0.05 and power of 0.95) based on B cell developmental data and BM cellularity determined that a minimum of n=7 mice per group was needed for our studies. The total sample size for each experiment was >7 performed in three independent experiments. Age-matched mice of both sexes were used. VhlcKO and control mice (C57BL/6 wild type and Vhl-floxed (Vhlfl/fl, Dmp1-Cre-negative mice) were used and no sex-specific differences in B cell development or other relevant characteristics to our studies were detected. Student’s t-test and nonparametric Bonferroni-corrected Mann-Whitney U-test was used to test differences between mean and median values with Graph-Pad Prism and were considered significant if p<0.05. Outlier analysis was also performed with Graph-Pad Prism and any outliers identified were not included in the data graphs.

Experimental Animals

Mice on the C57BL/6 background were used. Stock #023047 B6.N.FVB-Tg1Ifque/BwdJ (Dmp1-Cre) (45) and Stock #012933 B6.129S4(C)-Vhl tm1Jae/J (46) were purchased from The Jackson Laboratory. These two lines of mice were crossed to generate Vhl conditional knockouts in Dmp1-expressing cells (VhlcKO). Genotyping was confirmed following protocols from the Jackson Laboratory. Stock #002014 B6.SJL-Ptprca Pepcb/BoyJ mice were used for reciprocal bone marrow transplantation.
studies. Mice were housed under specific pathogen-free conditions in the University of California, Merced’s vivarium with autoclaved feed and water, and sterile microisolator cages. The University of California Merced Institutional Animal Care and Use Committee approved all animal work.

Bone Marrow Transplantation
Recipient mice were 10 weeks of age at the time of transplantation. Whole bone marrow B6.SJL (CD45.1+) donor cells (1x10⁶) were injected retro-orbitally into lethally irradiated (1000 rads using a Cesium-137 source, JL Shepherd and Associates, San Fernando, CA, USA) recipient CD45.2+ Vhl−/− mice or control (Cre-negative; Vhlfl/fl) littermates under isoflurane anesthesia. Reciprocal Vhl−/−→WT (B6.SJL, CD45.1+) chimeras were also prepared. Animals were supplemented with neomycin in the drinking water for 14 days post-transplant as described (47).

Sample Collection: Bone Marrow, Peripheral Blood, Spleen and Serum
Bone Marrow Collection
Mice were euthanized by the inhalation of carbon dioxide followed by cervical dislocation. Femurs and tibias were dissected, and muscles were removed. To release the BM, bones were crushed with a mortar and pestle in M199+ (M199 with 2% FBS). BM cells were collected into 15mL conical tubes after being rinsed away from bone chips with M199+, resuspended by trituration, filtered through 70-micron nylon mesh into a 50 mL conical tube, and centrifuged for 5 mins at 1500 rpm and at 4°C. Cell pellets were resuspended and treated with ACK lysis buffer to remove erythrocytes. Cells treated with ACK were washed and resuspended in M199+. Cell counts were obtained using a hemocytometer and Trypan Blue staining to exclude dead cells.

To collect BM supernatant, femurs were cleaned of any muscle tissue and the epiphyses were cut off and discarded. The bone shaft was then placed into a 0.2 mL tube in which a hole was introduced using a needle. Thirty µL of 1x phosphate buffered saline (PBS) was placed on the top end of the bone shaft, and the tube containing the bone was placed into a 0.2 mL microcentrifuge tube and centrifuged for 30 seconds at 15,000rpm. The BM supernatant was collected and stored at -80°C until analysis.

Peripheral Blood Collection
Mice were heated under a heat lamp to increase blood circulation and then restrained. Blood collection was performed via tail bleeds by making an incision with a scalpel blade over the ventral tail vein. No more than ten drops were collected (<0.5 mL) in a 1.5 ml Eppendorf tube with 50 µL of heparin. To obtain blood serum, blood was collected in 1.5 ml tubes without heparin and allowed to clot for 30 minutes at room temperature. The samples were then centrifuged for 10 minutes at 4000 rpm at 4°C. Blood serum was collected and stored at -80°C until the day of analysis.

Spleen Cell Collection
Dissected spleens were processed and mashed in 1 mL of ACK lysis buffer in a petri dish for no more than one minute. Five mL of M199+ were added into the dish to dilute the ACK lysis buffer and to stop red cell lysis. Spleen cells were aspirated into a 5mL syringe to create single cell suspensions by passing the cells through the syringe several times then filtering through a 70-micron nylon mesh into a 15 mL conical tube. Cells were centrifuged at 2000 rpm at 4°C for 3 minutes. Cell pellets were loosened by gently tapping the tubes by hand before resuspending the cells in 5 mL of M199+. Live cell counts were determined using a hemocytometer and Trypan Blue staining.

Quantification of Cytokines
Cytokine measurements were performed using a customized bead-based multiplex (13-LEGENDplex assay) from Biolegend, Inc. with the analytes IL-3, IL-5, IL-6, IL-7, IL-15, IL-34, M-CSF, TPO, GM-CSF, LIF, EPO, CXCL12, SCF for the analysis of BM serum and peripheral blood serum of Vhl−/− and control mice. Concentrations of cytokines were determined from samples following manufacturer’s instructions and software.

Flow Cytometry Analysis and Antibodies
Cells were stained for flow cytometry and included a pre-incubation step with unconjugated anti-CD16/32 (clone 32) to block Fc receptors as previously described (47, 48). The antibody cocktails used for different sets of stains are listed in Supplementary Table 1. For viability staining, DAPI (Sigma-Aldrich, 0.005 µg/ml final concentration) or propidium iodide (Sigma-Aldrich, 0.025 µg/ml final concentration) was used. Single color stains were used for setting compensations and gates were determined with fluorescent-minus one controls, isotype-matched antibody controls, or historical controls. Intracellular staining of Ki67 was performed using the eBioscience Foxp3/Transcription Factor Staining Buffer Set following the manufacturer’s instructions. For cell cycle analysis, DAPI was used at a final concentration of 0.1 µg/ml per sample. Apoptosis staining was performed using Biologend Annexin V Apoptosis Detection Kit with 7AAD. Flow cytometry data was acquired on the BD LSR II. The data was analyzed using FlowJo Software version 10.7.1.

Preparation of Long Bones for Imaging
To label blood vessels, mice were injected with fluorescent antibodies (Supplementary Table 1) through the retro-orbital venous sinus. After 20 minutes of incubation, intracardial perfusion was performed with 1X PBS following by cold and fresh 4% paraformaldehyde (PFA). Subsequently, femurs were harvested and fixed in the 4% PFA for 30 minutes, at 4°C. The bones were then washed with 1X PBS, immersed in 30% sucrose for 1 hour, frozen in optimal cutting temperature (OCT) compound and kept at ~ 80°C. Samples were shaved using a cryostat (LEICA CM1860) equipped with a high-profile blade (Leica; 3802121).

To optically clear long bones, a modified uDISCO clearing protocol was used (49). After intracardial perfusion as described above, long bones were immersed in 4% PFA overnight and put through a series of tert-butanol (Sigma-Aldrich, SHBM5332) dehydration steps at 30% (4 hours), 50% (4 hours), 70% (overnight), 80% (4 hours), 90% (4 hours), and 100%
in a chamber sealed with solvent-resistant silicone gel (DOWSIL™) as previously described (11, 50). The secured mouse was anesthetized with isoflurane (3-4% induction, 1.5% maintenance at 1L/min) and the top of the head shaved. The skin was cleaned with 70% alcohol wipes before surgery. The skull was incised along the sagittal and lambda suture of the skull and the skin retracted to expose the calvarial bones in B6 C57BL/6 (B6) background and we performed a thorough genetic background (21). For our studies, we required a pure genetic background (21). For our studies, we required a pure

Two-Photon Microscopy

Imaging was performed with a custom-built two-photon video-rate microscope (Bliq Photonics) equipped with two femtosecond lasers (Spectra Physics; Insight X3, Spectra Physics; MaiTai eHP DS). During intravitral imaging, the Spectra Physics Insight X3 and MaiTai laser wavelengths were tuned to 840 nm and 1040 nm, respectively, and for ex vivo imaging only the Insight X3 was tuned to 1220 nm. Three fluorescent channels were acquired (503-538 nm, 572-608 nm, and 659-700 nm). For all two-photon imaging, a 25x water immersion objective (Olympus; XLPLN25XWMP2) with 1.05 numerical aperture was used to image a 317 µm by 159 µm field of view. Videos were recorded at 30 frames per second and images were generated by averaging of 30 frames from the live video mode.

For in vivo imaging of calvarial bone marrow, mice were anesthetized with isoflurane (3-4% induction, 1.5% maintenance at 1L/min) and the top of the head shaved. The skin was cleaned with 70% alcohol wipes before surgery. The mouse was placed on a heating pad and secured in a custom head mount. An incision was made along the sagittal and lambda suture of the skull and the skin retracted to expose the calvarial bones as previously described (11, 50). The secured mouse was then placed on the microscope stage for two-photon microscopy (11, 50). In order to measure BM blood vessel permeability, leakage and flow velocity in the calvaria BM during in vivo imaging, 70 kDa Rhodamine-B-Dextran (ThermoFisher, D1841) was injected retro-orbitally while the mouse was on the stage.

For ex vivo imaging, optically cleared long bones were mounted in a chamber sealed with solvent-resistant silicone gel (DOWSIL™ 730) and shaved long bones were mounted on a wet sponge to prevent the sample from drying during imaging. Slides were imaged with similar acquisitions settings as the in vivo imaging.

Image Quantification

For in vivo image analysis, image processing and permeability/leakage measurements were performed with Fiji (ImageJ 1.53k) and BM blood flow velocity was quantified with custom scripts in MATLAB (2020a). To measure permeability in the calvaria, live two-photon microscopy video was recorded for the first 30 seconds after Rhodamine-B Dextran was injected. The blood vessel permeability was calculated based on the change in fluorescence intensity outside of blood vessels over time as previously described (51, 52). For leakage measurements, z-stacks (2 µm step size) were recorded randomly around the calvarium BM 10 minutes after injection. Leakage values were calculated by dividing the fluorescence intensity of the perivascular space adjacent to a vessel by the fluorescence intensity inside the blood vessel. Representative examples of BM leakage were generated by taking maximum intensity projections (MIPs) of BM regions with image contrast/enhancement applied. Blood flow velocity was calculated by recording 30 second videos of blood flow in the BM calvaria and then utilizing the Line Scanning Particle Image Velocimetry (LSPIV) method implemented in a custom MATLAB script to calculate blood flow velocity as previously described (53, 54). ImageJ (ImageJ 1.53k) was used to adjust video and image contrast for figure presentation.

In long bone images, as required, 3D z-stacks were rotated with the “Transform” plugin in ImageJ to exclude the non-relevant signals and final images were generated by taking maximum intensity projections (MIPs) of BM regions and adjusting the image contrast/enhancement. To generate a depth-dependent profile of vessel diameter in long bones, measurements were taken at 0-30 µm (shallow BM), 75-105 µm (middle BM), and 150-180 µm (deep BM) below the endosteum. To measure vascular density, image brightness/contrast was first adjusted in Fiji (ImageJ 1.53k) and then images were converted to binary. Next, noise reduction was performed via Despeckle, and binary Fill Hole was applied. Finally, using analytical coding developed in Python (3.7.6), the ratio of the total blood vessel pixels to total BM pixels was determined for BM vessel density measurements.

RESULTS

Vhl Deletion in Dmp1-Expressing Cells Dysregulates Hematopoiesis

Previous studies of VhlcKO mice utilized mice on a mixed genetic background (21). For our studies, we required a pure C57BL/6 (B6) background and we performed a thorough comparison of our B6 VhlcKO mice to previous published results. Similar to previous reports (21), we found that long bones in B6 VhlcKO mice display abnormally high bone mass and density and the BM cavity is severely occluded with bone (Figure 1A, accompanied by stunted B cell development, splenomegaly (Supplementary Figures 1A-E), and reduced BM cellularity compared to controls (Figure 1B). In the B6 VhlcKO, we extended our analysis to be longitudinal, examining hematopoietic lineage at multiple ages. Analysis of specific hematopoietic cell lineages in the BM revealed a decrease in B cells, no change in T cell frequency, and an increase in CD11b+ Gr1- cells (enriched for monocytes) and CD11b+ Gr1+ cells (enriched for Ly6G+ granulocytes, but also may include CD115+ and Ly6C+ monocytes) in 6-week-old, 10-week-old and 6-month-old mice (Figures 1C, D). Furthermore, an overall reduction in the absolute numbers of all hematopoietic lineages in the BM of VhlcKO mice was observed (Table 1). Lineage analysis in the spleen at 10 weeks revealed a decrease in B cells, no change in T cells, and an increase in CD11b+ Gr1+ cells that became more prominent as mice aged to 6 months. CD11b+ Gr1- cells in the VhlcKO spleen at 6-weeks-old were slightly

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reduced, similar to controls at 10-weeks-old, and were increased at 6-months-old (Supplementary Figure 1E). Peripheral blood of the Vhl cKO mice showed no change in B cells at 6 weeks, but B cells were decreased at 10 weeks and 6 months. In contrast, CD11b+ Gr1- cells were increased at 10-weeks-old, and CD11b+ Gr1+ cells at 6-months-old only (Supplementary Figure 1F).

**Increased Frequencies of Hematopoietic Progenitor Cells in the Vhl cKO BM**

To further investigate if the defect in hematopoiesis occurred upstream of lineage-committed cells, we analyzed the hematopoietic progenitor compartments in the BM of Vhl cKO mice. Long-term hematopoietic stem cells (LT-HSCs: LSK, CD150+, CD48-), short term hematopoietic stem cells (ST-HSCs: LSK, CD150-, CD48-), multipotent progenitors (MPP2: LSK, CD150+, CD48+; MPP3: LSK, CD150-, CD48+; and MPP4: LSK, CD150-, Flk2+, CD48+), and common lymphoid progenitors (CLPs: Lineage-, cKit+/Sca1+, CD127+ Flk2+) from Vhl cKO and control mice were quantified using flow cytometry (Figures 2A, B). The results showed an increase in the frequency in LT-HSCs, ST-HSCs, MPP2, MPP3, and CLPs at 6-weeks, 10-weeks and 6-months-old (Figure 2C). MPPs are heterogeneous with different lineage-biased potential. MPP2/3 are myeloid-biased while MPP4 are lymphoid-primed (55, 56). In our results, MPP4 frequency was increased starting at 10-weeks-old (Figure 2C). These results show that deletion of Vhl in Dmp1-expressing cells increases progenitor frequencies and indicates that downstream differentiation of B cells may be blocked. However, examination of MPP4 absolute numbers showed decreased MPP4s in 6-week-old Vhl cKO, an increase at 10-weeks-old, and numbers similar to controls at 6-months old. In 6-week-old Vhl cKO mice, the absolute numbers of LT-HSCs and MPP3 were increased, whereas at 6-months-old, LT-HSCs and CLPs were decreased (Figure 2D).

**Vhl Deletion in Dmp1-Expressing Cells Dysregulates B Cell Development in the BM**

To further explore the effects of Vhl deletion in OBs and OCYs on B cell development and to identify at which stage B cell development was stunted in the BM, we determined the frequencies of Hardy Fractions A-F (Figures 3A, B) using flow cytometry (1, 57). Vhl cKO mice regardless of age retained
### TABLE 1 | Hematopoietic lineage mean±SD and absolute number

| Lineage Population | Bone Marrow | Spleen | Bone Marrow | Spleen |
|-------------------|-------------|--------|-------------|--------|
| **CD45+ Population (mean% ± SD)** |          |        |              |        |
| Control cKO       | 44.16 ± 7.16| 33.69 ± 8.49*** | 60.71 ± 2.64 | 58.57 ± 3.51 |
| Vhl cKO Control   | 1.45 ± 0.79 | 1.61 ± 0.62 | 23.65 ± 3.56 | 23.49 ± 3.42 |
| CD11b+ Gr1-       | 2.58 ± 0.37 | 3.28 ± 0.74** | 2.55 ± 0.57 | 1.95 ± 0.15** |
| Control cKO       | 33.8 ± 4.55 | 15.83 ± 7.01**** | 60.71 ± 7.63 | 46.37 ± 7.53**** |
| Vhl cKO Control   | 45.93 ± 6.89 | 58.11 ± 7.78*** | 1.15 ± 0.50 | 6.27 ± 2.93**** |
| CD11b+ Gr1+       | 56.01 ± 7.91 | 69.31 ± 3.49** | 2.55 ± 0.67 | 14.33 ± 3.33** |

We hypothesized that the observed reduction of B cells was due to increased apoptosis and diminished B cell proliferation in the BM. To test this, B cells were stained with Annexin V and 7AAD to identify cells that were live, in early stage apoptosis or late stage apoptosis (Figure 5A, left panels). Normally, apoptosis is the most extensive in Fraction A (pre-pro-B cells) amongst the B cell fractions (60). The frequencies of VhlKO Fraction A cells in live, early and late apoptosis stages was comparable to controls at all ages examined (Figure 5B). Apoptosis in Fraction B-C in VhlKOS was similar to controls at 6-weeks-old. At 10-weeks-old, the frequency of live Fraction B-C cells increased and those in early apoptosis decreased in the VhlKO. At 6-months-old, there was no difference in the frequencies of live and early stage apoptosis.
apoptotic Fraction B-C cells, but their frequency in late stage apoptosis was increased (Figure 5B). No differences in the stages of apoptosis were observed between controls and Vhl cKOs for Fractions D, E and F at all ages examined, with the exception of increased Fraction F cells in late stage apoptosis at 6-months-old (Supplementary Figure 4).

B cell development leads to the assembly and signaling of the B cell antigen receptor (BCR). CD43+ Fraction A-C (pre-pro-B and pro-B cells) normally have higher proliferation rates compared to CD43- Fraction D-E (Pre-B cells and immature B cells) (5, 61). Proliferation is halted at Fraction D (small pre-B cell) to allow light (L) chain gene rearrangement, subsequently expressing a complete IgM surface molecule (Fraction E) (5, 62). Cell cycle analysis in Vhl cKO B cells was performed using Ki67 and DAPI staining (Figure 5A, right panels). There were no differences in the distribution of cells in G0 (quiescent, DAPI- Ki67-), G1 (DAPI- Ki67+, or S/G2/M (DAPI+ Ki67+) phases between Vhl cKO and control mice amongst all Hardy Fractions at 6-weeks-old (Supplementary Figure 4).

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B cell development at each stage requires specific signaling molecules from a variety of niche cells (5, 63). To further explore the dysregulated niche, BM supernatant was analyzed for levels of CXCL12 and SCF, which are critical for B cell development (1, 27, 28, 31). CXCL12 levels were reduced in the Vhl cKO BM serum, while SCF levels were unaffected (Figure 5D). This suggested that increased apoptosis and reduced proliferation of Fraction B-C cells are caused by reduced CXCL12 levels in the Vhl cKO BM.

**Increased Bone Marrow Blood Vessel Diameter and Density in Vhl cKO Microenvironments**

We attempted to quantify MSC, OB and EC subsets using flow cytometry of collagenase-digested bones (64), but we concluded...
that the high bone mass of Vhl cKO mice prevented complete digestion to accurately enumerate these populations (Supplementary Figure 6). To more precisely examine the changes in the microenvironment of Vhl cKO mice, we imaged femurs that were shaved to remove cortical bone (for analysis of the metaphysis) or optically cleared with a modified uDISCO protocol (for analysis of the fully intact diaphysis) (Supplementary Videos 1, 2) (49). We measured the vessel diameter and frequency in the cleared long bones and found that regardless of their position in the BM, blood vessels in Vhl cKO mice were significantly larger in diameter than the control group (Figures 6A–C) while generally no difference was observed in the vessel frequency (Supplementary Figure 7A). Metaphyseal and diaphyseal BM and bone vessel density measurements revealed that in VhlcKO, blood vessels occupy a larger volume than controls (Figures 6D–F and Supplementary Figure 7B, C).

Furthermore, we observed an apparent decrease in endosteal lining arterioles in the diaphysis of 6-month-old VhlcKO femurs compared to controls (Supplementary Figure 7D). Taken together, these data reveal a striking alteration in the overall architecture of the BM vascular network in VhlcKO mice.

VhlcKO Bone Marrow Blood Vessels Display Increased Permeability

While it has been shown that the bone and vascular system undergoes significant remodeling in VhlcKO mice, there has been a lack of information regarding potential functional changes to BM blood vessels. To examine changes to the BM vasculature system which could negatively impact B cell development, we sought to quantify changes to the vascular permeability, leakage and blood flow velocity via intravital two-photon microscopy of the calvaria. Vessel permeability reflects the rate at which small molecules exit...
blood vessels and fill the surrounding perivascular space, whereas leakage is the ratio of fluorescent dye in the perivascular space and vascular lumen after reaching equilibrium. Blood vessel leakage and permeability was calculated by administering Rhodamine B Dextran (70kDa) via a retro-orbital injection. We found that VhlKO mice displayed greater vascular leakage overall, and that vascular leakage increased in both control and VhlKO mice with age (Figures 7A, B and Supplementary Videos 3–8). Similarly, we observed an increase in vascular permeability in VhlKO mice, which significantly increased with age (Figure 7C and Supplementary Videos 9, 10). We observed a decrease in blood flow velocity in VhlKO mice compared to controls for 6-week-old and 10-week-old mice (Figure 7D). Lastly, we observed an age-related reduction in blood flow in both VhlKO and control mice (Figure 7D), which is consistent with previously published changes in BM vascular flow rate with age (65).

**Evidence for Age-Related Reduction in Oxygen Levels Within Local Niches in the VhlKO Bone Marrow**

Hypoxic niches in the BM microenvironment are crucial for hematopoietic development but BM oxygenation can be altered through changes in vascular supply and/or cellular consumption (11). Dynamic regulation of HIF-1α levels is required for normal B cell development such that HIF activity is high in B cell precursors and must decrease in the immature B cell stage in the BM (66). In wild type mice at 10-16 weeks of age, studies using the hypoxic marker pimonidazole (PIM) revealed that HSCs in the BM stain positively with PIM, indicating a hypoxic niche (67). In contrast, low PIM staining in BM B220+ cells was observed in 6-12 week old mice, indicating a relatively normoxic niche for B220+ cells in wild type mice (68). To evaluate hypoxia in distinct B cell developmental stages, VhlKO and control mice were injected with PBS or 120 mg/kg PIM. PIM staining of LSKs in the BM was positive, as previously reported (67), but this staining was more intense in LSKs of control and VhlKO mice at 6 months of age (Figure 8A top panels). Remarkably, PIM staining in VhlKO LSKs was significantly higher than control LSKs at 6 months (Figure 8B). CD45+ B220+ cells [which include all Hardy Fractions, in addition to other hematopoietic progenitors, natural killer cells, dendritic cells and T cells (69–73)] displayed negative or low staining with PIM in both control or VhlKO mice at 10 weeks old, but the PIM staining in B220+ cells in VhlKO mice at 6 months was significantly elevated compared to controls (Figure 8A, bottom panels and Figure 8B).

Next, we performed PIM staining in order to determine if specific Hardy Fractions were experiencing hypoxia in the VhlKO bone marrow. This analysis revealed that in general, the Fraction A cells stain with PIM at a higher level than the Fractions B through F stain with PIM at a higher level than the Fractions B through F. This is consistent with previous reports (67). Similarly, we observed an increase in vascular permeability in VhlKO mice compared to controls for 6-week-old and 10-week-old mice (Figure 7D). Lastly, we observed an age-related reduction in blood flow in both VhlKO and control mice (Figure 7D), which is consistent with previous published changes in BM vascular flow rate with age (65).

**DISCUSSION**

Here, we report that deletion of the Vhl gene in Dmp1-expressing cells results in cell-extrinsic changes in the bone marrow microenvironment that deleteriously affect B cell development.
as early as 6 weeks of age. Specifically, we observed reduced CXCL12 levels in the bone marrow, which could result in the inability of Fraction B-C to proliferate. We also observed elevated levels of EPO, and an increase in the blood vessel diameters and vessel density in the \( Vhl \) cKO at all ages examined, consistent with a response to hypoxia. To our knowledge, our report is the first to show pimonidazole binding on Fraction A cells in wild type mice, indicating that in general, Fraction A cells reside in hypoxic niches of the BM, similar to LSKs. Burrows et al., 2020 utilized EF5, a hypoxia probe similar to pimonidazole (66), and reported high EF5 staining of “pro-B/pre-B” (B220+ IgM- IgD-) cells, which includes Hardy Fractions A-D, but they did not distinguish EF5 staining on clearly delineated Hardy Fractions, as we have in our current study. In addition, our staining of Hardy Fraction cell subsets with pimonidazole revealed that Fraction A cells in the \( Vhl \) cKO experienced more extreme hypoxia at 6 months of age. Collectively, our analyses demonstrate that the B cell developmental defects in the \( Vhl \) KO bone marrow microenvironment observed at younger ages (6 weeks and 10 weeks) are not due to dysregulation of oxygen levels in their local niches. However, the B cell defects could be exacerbated by hypoxia as the mice age to 6 months.

Evidence from several groups, including our own (2, 47, 74) supports that distinct BM cell subsets, including perisinusoidal cells (which are a subset of MSCs), osteoprogenitor cells (OBPs), OBs and OCYs support different stages of B cell maturation by providing CXCL12 (75, 76) and IL7 (1), both of which are important for proliferation and survival of Hardy Fractions A, B and C (a.k.a. pre-pro-B and pro-B cells) (4). Hematopoietic stem cells and progenitors are localized in the relatively hypoxic sinusoidal regions of the marrow (11, 12) which are anatomically and physically separate from the endosteal niches. Osteolineage cells

FIGURE 5 | \( Vhl \) cKO mice display increase apoptosis and reduced cell proliferation during early B cell development. (A) Representative FACS plots of apoptotic phases (live, early apoptosis, and late apoptosis) in B220+ cells (left) and cell cycle phases (G0 (quiescent), G1, and S/G2/M) in B220+ cells (red:CD43+ blue: CD43-) (right) in 10-weeks-old mice; (B) frequency of apoptotic phases in Fractions A and B-C in 6-weeks-old, 10-weeks-old and 6-month-old mice; (C) frequency of cells in each cell cycle phase within Fractions A and B-C at 6-weeks-old, 10-weeks-old old and 6-month-old mice. Our cell cycle analysis antibody panel (Supplementary Table 1) did not include anti-IgM and anti-IgD, so we could not remove these cells from the CD43+ population. However, these cells are very low in frequency in the \( Vhl \) cKO (Figure 3) and do not significantly change the proliferation results in controls (data not shown); (D) CXCL12, SCF, TPO, and EPO cytokine level measurements in bone marrow supernatant of combined 10-weeks-old (filled) and 6-months-old (open) control or \( Vhl \) KO mice. p<0.05*, p<0.01** two-tailed Student’s t-test. N.S., not statistically significant.
originate from MSCs, which then differentiate to OBPs, early OBs, late OBs and mature OCYS. MSCs and HSCs are found in close proximity to each other (77) and might also be located within the BM cavity in direct contact with B cell progenitors (1). Osteoblast depletion studies in vivo demonstrated OBs as a key regulator of B cell development (78) and this was later supported later by independent studies in mice, in which OBs that lack expression of Gsα (79) and that OBs defective in the mTORC1 signaling pathway (80) could not support full B cell development. The role of MSCs in the regulation of B cell proliferation, survival, and

FIGURE 6 | Ex vivo two-photon imaging of long bones in VhlKO and controls. (A) Representative macroscopic images of the femur diaphyseal BM (scale bars: ~200 μm), (B) magnified z-stacks (scale bars: ~100 μm), and (C) statistical analysis after uDISCO clearing show an increase in the VhlKO vascular diameter relative to the controls; (D) ex vivo images of femur metaphyseal BM after max intensity projection reveal bone replacement and vascular alteration in VhlKO; (E, F) quantification of the metaphyseal and diaphyseal vascular density (scale bars: ~200 μm). Red: blood vessels (labeled with Alexa647 conjugated antibodies against CD31, CD144, and Sca-1), Blue: bone (SHG: Second harmonic generation). *p<0.05, **p<0.01, ****p<0.0001, two-tailed Student’s t-test.

FIGURE 7 | Disruption in blood-bone marrow barrier revealed by intravital microscopy. Blood vessel microenvironment comparisons of control and VhlKO mice at 6-week (n=4), 10-week (n=4) and 6-month (n=5) timepoints. (A) Representative contrast adjusted max intensity projections of the calvarial BM in control and VhlKO mice by age; White: blood vessel (Rhodamine B Dextran, 70 kDa); scale bar: 50 μm; quantification of calvarial BM (B) blood vessel leakage, (C) vascular permeability, and (D) blood flow velocity. *p<0.05, **p<0.01, ****p<0.0001, Mann-Whitney test.
differentiation appears to be highly context-dependent (81–83), and new reports of novel CD51+ MSC subsets and their differential ability to support B lymphopoiesis in the BM (25, 37) will require further scrutiny in the context of altered bone homeostasis.

One caveat to the identification of the “true” niche cells that support B cell development is new information on off-target gene deletion in Dmp1-Cre mice. We utilized Dmp1-Cre for our Vhl deletion studies as they are the main model currently available to target osteocytes. However, despite its widespread use, Dmp1-Cre displays off-target expression (43, 84, 85). Broad MSC targeting of Vhl in Prx-Cre;Vhlfl/fl mice resulted in delays in BM cavity development, increases in trabecular bone with dilated BM vessels and few hematopoietic cells in perinatal mice (86). Similar phenotypes were observed in Osx-Cre;Vhlfl/fl mice at the mature OBs, displayed similar bone and hematopoietic phenotypes plus angiogenesis in the long bones and changes in OCY morphology with fewer dendrite connections (23). Taken together, these studies indicate that deletion of Vhl at the MSC, OBs and OCY phases from ontogeny results in physical changes in bone microenvironment and altered hematopoiesis, and implies that the phenotypes observed could have been generated at an early osteoprogenitor stage and erroneously attributed to more mature osteolineages. Single cell RNA-Seq data on bone marrow stromal cells (88–90) could provide information on non-overlapping mRNAs between MSCs, early OBs, late OBs, in order to create new mouse models for studies of HSC and B cell bone marrow niches, and permit discovery of the specific contributions of MSCs and OBPs to B cell development.

Our studies show an effect of Vhl-deletion in Dmp1-expressing cells on ECs. Our imaging results suggest that there is an increase in bone ECs, which is consistent with previous studies in Osx-Cre; Vhlfl/fl mice where endomucin staining showed that Vhl deletion increased bone vasculature with dilated blood vessels (21). We also observed larger vessels in the BM across all ages and an increase in BM blood volume. These changes, along with the observed decrease in endostal arterioles in the long bones of 6 month old mice and an increase in PIM staining, suggests that oxygenation of the VhlKO marrow may be lower than normal, which may play a role in dysregulation of B cell development in older mice. Future studies will be needed to clarify this and to identify other changes in specific types or locations of blood vessels in the VhlKO model as a function of age.

Given the connection between Vhl and hypoxia response, it was interesting that EPO levels were high in the BM supernatant of the VhlKO mice. High Epo mRNA was also observed in the bones of Osx-Cre;Vhlfl/fl (8) mice. Deletion of Vhl at the mature

FIGURE 8 | Hypoxia cell marker pimonidazole indicates difference by age and amongst B cell fractions in control and VhlKO mice. VhlKO and control mice were injected with PBS or 120 mg/kg pimonidazole (PIM). PIM staining of (A) Live, Lin-, CD45+, Sca1+, cKit+ (LSKs) (top) and Live, B220+ cells (bottom) in the BM of 10-weeks-old and 6-months-old mice; dashed line: isotype control; blue line: anti-PIM staining in controls; red: anti-PIM staining in VhlKO; (B) summary of PIM staining in VhlKOs (red) normalized to the mean fluorescence intensity (MFI) in controls (blue); results from 4 independent experiments are shown; (C) representative anti-PIM staining plots of individual B cell Fractions (A–F) from a control (blue) and a VhlKO mouse (red) at 10-weeks-old (top) and at 6-months-old (bottom). *p<0.05, two-tailed student’s t-test.
TABLE 2 | Mode Fluorescence Intensity of PIM staining on B cell fractions in control and Vhl cKO mice.

| Age      | Genotype | Treatment | MFI (mode) |
|----------|----------|-----------|------------|
|          |          | Fr A      | Fr B-C     | Fr D      | Fr E      | Fr F      |
| 10-weeks-old | Vhl cKO  | Isotype   | 336        | 187       | 146       | 125       | 146       |
|          | Vhl cKO  | Isotype   | 358        | 166       | 146       | 166       | 208       |
|          | Control PIM | Fr A    | 3561       | 1177      | 638       | 638       | 613       |
|          | Control PIM | Fr B-C  | 3678       | 1501      | 638       | 638       | 689       |
|          | Control PIM | Fr D     | 2940       | 742       | 470       | 493       | 402       |
|          | Control PIM | Fr E     | 3926       | 1547      | 715       | 824       | 663       |
|          | Control PIM | Fr F     | 1371       | 293       | 229       | 229       | 229       |
|          | Control PIM | Fr A    | 715        | 250       | 187       | 187       | 187       |
|          | Control PIM | Fr B-C  | 2514       | 796       | 380       | 402       | 424       |
|          | Control PIM | Fr D     | 3034       | 912       | 493       | 540       | 588       |
|          | Control PIM | Fr E     | 3152       | 1371      | 493       | 564       | 564       |
|          | Control PIM | Fr F     | 3561       | 943       | 540       | 564       | 516       |
|          | Vhl cKO  | Isotype   | 1106       | 424       | 336       | 336       | 358       |
|          | Vhl cKO  | Isotype   | 882        | 271       | 206       | 187       | 208       |
|          | Control PIM | Fr A    | 293        | 146       | 104       | 125       | 125       |
|          | Control PIM | Fr B-C  | 293        | 83.5      | 83.5      | 125       | 146       |
|          | Control PIM | Fr D     | 4057       | 1290      | 613       | 689       | 613       |
|          | Control PIM | Fr E     | 1413       | 470       | 388       | 380       | 358       |
|          | Control PIM | Fr F     | 1744       | 516       | 338       | 338       | 338       |
|          | Control PIM | Fr A    | 2292       | 447       | 336       | 336       | 358       |
|          | Control PIM | Fr B-C  | 1413       | 493       | 314       | 355       | 314       |
|          | Control PIM | Fr D     | 882        | 293       | 206       | 229       | 229       |
|          | Control PIM | Fr E     | 1006       | 336       | 229       | 250       | 229       |
|          | Vhl cKO  | Isotype   | 3800       | 1106      | 824       | 742       | 882       |
|          | Vhl cKO  | Isotype   | 2292       | 742       | 424       | 564       | 613       |
|          | Control PIM | Fr A    | 4481       | 1594      | 769       | 1038      | 974       |
|          | Control PIM | Fr B-C  | 3926       | 1106      | 824       | 796       | 974       |
|          | Control PIM | Fr D     | 1594       | 493       | 271       | 271       | 338       |
|          | Control PIM | Fr E     | 1594       | 516       | 293       | 336       | 424       |
|          | Vhl cKO  | Isotype   | 1330       | 271       | 293       | 271       | 380       |

OB stage using the Osx-Cre (8) and Ocn-Cre (22) (targeting osteoprogenitors) and in MSCs, OBs and OCYs using Dmp1-Cre (23), increased bone mass and angiogenesis, likely through HIF1α-regulated expression of VEGF and EPO. If elevated EPO levels directly affect B cell development in the Vhl cKO BM has not yet been verified. However, it has been reported that ECs in the BM suppress levels of CXCL12 expression in response to increased EPO levels (91). We also observed decreased CXCL12 in the BM supernatant of Vhl cKO mice. CXCL12 is required for proper development and retention of B cells in the BM (29, 76). This suggests that altered vascular components in the Vhl cKO bone and BM microenvironments impair B cell development possibly through the effects of EPO on EC function.

Permeability of the BM vasculature in the Vhl cKO mice was also compromised. We found an increased vascular leakage and permeability in the Vhl cKO BM compared to controls regardless of age. In addition, vascular permeability appeared to increase with age, with the highest vascular permeability and leakage being observed in 6-month-old Vhl cKO mice when compared with 6-week-old mice. Interestingly, it was observed that vascular blood flow velocity decreased in 6-week-old and 10-week-old Vhl cKO mice but was not affected in 6-month-old Vhl cKO mice. An increase in blood flow velocity would normally explain an increase in permeability and leakage, but that is not evident in our data. Instead, the more likely explanation is that the blood-bone marrow barrier is compromised, increasing the exposure of the BM to plasma components.

Deletion of Vhl in B cells stabilizes Hif1α levels and affects mature B cell function by impairing cell proliferation, antibody class-switching, generation of high affinity antibodies, antibody responses, and impairs metabolic balance essential for naïve B cell survival and development (58, 59, 92). Dynamic regulation of HIF-1α levels was also found to be a crucial step in B cell development in the BM (66). Burrows et al. found decreased Hif1α activity at the immature B cell stage in the BM and that HIF-1α suppression was required for normal B cell development (66). This dynamic regulation of HIF-1α activity during B cell development is consistent with our results, which revealed that Fraction A cells stain highly with PIM, and PIM staining was reduced as B cell development progressed to Fraction F. Together, our findings and that of Burrows et al. suggest that the earliest B cell stages (e.g. pre-pro B, Fraction A) might prefer a more hypoxic niche compared to the later B cell stages. Although Vhl is deleted in Dmp1-expressing cells in our model, we cannot yet rule out if this deletion is artifically causing changes that would be found in a true hypoxic state through Hif1 stabilization, when in fact the oxygenation of the BM of the Vhl cKO is not altered. In addition, PIM cannot provide true quantification of dissolved oxygen concentration in tissue. PIM adduct staining results could reflect inadequate oxygen supply to the BM, faulty rates of intracellular oxygen consumption, or both. Direct in vivo measurement of oxygen tension using two-photon phosphorescence lifetime microscopy would help answer this question (11).

The information generated in this study helps define the role of Vhl and altered bone homeostasis on immune cell development. Our results suggest the following working model of the interactions in the BM microenvironment that controls B cell development (Figure 9): Vhl in Dmp1-expressing MSCs, OBs and OCYs plays a significant role in the BM microenvironment, indirectly regulating B cell development through a decrease in CXCL12, an increase in EPO, increased vasculature and vascular permeability. However, the oxygen levels in the Vhl cKO appear to be dynamic, such that developing Fraction A cells experience hypoxia in older, but not younger mice. We conclude that the B cell developmental defects in the BM of Vhl cKO mice are not initiated by dysregulated oxygen levels in the BM. However, direct measures of oxygen tension in the local niches of each Hardy B cell Fraction is yet to be performed (and is a goal for our future studies). Our results demonstrate the significant changes of the physical niche in Vhl cKO mice and their effects on B cell development. Whether the physical space, niche cells, or molecular signals all play a direct or indirect role on B cell development remains to be explored and defined, with the possibility that these events are completely independent of each other. The results of this work could contribute to the development of new therapies or new targets for exogenous CXCL12 and EPO antagonists, to preserve and improve bone marrow function during microenvironmental niche changes or stress.
DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by University of California, Merced IACUC.

AUTHOR CONTRIBUTIONS

BC, NA, CB, JS, and JM contributed to experimental design, data collection, analysis, and manuscript writing. HT contributed to data collection and analysis. JS and JM approved the final manuscript and are joint senior authors.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.780945/full#supplementary-material

Supplementary Videos 1, 2 | Representative ex vivo Videos recorded in the uDISCO cleared long bone BM.

Supplementary Video 1 | Representative 10-week-old Control uDISCO cleared long bone Z stack (scale bar ~200 μm).

Supplementary Video 2 | Representative 10-week-old Control uDISCO cleared long bone 3D view (scale bar ~100 μm).

Supplementary Videos 3-8 | Representative Leakage Videos recorded in the calvaria BM. Representative zstacks of the calvaria BM recorded 10 minutes after Rhodamine B Dextran injection. Z step size is 2 μm and scale bars ~50 μm. Green Channel = bone (SHG), Blue = Rhodamine-B-Dextran (70 kDa). Brightness/Contrast adjusted for display only.

Supplementary Video 3 | Representative 6-week-old Control Leakage Zstack.

Supplementary Video 4 | Representative 6-week-old VhlcKO Leakage Zstack.

Supplementary Video 5 | Representative 10-week-old Control Leakage Zstack.

Supplementary Video 6 | Representative 10-week-old VhlcKO Leakage Zstack.

Supplementary Video 7 | Representative 6-month-old Control Leakage Zstack.

Supplementary Video 8 | Representative 6-month-old VhlcKO Leakage Zstack.

Supplementary Videos 9, 10 | Representative permeability videos recorded in the calvaria BM. Representative video of the calvaria BM permeability recorded immediately after Rhodamine-B-Dextran injection. Scale bars ~50 μm. Brightness/Contrast adjusted for display only.

Supplementary Video 9 | Representative 6-week-old Control Permeability Video.

Supplementary Video 10 | Representative 6-week-old VhlcKO Permeability Video.
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