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Androgens Regulate Bone Marrow B Lymphopoiesis in Male Mice by Targeting Osteoblast-Lineage Cells

Anna S. Wilhelmson, Alexandra Stubelius, Anna E. Börjesson, Jianyao Wu, Anna Stern, Stephen Malin, Inga-Lill Mårtensson, Claes Ohlsson, Hans Carlsten, and Åsa Tivesten

From the Wallenberg Laboratory for Cardiovascular and Metabolic Research, Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden (A.S.W., Å.T.); Centre for Bone and Arthritis Research (CBAR), Institute of Medicine, Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden (Al. S., A.B., J.W., C.O., H.C.); Department of Rheumatology and Inflammation Research, University of Gothenburg, Gothenburg, Sweden (An. S., I.L.M.); and Department of Medicine, Center for Molecular Medicine, Karolinska Institute, Karolinska University Hospital, Stockholm, Sweden (S.M.).

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Corresponding author and person to whom reprint requests should be addressed: Åsa Tivesten, Wallenberg Laboratory for Cardiovascular and Metabolic Research, Sahlgrenska University Hospital, Bruna Stråket 16, SE-413 45 Gothenburg, Sweden. Telephone +46 31 3422913, Fax +46 31 823732, E-mail asa.tivesten@medic.gu.se

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Abstract

Testosterone has profound immune-modulatory actions, which may be important for the sexual dimorphism in immune-related disorders, such as autoimmune diseases. A well-known effect of androgens is inhibition of bone marrow B lymphopoiesis; however, a plausible target cell for this effect has not yet been presented. The aim of this study was to determine the target cell for androgen-mediated regulation of bone marrow B lymphopoiesis in males. We confirm higher number of bone marrow B cells in male mice with global inactivation of the androgen receptor (AR) and these G-ARKO mice had increased number of B cell precursors from the pro-B stage. Since osteoblast-lineage cells are known to support B lymphopoiesis at the pro-B stage, we investigated the effect on B lymphopoiesis in osteoblast-lineage cell-specific ARKO (O-ARKO) mice; O-ARKO mice had increased number of B cells in the bone marrow and the number of B cell precursors was increased from the pro-B stage, demonstrating that O-ARKO mimics the bone marrow B lymphopoiesis pattern of G-ARKO mice. By contrast, O-ARKO mice displayed only minor changes in B cell numbers in the splenic compartment compared to G-ARKO. Further, O-ARKO mice had moderately reduced number of bone trabeculae in the vertebrae while cortical bone was unaffected. In conclusion, androgens exert inhibitory effects on bone marrow B lymphopoiesis in males by targeting the AR in osteoblast-lineage cells. The identification of the likely target cell for androgen-mediated regulation of bone marrow B lymphopoiesis will contribute to elucidation of the mechanisms by which androgens modulate immune-related disorders.

Introduction

The sex difference in the pathogenesis of many immune-related disorders, such as autoimmune diseases (1), may be related to the profound immune-modulatory actions of sex hormones. While the complex actions of estrogens in the immune system have been studied in considerable detail (2), less attention has been focused on the actions of androgens, which also are powerful regulators in the immune system (3). For example, androgens modulate humoral immunity (4-6) and bone marrow B lymphopoiesis in males (6-13).

Identification of the target cells for the diverse actions of sex hormones may provide important insight as well as tools to elucidate the specific roles of sex hormones in immune-related disorders. Several studies have attempted to dissect how androgens suppress bone marrow B lymphopoiesis, e.g. by using mouse models of androgen deficiency (castration) or androgen receptor (AR) knockout (ARKO) mice (6,11). However, the results are contradictory and a plausible mechanism for the regulation of B lymphopoiesis by androgens has not been presented. For example, there are conflicting data whether this is an intrinsic effect of the hematopoietic compartment or an extrinsic effect mediated via non-hematopoietic stromal cells (6,11).

The aim of this study was to determine the target cell for androgen-mediated regulation of B lymphopoiesis in male mice. Based on previous data identifying osteoblast-lineage cells as important regulators of B lymphopoiesis (11,14), we hypothesized that androgens regulate bone marrow B lymphopoiesis by targeting cells of the osteoblast lineage.

Material and Methods

Animals

Mice carrying on their X chromosome an AR gene with a floxed exon 2 were generated as previously described (15). To produce global androgen receptor knockout (G-ARKO) male mice, female mice heterozygous for the floxed AR allele were mated with male mice expressing the Cre recombinase ubiquitously under the control of the strong phosphoglycerate kinase (Pgk) promoter (Pgk-Cre^{+} mice) (16) as previously described (15,17). Osteoblast-lineage cell-specific ARKO male mice (O-ARKO) were generated by breeding $AR^{+/flox}$ female mice with male osterix (Osx1)-Cre^{+} mice (Jackson laboratory, Bar Harbor, Maine, USA) (18). In these mice, Cre is expressed from the osteoprogenitor stage resulting in deletion of AR in osteoprogenitors as well as osteoblast precursors, mature osteoblasts and osteocytes that stem from the osteoprogenitors (18). Due to effects on the skeleton and body weight of Osx1-Cre (19,20), Cre^{+} littermates without the AR^{flox} construct were used as controls for the experiments. We assessed presence of AR, Cre, and Zfy (for gender) by PCR amplification of genomic DNA (15). In all experiments the ARKO mice were compared to littermate controls and all mice were on C57BL/6J background.

The mice were housed in a temperature- and humidity-controlled room with a 06:00–18:00 h light cycle and fed a soy-free diet (R70, Lantmännen, Stockholm, Sweden) and tap water *ad libitum*. Due to congenital malocclusion resulting in growth retardation in Osx1-Cre^{+} mice, Osx1-Cre^{+} controls and O-ARKO mice were fed soaked chow and their teeth were cut to maintain chewing ability. The Ethics Committee on Animal Care and Use in Gothenburg approved all procedures.

Tissue collection and cell isolation

All mice were sacrificed at 10–14 weeks of age. The mice were anesthetized (Isoflo® vet, Orion Pharma animal health, Sollentuna, Sweden) and then perfused with saline at physiological pressure. Bone marrow was flushed out from one femur and cells were kept in PBS on ice. Further, tissues were dissected and either snap-frozen in liquid nitrogen for DNA quantification or fixed in formaldehyde for bone morphometry.

Single cell preparation and immunophenotyping by flow cytometry

Bone marrow cells were washed with PBS and put on ice until the total number of nucleated cells was determined using an automated cell counter (Sysmex, Kobe, Japan). Expression of cell surface markers was detected using fluorochrome conjugated antibodies following Fc-blockage (CD16/CD32): B220, CD19, CD25, CD43, CD93, c-kit (CD117), and IgM for bone marrow B cell precursors; and CD19, CD21, CD23, CD43, and CD93 for splenic B cells (Supplemental Antibody Table). Fluorochrome-minus-one was used as control. Cells were analyzed by FACSCantoII or Accuri (BD Bioscience, San Jose, CA, USA) and data were analyzed using FlowJo software (Three Star Inc., Ashland, VA, USA). The gating strategies, using established markers for the B cell populations in bone marrow and spleen (21-23), are presented in Supplemental Figures 1A-B. Hematopoietic cell subsets were defined using forward and side scatter to assess cell size and granularity; lymphocytes, monocytes and granulocytes were separated as presented in Supplemental Figure 2.

DNA quantification

As exon 2 of the AR gene is excised in the ARKO mouse model (15), quantification of exon 2 vs. 3 was used to assess the efficacy and specificity of the cell-specific AR deletion. Genomic (*g*) DNA from organs/tissues was isolated using DNeasy blood and tissue Kit (Qiagen) according to the manufacturer's instructions. *g*DNA amplification was detected using SyBR green master mix (Qiagen) in an ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The following primer pairs were used: AR exon 2; forward GGACCATGTTTTACCCATCG and reverse CCACAAGTGAGAGCTCCGTA, and AR exon 3; forward TCTATGTGCCAGCAGAAACG and reverse CCCAGAGTCATCCCTGCTT. Ct values for AR exon 2 were normalized to Ct values for AR exon 3 using the $2^{-\Delta\Delta ct}$ method (24).

Bone morphometry by high-resolution µCT

High-resolution micro–computed tomography (µCT) analyses were performed on the distal femur and lumbar vertebrae (L5) by using an 1172 model µCT (Bruker micro-CT, Aartselaar, Belgium). The bone

was imaged with an X-ray tube voltage of 50 kV and current of 201 μA, with a 0.5-mm aluminum filter. The scanning angular rotation was 180° and the angular increment 0.70°. The voxel size was 4.48 μm isotropically. The NRecon (version 1.6.9) was employed to perform the reconstruction following the scans. In the femur, the trabecular bone proximal to the distal growth plate was selected for analyses within a conforming volume of interest (cortical bone excluded) commencing at a distance of 426.5 μm from the growth plate, and extending a further longitudinal distance of 134.5 μm in the proximal direction. Cortical measurements were performed in the diaphyseal region of the femur starting at a distance of 5.08 mm from the growth plate and extending a further longitudinal distance of 448.5 μm in the proximal direction. For BMD analysis, the equipment was calibrated with ceramic standard samples. In the vertebra, the trabecular bone in the vertebral body caudal of the pedicles was selected for analyses within a conforming volume of interest (cortical bone excluded) commencing at a distance of 4.48 μm caudal of the lower end of the pedicles, and extending a further longitudinal distance of 134.5 μm in the caudal direction. Trabecular thickness and separation were calculated by the sphere-fitting local thickness method.

Dual Energy X-ray Absorptiometry (DXA)

Analyses of total body areal bone mineral density (aBMD) and bone mineral content (BMC) were performed by DXA using the Lunar PIXImus mouse densitometer (Wipro GE Healthcare, Madison, WI, USA)(25).

Bone turnover markers

The bone formation markers osteocalcin and bone resorption marker C-terminal cross-linked telopeptide of collagen type I (CTX-I) were measured in serum using ELISA kits (Mouse Osteocalcin ELISA kit, #60-1305, Immutopics Inc., San Clemente, CA, USA; RatLaps^{TM} EIA, Immunodiagnostic Systems Ltd, Boldon, United Kingdom) according to the manufacturer's instructions.

Statistical analyses

All data represent mean ± SEM. Statistical evaluations were performed with Prism software (version 6, GraphPad Software, Inc., San Diego, CA, USA) and the non-parametrical Mann-Whitney U test was used for two-group comparisons. A *p*-value of <0.05 was considered statistically significant.

Results

Increased bone marrow B lymphopoiesis in mice with global deletion of the androgen receptor (G-ARKO)

To confirm the importance of the AR for B lymphopoiesis in males, we first analyzed the bone marrow from mice with a global deletion of the AR (G-ARKO). There were an increased number of lymphocytes and a reduced number of granulocytes, while the total number of cells in the femoral bone marrow did not differ between G-ARKO and Pgk-Cre^{+} littermate controls (Supplemental Figure 2 and Supplemental Table 1).

The number of B220^{+}CD19^{+} B cells in the bone marrow of G-ARKO was increased compared to controls (+80%, Figure 1A). Analyzing the different subsets of B cell precursors in G-ARKO vs. control bone marrow (Figure 1B-C), the number of pre-pro-B cells was unchanged, while the numbers of pro-B cells (+128%), large pre-B cells (+110%), small pre-B cells (+108%), and immature B cells (+94%) all were significantly increased. Thus, G-ARKO mice had increased number of B cell precursors from the pro-B stage.

Characteristics of osteoblast-lineage cell-specific ARKO (O-ARKO) mice

Since osteoprogenitors/osteoblasts are known to support B lymphopoiesis at the pro-B cell stage (14,26), we hypothesized that androgens may exert their effect on B lymphopoiesis by targeting these cells. We therefore generated and characterized osteoblast-lineage cell-specific ARKO (O-ARKO) male mice. Analyzing the efficiency of AR deletion in these mice, we observed a 50% reduction of AR *g*DNA in the femur shaft of O-ARKO mice vs. Osx1-Cre^{+} littermate controls (Figure 2A), confirming a satisfactory deletion of AR in osteoblast-lineage cells. We could also corroborate a tissue-specific deletion, as none of the other analyzed tissues showed reduced AR *g*DNA levels (Supplemental Figure 3).

To assess the androgen status of G- and O-ARKO mice, we recorded the wet weights of the seminal vesicles and the testes. As previously published (15), the male G-ARKO mice had much lower seminal vesicle and testes wet weights compared to littermate controls, displaying a general androgen insensitivity

phenotype (Figure 2B). In O-ARKO, the seminal vesicle and testis weights did not differ from control mice (Figure 2B), indicating a normal androgen level and peripheral AR action in these mice.

Androgens are important regulators of bone homeostasis in males, and androgen/AR-deficient male mice are osteopenic, with a reduction of both trabecular and cortical bone mass (27). To verify the effects on bone in O-ARKO, trabecular and cortical bone parameters were analyzed. The trabecular number, assessed by μCT, was significantly reduced in vertebrae (Figure 2C-D) and a similar non-significant trend was observed in femur in O-ARKO compared to control mice (Figure 2C). In contrast, neither cortical bone mass, as indicated by femoral cortical bone thickness (Figure 2E) and cortical volumetric BMD (Supplemental Table 2), nor trabecular thickness in vertebrae/femur (Supplemental Table 2) were significantly affected in O-ARKO mice. Analyses of aBMD and BMC using DXA, by which trabecular and cortical bone compartments cannot be separated, did not reveal any significant reduction in bone mass in O-ARKO mice (Table S2). Bone turnover was assessed by measuring serum concentration of osteocalcin and CTX-I (type I collagen fragments), which are markers for bone formation and resorption, respectively. O-ARKO mice had increased serum levels of both osteocalcin and CTX-I in serum compared to control mice (Figure 2F), suggesting an elevated bone turnover in O-ARKO mice. Taken together, AR deficiency in osteoblast-lineage cells moderately reduced the number of bone trabeculae in the vertebrae while cortical bone mass was unaffected, supporting the notion that AR in osteoblast-lineage cells is involved in the regulation of trabecular but not cortical bone homeostasis (27).

O-ARKO mimics the bone marrow B lymphopoiesis pattern of G-ARKO mice

We next evaluated bone marrow B lymphopoiesis in O-ARKO male mice. Analyzing the bone marrow of O-ARKO mice vs. littermate controls (Osx1-Cre^{+}), O-ARKO mice had increased number of B lymphocytes (+60%, Figure 3A) in the bone marrow. Further, the total cell number was increased in bone marrow of O-ARKO mice, with increased number of lymphocytes but unaltered number of granulocytes compared to controls (Supplemental Figure 2 and Supplemental Table 1).

The O-ARKO mice had an increased number of B cell precursors from the pro-B stage (Figure 3B-C); the number of pre-pro-B cells was unchanged, while the numbers of pro-B cells (+54%), large pre-B cells (+65%), small pre-B cells (+79%), and immature B cells (+66%) all were significantly increased. Thus, an osteoblast-lineage cell-specific deletion of AR mimics the bone marrow B lymphopoiesis pattern of G-ARKO.

Increased splenic B lymphopoiesis in G-ARKO but not O-ARKO mice

We next assessed the splenic B cell pools in the G-ARKO and O-ARKO mice. Compared to littermate controls (Pgk-Cre^{+}), G-ARKO mice had a doubling of the number of splenic B cells (+233%, Figure 4A). All different subsets of splenic B cells were increased in G-ARKO, albeit to a varying degree (Figure 4B-C); T1 immature B cells were increased by 289%, T2 immature B cells by 231%, follicular B cells by 114%, marginal zone B cells by 110%, and B1 cells by 141%. By contrast, the O-ARKO mice had an unchanged total B cell number in spleen compared to control mice (Figure 4D). Analyzing the different B cell subsets in spleen of O-ARKO, only the immature B cells were modestly increased (Figure 4E-F): T1 immature B cells were increased by 61% and T2 immature B cells by 53%, while the mature B cell subsets were unaffected. Thus, G-ARKO mice had elevated number of both immature and mature B cell subsets in the spleen while O-ARKO mice only a showed minor increase in the immature subsets.

Discussion

In this study, we have addressed the controversial question of the target cell responsible for the androgen-mediated regulation of bone marrow B lymphopoiesis in males (6,11). We show that depletion of the AR in cells of the osteoblast-lineage results in increased bone marrow B lymphopoiesis, demonstrating a central role for the AR in osteoblast-lineage cells.

Previously published data on androgen- and AR-mediated inhibition of bone marrow B lymphopoiesis are consistent (6-11). However, there have been conflicting reports regarding the target cell(s) for this effect. Altuwaijri et al. suggested the effect to be B cell-intrinsic, using a B cell-specific ARKO mouse (CD19-driven Cre expression) (6). In contrast, Olsen et al. suggested a non-hematopoietic target cell, using a bone marrow transplantation approach (11). Our present data support the notion of a non-hematopoietic target cell. Furthermore, we could specify that AR in osteoblast-lineage cells regulates bone marrow B lymphopoiesis. As the O-ARKO bone marrow B lymphopoiesis pattern mimics that of G-ARKO, our data clearly show that the osteoprogenitor/osteoblast niche is a likely target for this androgen-mediated effect. Our data are supported by previous studies demonstrating a pivotal role of osteoblast-lineage cells supporting B lymphopoiesis (14,26,28-31). The exact mechanism for androgen/AR regulation of B lymphopoiesis remains to be elucidated, but cytokines (such as IL-7, SCF, or CXCL12) known to be secreted from the osteoblast-lineage cells are candidates for mediating the suppressive effect (14,32).

While previous studies have addressed androgenic suppression of bone marrow B lymphopoiesis, few of them have evaluated the effect on the progressive stages of developing B cells in the bone marrow. The present results clearly show a regulation at the pro-B stage (delineated as $B220^{+}CD19^{+}c\text{-}kit^{+}$, using markers and nomenclature from Osmond, Rolink, and Melchers (21)), in both G-ARKO and O-ARKO mice. Our data are consistent with a previously published report, demonstrating an inhibitory effect of androgens at the pro-B stage (10). In contrast, Altuwaijri et al. suggested an AR-mediated effect from the pre-B stage (delineated as $B220^{+}CD2^{+}IgM^{-}$)(6). This discrepancy might originate from the use of different

markers and/or nomenclature of B cell progenitors in these studies. Notably, our data are supported by the fact that osteoblast-lineage cells nurse B cell progenitors at the pro-B stage (29-31).

In the present study, we found that G-ARKO mice had unchanged total number of bone marrow nucleated cells, while these were increased in the O-ARKO model. Therefore, we analyzed the number of cells in other hematopoietic cell populations. While the use of forward and side scatter for the definition of bone marrow cell subsets should be considered as a study limitation, our finding of reduced number of granulocyte-like cells in the bone marrow of G-ARKO mice is in line with a previously published report showing that G-ARKO mice are neutropenic and have reduced granulopoiesis (33). Because this effect on bone marrow granulocyte number was not seen in O-ARKO mice, differential effects on granulopoiesis in the G-ARKO and O-ARKO models may contribute to the discrepancy in total bone marrow cell number. Further, these data suggest that the bone marrow granulopenia in AR deficiency is less likely to be osteoblast-lineage cell-dependent.

Our data showing that G-ARKO mice have increased number of B cells in spleen is consistent with previously published data that androgen- and AR-deficiency increase peripheral B cell number (6,7,10). However, despite increased B lymphopoiesis, AR deficiency specifically in osteoblasts neither altered the total splenic B cell number nor the mature B cell number. The fact that we have not identified the mechanism for the regulation of peripheral B cell number is a limitation of the present study. Of note, similar findings, i.e. that targeting of the osteoblast niche severely alters B lymphopoiesis in the bone marrow while the peripheral B cell number remains unaffected, has been reported by others (30). Our results suggest that additional target cells and/or mechanisms support the increased number of peripheral B cells in androgen/AR-deficient states. However, to our knowledge, there are no published data on suggestive mechanisms. Future mechanistic studies may address e.g. increased homing of developing B cells to the spleen as well as increased survival of peripheral B cells in androgen/AR deficiency. Further, a

potential role of splenic stromal cells, which are important regulators of peripheral B cell homeostasis (34), should be considered in future studies.

In the present study, we found that AR deficiency in osteoblast-lineage cells moderately reduced the number of bone trabeculae in the vertebrae while cortical bone mass was unaffected. As the cortical bone compartment is more important than the trabecular bone compartment for overall bone mass, it is not surprising that the total body BMC as analyzed by DXA was not significantly affected in the O-ARKO mice. Our data are consistent with results from previous cell-specific ARKO models (recently reviewed (27)), which have been generated to evaluate the effects of AR in osteoblasts and osteocytes, respectively. These models show a role of the AR in mature osteoblast-lineage cells for trabecular but not cortical bone (35-37). Our data add to previous results by comprising the immature osteoblast-lineage cells; hence, AR neither in early osteoprogenitors cells nor in mature osteoblast-lineage cells has an impact on cortical bone. O-ARKO mice had elevated serum levels of markers for both bone formation and bone resorption, suggesting that the reduced trabecular number primarily is the result of elevated bone resorption. Taken together, the present and previous results demonstrate that trabecular bone is regulated by AR in osteoblast-lineage cells, while the target cell for AR-mediated effects on cortical bone mass is still to be identified (27,38).

The bone and the bone marrow are intimately connected, both spatially and functionally, and androgens have a major impact on both tissues. B lymphocytes are thought to contribute to the bone loss caused by sex steroid deficiency (38), which is supported by the concurrent bone loss and increased bone marrow B lymphopoiesis after castration and IL-7 treatment, respectively. Immature, but not mature, B cells are suggested to mediate the osteopenic effect through RANKL, and RANKL-deficient B cells cannot induce bone loss after castration. Additional investigations are needed to evaluate if the osteoblast-lineage cell-specific AR effect on trabecular bone is mediated via inhibition of B lymphopoiesis. Further, it is yet unclear if and how B cell-intrinsic androgen signaling may affect cortical and/or trabecular bone.

The fact that androgen deficiency in men is associated with increased risk of autoimmunity indicates a clinically important role of androgens in regulating autoimmunity (39,40). This effect of androgen deficiency is mirrored in rodent experimental models of autoimmune disease (41,42). To date, the androgenic actions resulting in protection from autoimmunity remain unclear. However, it is reasonable to believe that the observed protection against autoimmunity may somehow be related to the actions of androgens as potent regulators of B cell homeostasis in both humans and rodents (13). Our novel finding that androgens regulate B lymphopoiesis by targeting osteoblast-lineage cells provides an experimental research tool (the O-ARKO model) to delineate the relation between androgen deficiency-induced increased bone marrow B lymphopoiesis and autoimmune disorders. Although such a link would be not mediated via an increased mature B cell number in the periphery (unchanged in O-ARKO), important clonal deletion and receptor editing of self-reactive B cells occur in the bone marrow, and defects in this checkpoint may cause autoimmunity (43).

In conclusion, androgens exert inhibitory effects on B lymphopoiesis in males by targeting the AR in the osteoblast-lineage cells. The identification of the likely target cell for androgen-mediated regulation of bone marrow B lymphopoiesis will contribute to elucidation of the mechanisms by which androgens regulate immune-related disorders.

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Figure Legends

Figure 1. Increased B lymphopoiesis in mice with global deletion of the androgen receptor (G-ARKO)

A) Number of $B220^{+}CD19^{+}$ double positive B cells in bone marrow (BM) in control ($Pgk\text{-}Cre^{+}$) and general (G-)ARKO mice. B) Illustration of Pro-B and Pre-B cells in FACS plots from control and G-ARKO. C) Early Pro-B cells measured as $B220^{+}CD19^{-}c\text{-}kit^{+}CD43^{+}$, Pro-B cells $B220^{+}CD19^{+}c\text{-}kit^{+}CD43^{+}$, Large Pre-B cells $FSC^{hi}B220^{+}CD19^{+}CD25^{+}$, Small Pre-B cells $FSC^{lo}B220^{+}CD19^{+}CD25^{+}$, and Immature B cells $B220^{+}CD19^{+}CD93^{+}IgM^{+}$ in BM from control and G-ARKO mice. Cell number is presented as 10^{6} cells. n=5-6/group. **=$p<0.01$

Figure 2. Characteristics of osteoblast-lineage cell-specific ARKO (O-ARKO) mice

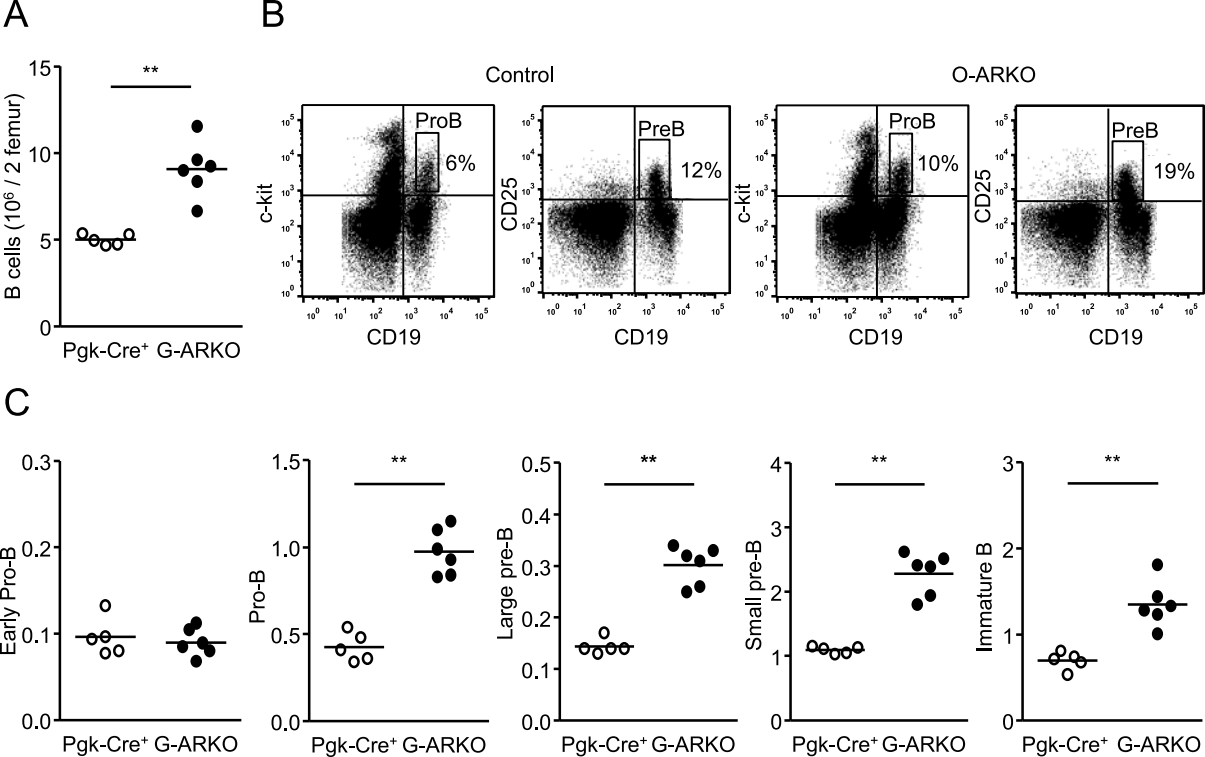
A) Relative AR exon 2 to exon 3 in DNA from femur shaft from control ($Osx1\text{-}Cre^{+}$) and osteoblast-specific (O-)ARKO mice. B) Wet weight of seminal vesicles and testes from $Pgk\text{-}Cre^{+}$, G-ARKO, $Osx1\text{-}Cre^{+}$, and O-ARKO mice. C) Number of trabecula in femur and vertebra L5 from control and O-ARKO. D) μCT images of L5 vertebra. E) Cross-sectional thickness of cortical bone in femur from control and O-ARKO mice assessed by μCT. F) Serum concentration of markers for bone turnover in control and O-ARKO mice; osteocalcin for bone formation and C-terminal cross-linked telopeptide of collagen type I (CTX-I) for bone resorption. n=7-18/group. $^{(*)}p=0.059$, *=$p<0.05$, ***=$p<0.001$

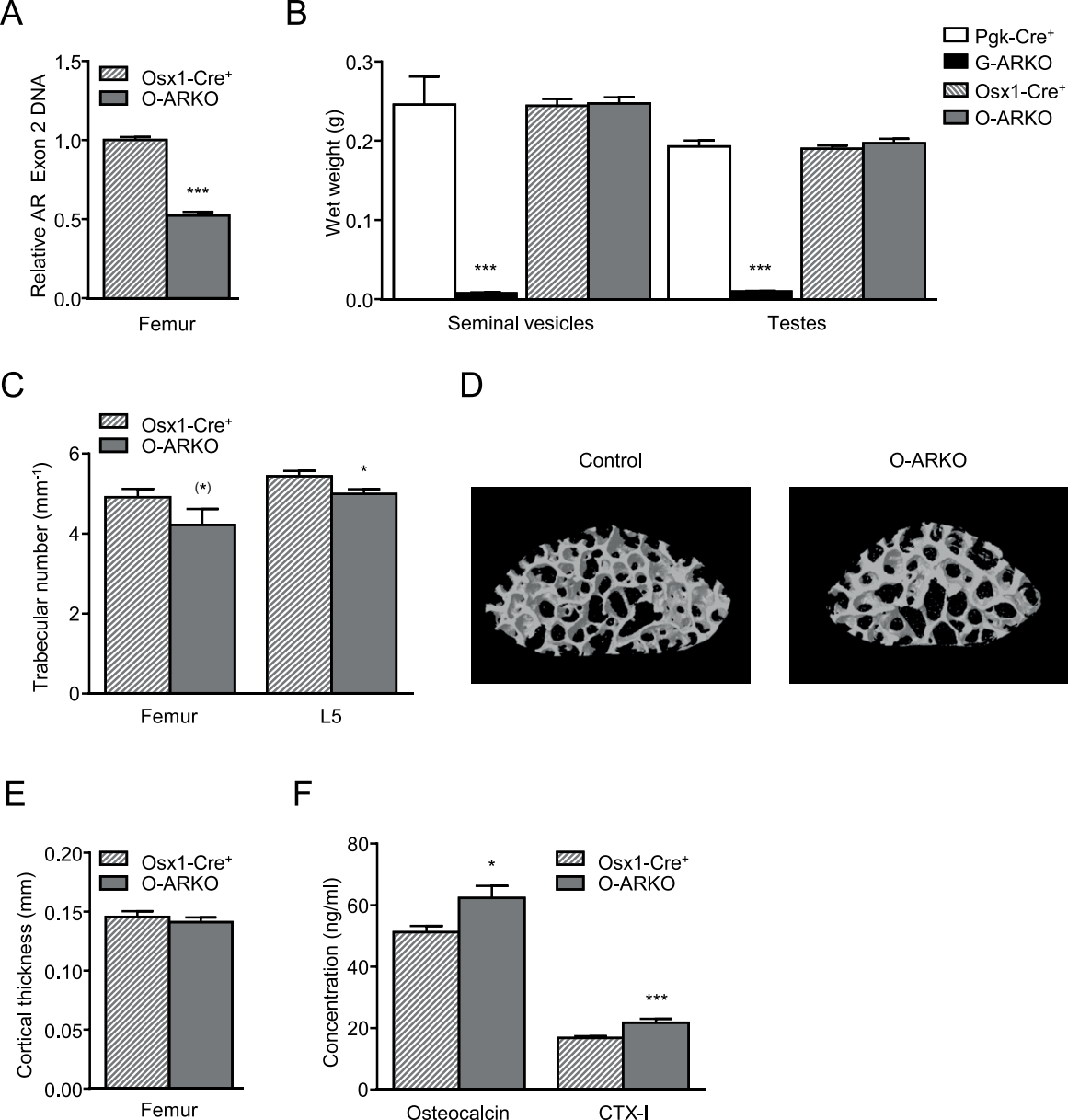
Figure 3. **O-ARKO mimics the B lymphopoiesis pattern of G-ARKO mice**

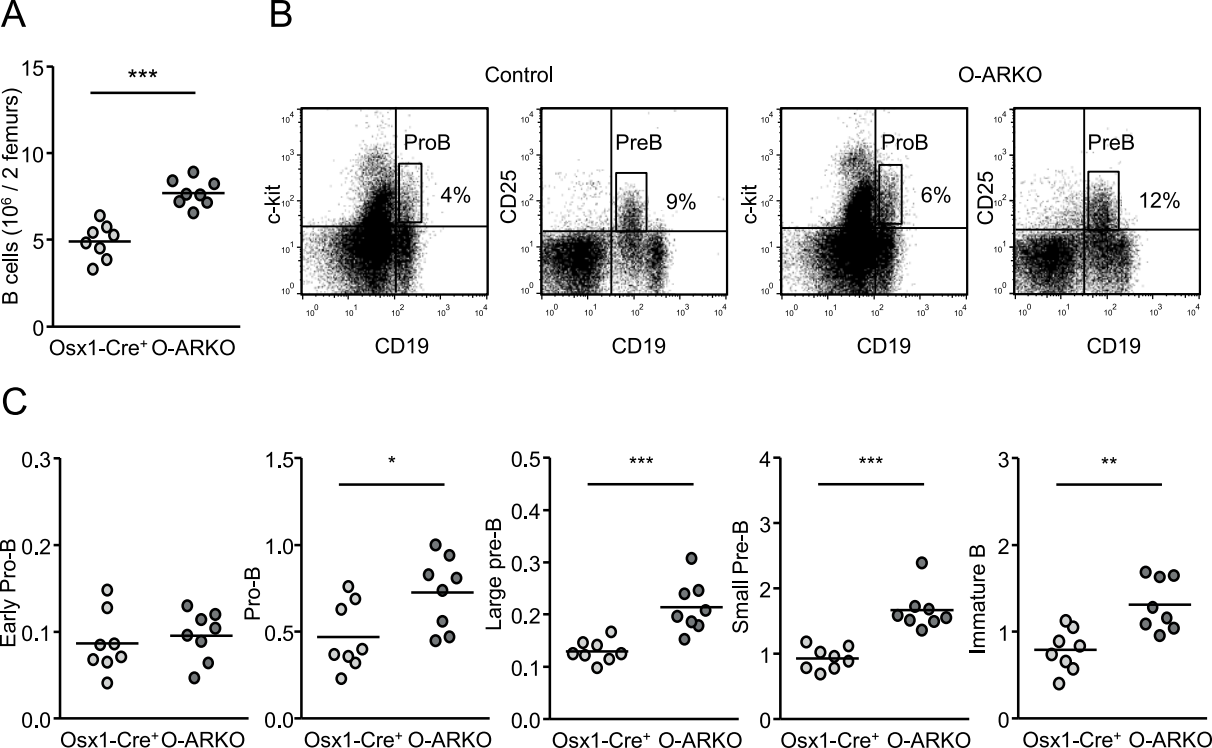
A) Number of $B220^{+}CD19^{+}$ double positive B cells in bone marrow (BM) from control ($Osx1\text{-}Cre^{+}$) and O-ARKO mice. B) Illustration of Pro-B and Pre-B cells in FACS plots from control and O-ARKO. C) Early Pro-B cells measured as $B220^{+}CD19^{-}c\text{-}kit^{+}CD43^{+}$, pro-B cells $B220^{+}CD19^{+}c\text{-}kit^{+}$, large Pre-B cells $FSC^{hi}B220^{+}CD19^{+}CD25^{+}$, small Pre-B cells $FSC^{lo}B220^{+}CD19^{+}CD25^{+}$, and immature B cells $B220^{+}CD19^{+}CD93^{+}IgM^{+}$ in BM from control and O-ARKO mice. Cell number is presented as 10^{6} cells. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$

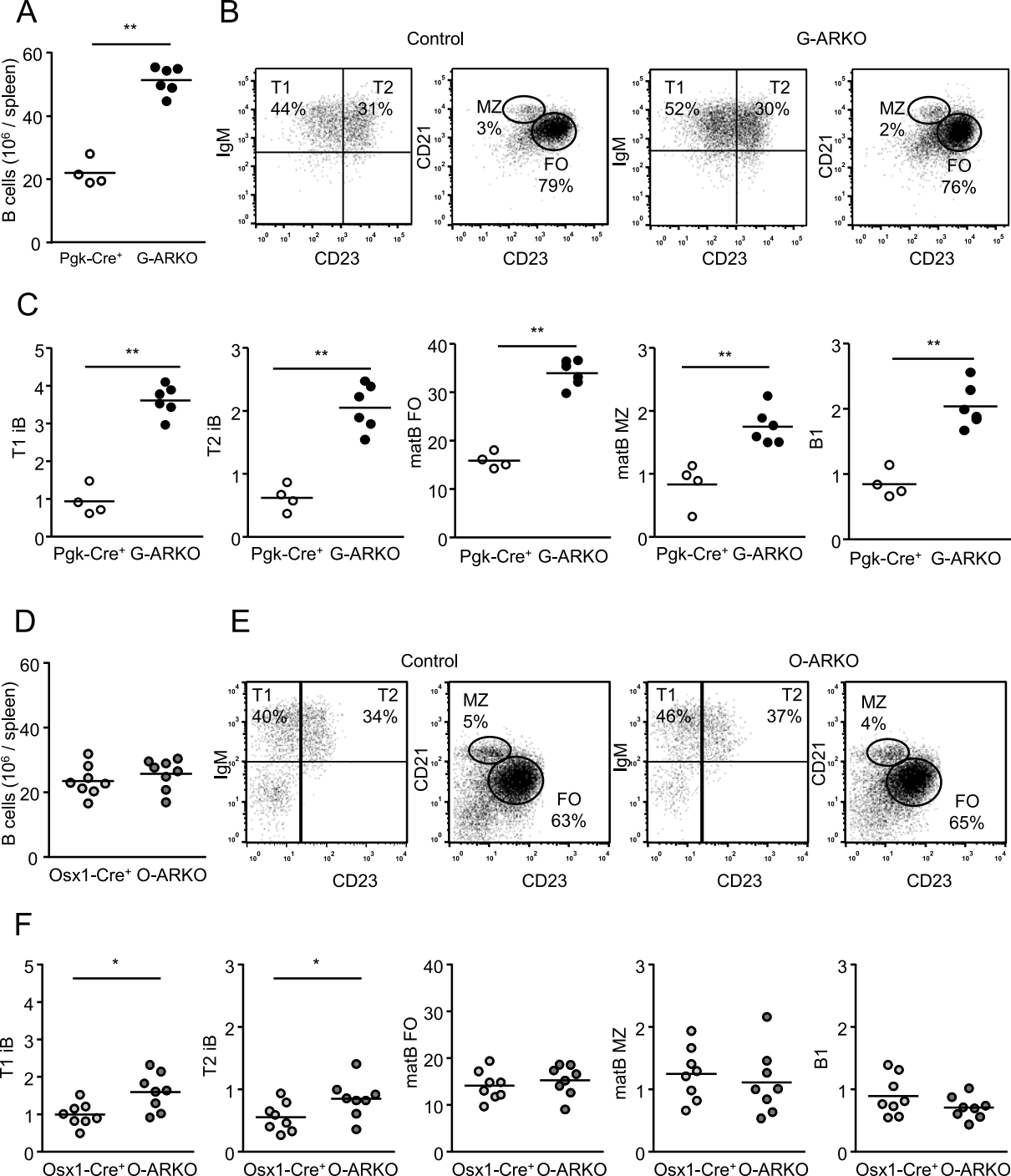
Figure 4. Increased splenic B lymphopoiesis in G-ARKO but not O-ARKO mice

A) Total number of $CD19^+$ B cells in spleen from control (Pgk-Cre^+) and G-ARKO mice. B) Illustration of immature and mature B cells in spleen in FACS plots from control and G-ARKO. C) T1 B cells $CD19^+CD93^+IgM^+Cd23^-$, T2 B cells $CD19^+CD93^+IgM^+Cd23^+$, follicular B cells $CD19^+CD93^-CD23^+CD21^{hi}$, marginal zone B cells $CD19^+CD93^-CD23^+CD21^{Int}$ and B1 cells $CD19^+CD43^+$. D) Total number of $CD19^+$ B cells in spleen from control (Osx1-Cre^+) and O-ARKO mice. E) Illustration of immature and mature B cells in spleen in FACS plots from control and O-ARKO. F) T1 B cells $CD19^+CD93^+IgM^+Cd23^-$, T2 B cells $CD19^+CD93^+IgM^+Cd23^+$, follicular B cells $CD19^+CD93^-CD23^+CD21^{hi,}$ marginal zone B cells $CD19^+CD93^-CD23^+CD21^{Int}$ and B1 cells $CD19^+CD43^+$. Cell number is presented as 10^6 cells. n=4-8/group. *=$p<0.05$, **=$p<0.01$


A
B
C
B cells (10^6 / 2 femur)
**
Pgk-Cre+ G-ARKO
Control
O-ARKO
ProB
6%
PreB
12%
10%
19%
c-kit
CD25
CD19
Early Pro-B
Pro-B
Large pre-B
Small pre-B
Immature B


A
Relative AR Exon 2 DNA
1.5
1.0
0.5
0.0
Osx1-Cre+
O-ARKO
***
Femur
B
Wet weight (g)
0.3
0.2
0.1
0.0
Pgk-Cre+
G-ARKO
Osx1-Cre+
O-ARKO
***
***
Seminal vesicles
Testes
C
Trabecular number (mm-1)
6
4
2
0
Osx1-Cre+
O-ARKO
(*)
*
Femur
L5
D
Control
O-ARKO
E
Cortical thickness (mm)
0.20
0.15
0.10
0.05
0.00
Osx1-Cre+
O-ARKO
Femur
F
Concentration (ng/ml)
80
60
40
20
0
Osx1-Cre+
O-ARKO
*
***
Osteocalcin
CTX-I


A
B
C
B cells (10^6 / 2 femurs)
***
Osx1-Cre+ O-ARKO
Control
O-ARKO
ProB
PreB
4%
9%
6%
12%
c-kit
CD25
CD19
Early Pro-B
Pro-B
Large pre-B
Small Pre-B
Immature B
*
**

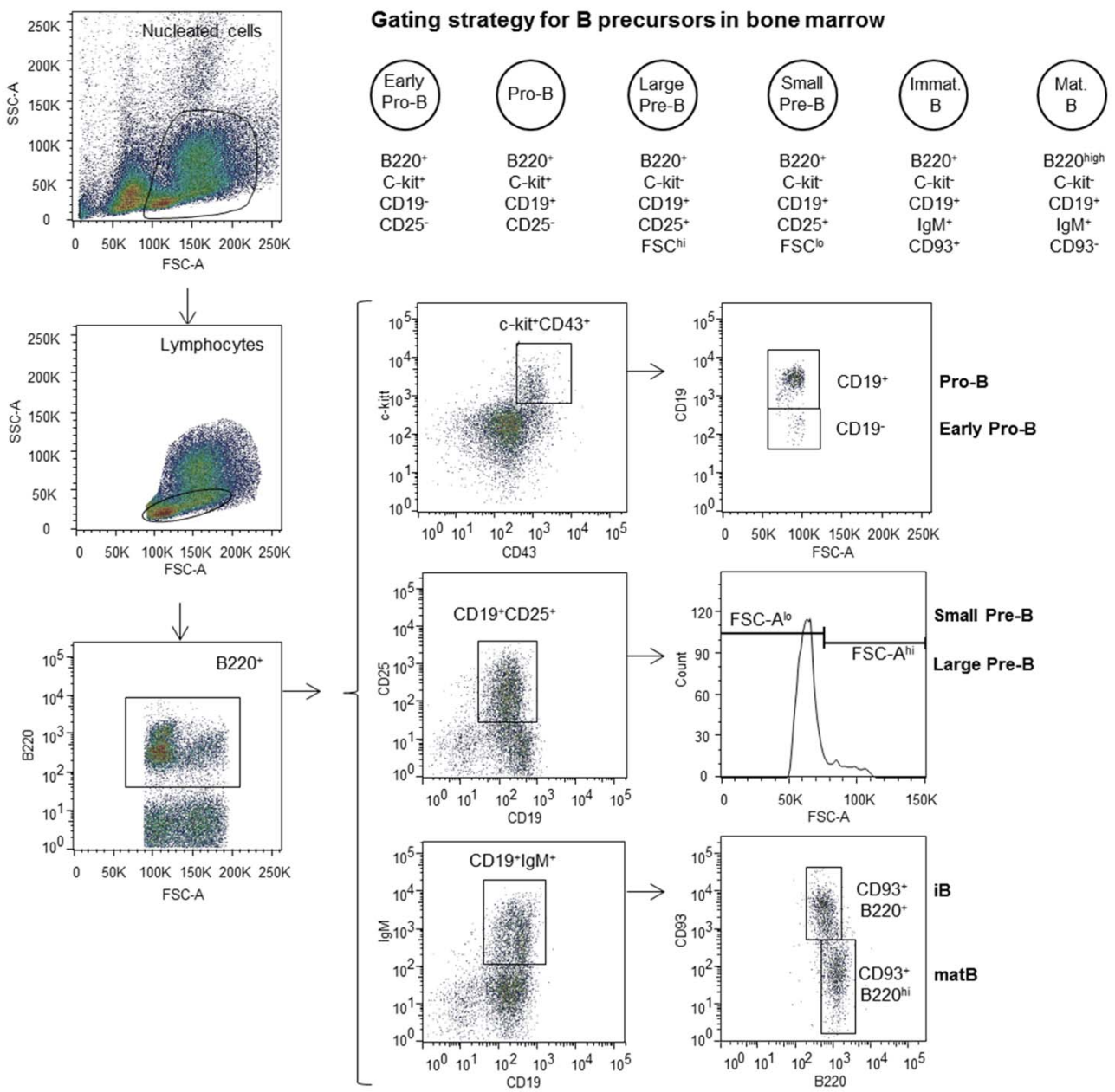

A
B
Control
G-ARKO
B cells (10^6 / spleen)
Pgk-Cre+
G-ARKO
T1
44%
T2
31%
MZ
3%
FO
79%
T1
52%
T2
30%
MZ
2%
FO
76%
IgM
CD21
CD23
C
T1 iB
T2 iB
matB FO
matB MZ
B1
D
E
Osx1-Cre+
O-ARKO
T1
40%
T2
34%
MZ
5%
FO
63%
T1
46%
T2
37%
MZ
4%
FO
65%
F

Androgens Regulate Bone Marrow B Lymphopoiesis by Targeting Osteoblast-Lineage Cells

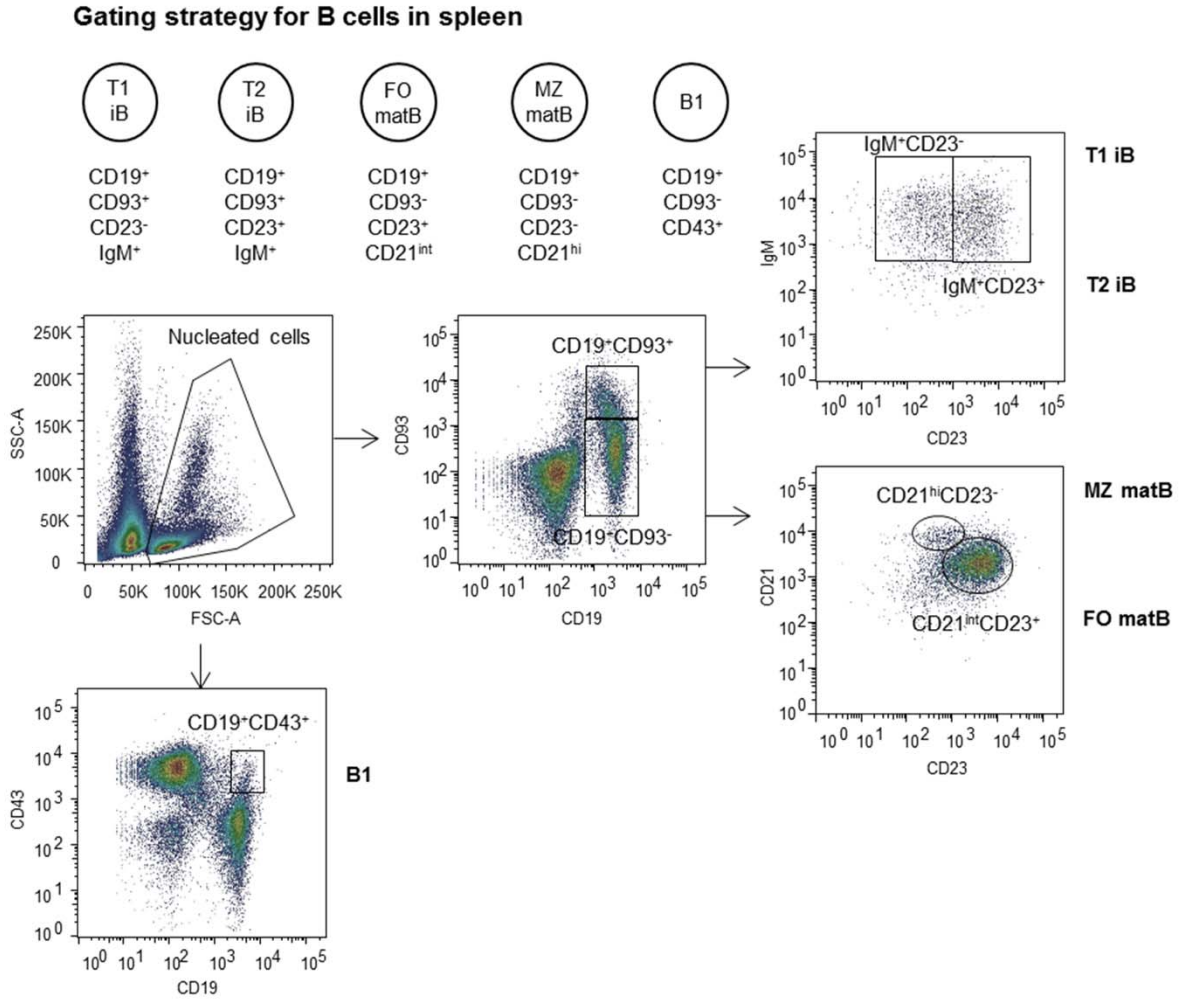
Anna S. Wilhelmson, Alexandra Stubelius, Anna E. Börjesson, Jianyao Wu, Anna Stern, Stephen Malin, Inga-Lill Mårtensson, Claes Ohlsson, Hans Carlsten, and Åsa Tivesten

Supplemental data

Supplemental figure 1A



Supplemental figure 1B



Antibody table

Peptide/protein target	Name of Antibody	Manufacturer, catalog #, and/or name of individual providing the antibody	Species raised in; monoclonal or polyclonal	Dilution used
CD16/CD32	Purified rat anti-mouse CD16/CD32	BD Bioscience, 553142, clone 2.4G2	Rat; monocolonal	1:100
B220	Anti-Human/Mouse CD45R (B220) APC-eFluor® 780	eBioscience, 47-0452-80, clone RA3-6B2	Rat; monocolonal	1:200
CD93	Anti-Mouse CD93 (AA4.1) APC	eBioscience, 17-5892-81, clone AA4.1	Rat; monocolonal	1:800
CD19	Anti-Mouse CD19 eFluor® 450	eBioscience, 48-0193-80 , clone 1D3	Rat; monocolonal	1:200
c-kit (CD117)	APC Rat Anti-Mouse CD117	BD Bioscience, 553356, clone 2B8	Rat; monocolonal	1:200
CD25	PE Rat Anti-Mouse CD25	BD Bioscience, 553866, clone PC61	Rat; monocolonal	1:400
CD43	Biotin Rat Anti-Mouse CD43	BD Bioscience, 553269, clone S7	Rat; monocolonal	1:400
CD23	Anti-Mouse CD23	eBioscience, 12-0232-81, clone B3B4	Rat; monocolonal	1:400
CD21(CR2/CR1)	Anti-Mouse CD21/CD35	BD Bioscience, 553818, clone 7G6	Rat; monocolonal	1:400
CD43	Anti-Mouse CD43	BD Bioscience, 553269, clone S7	Rat; monocolonal	1:400
IgM	Anti-Mouse IgM (μ-chain specific), F(ab′)2 fragment-Biotin antibody produced in goat	Sigma-Aldrich, SAB3701208, clone -	Goat; polyclonal	1:8000

Supplemental figure 2

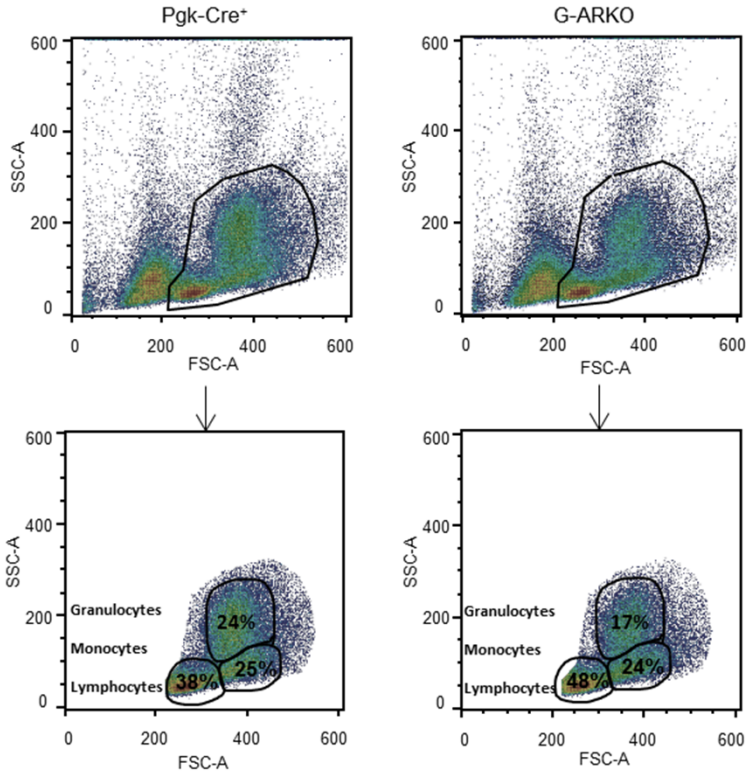


Figure S2. FACS plots with gating strategy for cell subsets from G-ARKO and control (Pgk-Cre^{+}) mice.

Supplemental table 1

Hematopoietic cell subsets in bone marrow of G- and O-ARKO mice

Number of cells	**Pgk-Cre^{+}**	**G-ARKO**	***p*-value**	**Osx1-Cre^{+}**	**O-ARKO**	***p*-value**
Nucleated cells (10^6)	29±0.92	31±1.9	0.33	37±1.9	45±1.4	*
Lymphocytes (10^6)	11±0.22	15±1.0	**	10±0.67	14±0.36	**
Monocytes (10^6)	7.2±0.26	7.5±0.41	0.75	7.8±0.35	8.8±0.27	0.08
Granulocytes (10^6)	6.9±0.40	5.4±0.25	**	14±0.90	16±1.0	0.16

*=p<0.05, **=p<0.01

Supplemental figure 3

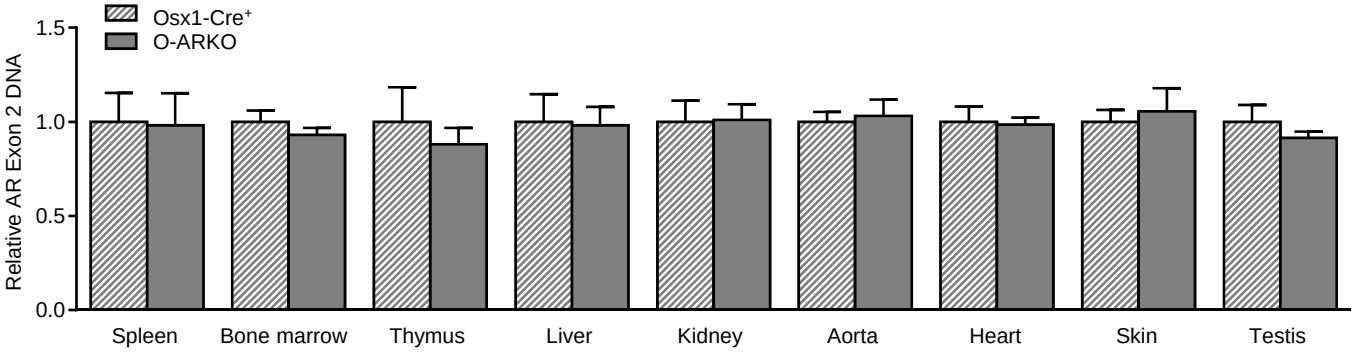


Figure S3. Relative AR exon 2 DNA from spleen, bone marrow, thymus, liver, kidney, aorta, heart, skin, and testis measured in O-ARKO and Osx1-Cre^{+} mice. n=7-8

Supplemental table 2

Bone parameters in O-ARKO mice

Bone parameter	**Osx1-Cre^{+}**	**O-ARKO**	***p*-value**
µCT			
Cortical volumetric BMD, femur (g/cm^3)	1.21±0.02	1.21±0.04	0.86
Trabecular thickness, femur (µm)	43±6.2	46±2.3	0.47
Trabecular thickness, vertebrae L_5 (µm)	46±0.9	51±2.0	0.07
DXA			
Total body areal BMD (g/cm^2)	0.047±0.001	0.047±0.001	0.62
Total body BMC (g)	0.41±0.02	0.39±0.04	0.46

µCT=micro–computed tomography, BMD=Bone Mineral Density, DXA=Dual Energy X-ray Absorptiometry, BMC= Bone Mineral Content