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Differential Expression of the Transcription Factor ARID3a in Lupus Patient Hematopoietic Progenitor Cells[1]

Michelle L. Ratliff[*], **Julie M. Ward**[*,†], **Joan T. Merrill**[‡], **Judith A. James**[†,§,¶], and **Carol F. Webb**[*,†,‖,2]

[*]Immunobiology and Cancer Research, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104

[‡]Clinical Pharmacology Program, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104

[§]Arthritis and Clinical Immunology, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104

[†]Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104

[¶]Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104

[‖]Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK 73104

Abstract

Although hematopoietic progenitor/stem cells (HPSCs) are used for transplantation, characterization of the multiple subsets within this population in man has lagged behind similar studies in mice. We found that expression of the DNA-binding protein, ARID3a, in mouse stem cells was important for normal development of hematopoietic lineages; however, progenitors expressing ARID3a in man have not been defined. We previously showed increased numbers of ARID3a$^+$ B cells in nearly half of systemic lupus erythematosus (SLE) patients, and that total numbers of ARID3a$^+$ B cells were associated with increased disease severity. Because expression of ARID3a in those SLE patients occurred throughout all B cell subsets, we hypothesized that ARID3a expression in patient HSPCs might also be increased relative to expression in healthy controls. Our data now show that ARID3a expression is not limited to any defined subset of HPSCs in either healthy controls or SLE patients. Numbers of ARID3a$^+$ HSPCs in SLE patients were increased over numbers of ARID3a$^+$ cells in healthy controls. While all SLE-derived HPSCs exhibited poor colony formation *in vitro* compared to controls, SLE HPSCs with high numbers of ARID3a$^+$ cells yielded increased numbers of cells expressing the early progenitor marker, CD34. SLE HPSCs with high numbers of ARID3a$^+$ cells also more readily generated autoantibody producing cells than HPSCs with lower levels of ARID3a in a humanized mouse model. These

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[2]Corresponding author: Carol F Webb, Immunobiology and Cancer Research Program, Oklahoma Medical Research Foundation, 825 NE 13th St., Oklahoma City, OK, 73104, USA. Telephone: (405) 271-7564; Fax: (405) 271-7128; carol-webb@omrf.org.

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data reveal new functions for ARID3a in early hematopoiesis and suggest that knowledge regarding ARID3a levels in HPSCs could be informative for applications requiring transplantation of those cells.

INTRODUCTION

Hematopoietic stem/progenitor cells (HSPCs) are lineage negative, $CD34^+$ (Lin^-CD34^+) cells (1), and are generally not abundant in peripheral blood (2). This progenitor population is heterogeneous and typically used for transplantation therapy, with the earliest HSPCs being hematopoietic stem cells (HSCs). Additional populations of hematopoietic progenitors included in the $CD34^+$ subset include multipotent progenitors (MPPs), multi-lymphoid progenitors (MLPs) and multi-myeloid progenitors (MMPs). Despite the fact that HSPCs are used for transplantation in many diseases, including severe autoimmune disease (3), a clear understanding of the intrinsic characteristics that influence the normal development and function of these cells in man is lacking (4).

The potential contribution of defects in HSPCs to various disease states is only becoming apparent. For example, dysfunction in HSPCs may also contribute to defects observed in systemic lupus erythematosus (SLE) (5–7). In addition, observations from a mouse lupus model indicated that HSPCs were greatly expanded in the periphery compared to wild type mice, and those HSPCs showed functional alterations including enhanced self-renewal properties and skewing toward the myeloid lineage (7). The use of HSPCs in bone marrow transplantation therapies for severe autoimmune disease underscores the need for better characterizations of human HSPCs in both patient and healthy control samples.

The ARID (A+T rich interacting domain protein) family of proteins consists of fifteen family members in man, each of which has unique functions including the ability to initiate epigenetic modifications and chromatin remodeling (8–10). ARID3a was first discovered in adult murine B cells where it was called Bright, for B cell regulator of immunoglobulin heavy chain transcription, and was shown to function in a complex with BTK and TFII-I to enhance immunoglobulin transcription in stimulated B cells (11–14). Observations from Bright dominant negative transgenic mice and $Bright^{-/-}$ mice suggested important roles for ARID3a/Bright in B lymphocyte development and function (15, 16). Mice deficient for ARID3a died between days 12 and 14 of gestation due to defects in erythropoiesis, and were severely depleted in hematopoietic stem progenitor cells (HSPCs) and hematopoietic stem cells (HSCs) (16). Expression of Bright/ARID3a in mice is tightly regulated during B cell differentiation such that transcription occurs in a subset of early HSPCs, and then is primarily limited to activated and innate-like B lineage cells (15–17). Expression in human B lymphocytes is also tightly regulated, such that the majority of naïve B cells in the periphery do not express it (18). However, nothing is known regarding expression of ARID3a in HSPCs in man.

Forced expression of ARID3a/Bright throughout all B lineage cells in mice resulted in the production of anti-nuclear antibodies and immunoglobulin deposition in renal glomeruli (19), common characteristics of patients with systemic lupus erythematosus (SLE). Systemic lupus erythematosus (SLE) is an autoimmune disease manifested by varying degrees of

disease severity (reviewed in 20). We found that 48% of 115 randomly selected SLE patients showed increased numbers of ARID3a$^+$ B cells compared to healthy controls, and that much like our transgenic mice, ARID3a expression occurred throughout all B cell stages in those patients (21). Furthermore, increased numbers of ARID3a$^+$ B cells in SLE, but not in rheumatoid arthritis patients, correlated with increased disease activity (21). These findings suggested to us that ARID3a expression in early HSPCs might also differ between healthy control and SLE samples.

Because ARID3a$^+$ B cells are associated with disease activity in SLE, and given the importance of ARID3a expression in hematopoietic progenitor development in mice, we sought to characterize ARID3a expression patterns in healthy control and SLE peripheral blood HSPCs and hematopoietic stem cells (HSCs) populations. Although human HSPCs are widely used as a source for hematopoietic transplants, they constitute a diverse set of progenitor cells and even the reconstituting HSC subsets may be heterogeneous (22). We hypothesized that progenitor populations might differ in ARID3a expression levels and that differential ARID3a expression might be associated with differences in developmental potential and/or functional attributes. Therefore, we assessed B lymphopoietic functions of HSPCs that differed in ARID3a expression levels both *in vitro*, and *in vivo*, utilizing a humanized mouse model. Our data suggest that numbers of ARID3a$^+$ cells are associated with maintenance of cells with a progenitor phenotype *in vitro*, and with increased autoantibody production *in vivo*.

MATERIALS AND METHODS

Cells

Peripheral blood mononuclear cells from a random cross section of 51 SLE patients (ranging in age from 21 to 72) and 19 healthy age and gender-matched controls were isolated from heparinized peripheral blood with Ficoll-Paque Plus (GE Healthcare). Lupus patients who met a minimum of four American College of Rheumatology Classification Criteria for SLE (23) were recruited after informed consent from the Oklahoma Medical Research Foundation (OMRF) Oklahoma Lupus Center (IRB compliance #09-07 and #06-19), in accordance with OMRF Institutional Review Board approval and with the Declaration of Helsinki.

Flow cytometry analyses

Mononuclear cell surface markers were stained with the following fluorochromes: human hematopoietic lineage markers (CD2, CD3, CD14, CD16, CD19, CD56, CD235a) APC (eBioscience), CD34 PE, CD38 Alexa Fluor 700, CD7 PE-Cy5, CD10 PE-Cy7, CD49f PerCP-Cy5.5, CD45RA Brilliant Violet 570, CD135-Biotin, CD127 (IL7Rα) PE-Cy7, SA-APC-Cy7 (BioLegend). Following surface marker staining, cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% Tween-20 and stained with goat anti-human ARID3a antibody (18), followed by rabbit anti-goat FITC (Invitrogen). Isotype controls (BD Biosciences, eBiosciences, BioLegend) were used for gating hematopoietic progenitor subsets as described (22, 24) . Doublet exclusion was used to ensure analyses of single cells prior to forward/side scatter gating. Data were collected using an LSRII (BD Biogenics) and

FACSDiva (BD Biosciences) software version 4.1, and were analyzed using FlowJo (Tree Star) software version 10.

Stromal-cell free cultures for B lineage cells

$CD34^+$ hematopoietic progenitors were isolated from mononuclear cells using the EasySep Human CD34 Positive Selection Kit (StemCell Technologies), and plated in triplicate at 10,000 cells per well as described (25, 26). Briefly, cells were cultured in QBSF®60 (Quality Biological, Inc.) supplemented with 10% FBS (Atlanta Biologicals), 100U Penicillin/Streptomycin (Gibco), 10% hMSC conditioned media (graciously provided by Dr. J. Gimble) and containing stem cell factor, 10ng/ml granulocyte stimulating factor, 5ng/ml FLT-3 ligand, and 5ng/ml IL-7 (R&D). Half of the media was replaced once a week for the duration. After 4 weeks of culture, cells were harvested, counted and assessed for the following markers by flow cytometry: CD34 PE, CD10 Pacific Blue, CD19 PE-Cy5 (all from BioLegend), and CD33 APC (BD Biosciences). For proliferation assays, cells were labeled with CFSE (10μM, Invitrogen) in PBS for 10 minutes, followed by 4X volume of RPMI supplemented with 20% FBS for 5 minutes on ice to quench CFSE uptake. Cells were washed once with RPMI containing 20% FBS, and twice with PBS supplemented with 20% FBS prior to *in vitro* culture. After 6 days, cells were harvested and assessed for CD34 and CFSE expression levels via flow cytometry.

Mice and HSC transplantation

All studies were approved by the Oklahoma Medical Research Foundation Institutional Animal Care and Use Committee. Adult (6–10 weeks) NOD-*scid IL2r*γ^{null} were obtained from Jackson Laboratory (Bar Harbor, ME). One week prior to conditioning, mice were administered antibiotic water, which was continued through the duration of the study. Mice were sublethally irradiated with 200 cGy 137Cs source (Mark I irradiator; J. L. Shepard and Associates). Twenty-four hours later, 50,000 $CD34^+$ PBMCs from individual patient samples were injected via tail vein. At 8 and 12 weeks post-transplant, approximately 200ul of peripheral blood was collected from the sub-mandibular superficial temporal vein into heparinized microcentrifuge tubes. PBMCs were isolated with Cell Separation Media (1-Step™ 1.077/265 Animal, Mononuclear Cells, Accurate Chemical and Scientific Corporation) and analyzed for expression of human CD45 by flow cytometry to assess levels of human engraftment. Spleen and bone marrow were assessed at 16 or 22 weeks for presence of human B cells and B lineage subsets using the following stains: hCD45 PE Texas Red, CD10 PE (Caltag), CD19 PE-Cy5, CD43 APC, CD20 APC-Cy7, CD38 Alexafluor 700, CD34 PE, CD10 Brilliant Violet 421 (BioLegend), CD27 PE-Cy7, IgM V450, IgD PerCP-Cy5.5, and CD33 APC (BD Bioscience). Healthy control PBMCs and wild type mouse spleen cells were used for positive and negative control stains, respectively.

Anti-nuclear Ab Detection

Serum from recipient mice were diluted 1:4 or 1:8 in PBS (27) and applied to fixed and permeabilized HEp-2 anti-nuclear Ab (ANA) slides using the NOVA Lite HEp-2 ANA kit (INOVA Diagnostics) to test for the presence of human Ig as previously described (28). Antibodies to nuclear antigens were detected using a secondary FITC-conjugated anti-

human Ig antibody (Jackson Immunoresearch Laboratories) and visualized using a Zeiss Axioplan 2i inverted microscope with an AxioCam HRm camera (Carl Zeiss). Positive and negative controls provided with the NOVA Lite kit were used.

Statistics

Data were statistically evaluated using the non-parametric Mann Whitney U test to compare distribution of variables between pairs of groups. The non-parametric ANOVA (Kruskal-Wallis) followed by Dunn post hoc tests were used for 3 group comparisons. Fisher's exact test was used to compare frequency within groups. Statistical analysis was performed with Prism (Graphpad) software version 6.03. P values of less than 0.05 were considered significant.

RESULTS

Because nothing was known about normal levels of ARID3a expression in peripheral blood progenitor populations, and nearly half of SLE patients had expanded numbers of ARID3a$^+$ B cells in our previous study (21), we hypothesized many patients would also have increased numbers of circulating ARID3a$^+$ CD34$^+$ blood cells. Therefore, we first determined ARID3a expression levels in the heterogeneous HSPC population in peripheral blood of 19 healthy control and 51 SLE patient samples utilizing CD34$^+$ selection followed by flow cytometry. We found that both numbers of cells expressing ARID3a, and intensities of ARID3a expression were increased above that of healthy controls in some SLE patient samples. Patient samples with total numbers of ARID3a$^+$ cells > 2 standard deviations above the average number of ARID3a$^+$ cells present in healthy controls (irrespective of the MFI) were designated as ARID3a^H (high) (Figure 1A, open circles), while the remaining SLE samples are defined as ARID3a^L (low) (Figure 1A, closed circles). The mean fluorescent intensity (MFI) of ARID3a in ARID3a^H patient samples was 666 (range of 232–1813), versus 194 (range 68–304) for healthy controls. ARID3a^L samples showed similar levels of the ARID3a protein as controls, with a mean MFI of 139 (range 52–260). Forty seven percent of SLE patient samples (24 of 51) showed increased numbers of ARID3a$^+$ HSPCs that were more than 2 standard deviations above the mean of the healthy controls (Fig. 1A). Two healthy control samples also fell in the ARID3a^H category by this definition, albeit only marginally. However, irrespective of the numbers of ARID3a$^+$ HSPCs, average total numbers of HSPCs did not differ between the SLE and control groups (Fig. 1B). These data indicate that numbers of ARID3a$^+$ HSPCs in SLE samples can be as much as 40-fold higher than in healthy controls.

Using surface marker expression defined by Notta et. al. (24), we next analyzed ARID3a expression levels in the self-renewing HSC population (lin$^-$CD34$^+$CD38$^-$CD45RA$^-$CD49f$^+$) important for long term survival of HSPC transplant grafts. Enumeration of CD34$^+$ HSCs via flow cytometry from 19 healthy control and 51 SLE patient samples showed increased numbers of those cells in patient samples compared to the healthy controls (Fig. 2A–B). Consistent with the known heterogeneity of HSCs, the average numbers of cells that expressed intracellular ARID3a in control samples was less than 10% of the average numbers of total HSCs (Fig. 2C). However, numbers of HSCs

expressing ARID3a in SLE samples were greatly increased in some cases, and histograms of individual patient samples revealed variable levels of ARID3a expression compared to representative healthy controls (Fig. 2C–D). Indeed, the average number of ARID3a^{+} HSCs in SLE patients was a log fold higher, and in some cases was >75-fold higher, than the average number of ARID3a^{+} HSCs in healthy controls. However, absolute numbers of HSCs in ARID3a^{H} and ARID3a^{L} samples were equivalent (Fig. 2E). These data indicate that numbers of ARID3a^{+} HSCs in SLE samples can be substantially higher than in healthy controls.

Because numbers of ARID3a^{+} cells were increased in circulating SLE B lymphocytes (21), we queried whether the ARID3a^{H} HSPC samples preferentially generated B lymphocytes *in vitro* compared to hematopoietic progenitors from ARID3a^{L} samples. Therefore, we determined the ability of HSPCs from ARID3a^{H}, ARID3a^{L} and control samples to develop into B lineage cells over a four week period using feeder free liquid cultures (25, 26). At the end of the culture period, increases in cell numbers were determined in 10 healthy control, 13 ARID3a^{L}, and 10 ARID3a^{H} hematopoietic progenitor samples. While healthy control and ARID3a^{H} samples showed equivalent expansion of cell numbers, ARID3a^{L} samples were, on average, 4-fold less (Fig. 3A). Microscopic examination of the cultures also showed discrete differences in the three culture samples. Healthy control samples showed typical colony formation with few single cells, while the ARID3a^{H} cultures had increased numbers of smaller colonies and increased numbers of single cells (Fig. 3B). ARID3a^{L} cultures showed both smaller colonies and fewer single cells than the other two cultures (Fig. 3B). We hypothesized that ARID3a^{L} cultures might eventually resemble the ARID3a^{H} culture numbers if given more time. However, after eight weeks in culture, the ARID3a^{L} cells numbers had not increased substantially over those observed at 4 weeks (not shown). Together, these data suggest that hematopoietic properties of ARID3a-enriched SLE progenitors differ from both healthy controls and from SLE samples with lower numbers of ARID3a^{+} cells.

To further explore the reasons for decreased numbers of ARID3a^{L} SLE HSPCs compared to both ARID3a^{H} SLE and healthy control HSPCs, we assessed proliferation of the cultures using the vital dye, CFSE, for the first six days of culture. While we observed no differences in viability among the three cultures at any time point, CFSE analyses indicated that ARID3a^{L} CD34^{+} SLE cells proliferated more slowly (an average of 1.2 cell divisions), compared to either ARID3a^{H} SLE or healthy control CD34^{+} cells, which averaged 3.4 and 3.2 cell divisions respectively, during the same time period (Fig. 4A). Because the cytokine cocktail used contained the stimulatory cytokine IL7, we further analyzed the HSPCs for relative expression levels of the IL7 receptor, IL7Rα . ARID3a^{L} SLE HSPCs showed decreased expression, compared to both ARID3a^{H} SLE and healthy control HSPCs (Fig 4B), suggesting that low IL7Rα expression might contribute to the reduced proliferation of ARID3a^{L} SLE HSPCs *in vitro*.

All three types of cultures were further evaluated for phenotypic characteristics by flow cytometry (Fig. 5A). While both healthy control samples and ARID3a^{L} SLE samples gave rise to equivalent percentages of CD34^{+} cells, the average percentage of cells retaining CD34 expression in ARID3a^{H} cultures were increased >5-fold over average percentages of

$CD34^+$ cells in control or $ARID3a^L$ cultures (Fig. 5B). Conversely, percentages of the $CD33^+$ monocyte lineage cells in $ARID3a^H$ cultures were less than half of those observed in the healthy control and $ARID3a^L$ SLE cultures (Fig. 5B). B lineage cells developed similarly in all culture sets, although total numbers of B lineage progenitors present in individual samples at four weeks varied (Fig. 5C). Notably, total numbers of $CD34^+$ cells and all B cell subsets were slightly increased in the $ARID3a^H$ cultures compared to the other culture types.

Typically, pre-pro-B cells have been characterized as $CD34^+$ $CD10^+$ $CD19^-$ cells, but Sanz et al. defined an alternative type of $CD19^+$ pre-pro-B cell that lacks surface CD10 (29). Because $ARID3a^H$ cultures had increased numbers of $CD34^+$ cells, we asked if some of those cells might also express CD19 without CD10. Although percentages were highly variable in individual cultures from $ARID3a^H$ samples (Fig. 6A), total numbers of "alternative pre-pro-B" cells were increased nearly 10-fold in these cultures over control cultures, and nearly 100-fold over average numbers in $ARID3a^L$ cultures (Fig. 6B). Total numbers of conventional pre-pro-B cells were also increased in $ARID3a^H$ cultures compared to the other cultures, but percentages of these cells were not significantly different among the three cultures (Fig. 6). These data suggest that $ARID3a^H$ SLE samples give rise to increased numbers of B lineage cells that retain CD34 with CD19 compared to healthy control samples, or SLE samples with lower ARID3a expression.

$CD34^+$ HSPCs, which are typically used for transplantation therapy, contain other types of hematopoietic progenitors in addition to HSCs, including multipotent progenitors (MPPs), multi-lymphoid progenitors (MLPs) and multi-myeloid progenitors (MMPs). Because percentages of $ARID3a^+$ cells were increased in HSPCs, HSCs (Figs. 1B, 2C) and mature B lineage cells in peripheral blood of SLE patients (21), we asked if other progenitor populations might also have increased numbers of $ARID3a^+$ cells in SLE samples. We reasoned that MPPs and MLPs, both precursors of B lineage cells, might show increased numbers of $ARID3a^+$ cells compared to MMPs, which give rise to myeloid lineage cells that were not increased in our $ARID3a^H$ *in vitro* cultures relative to $ARID3a^L$or control cultures (Fig. 5B). Using flow cytometry to distinguish MPP ($lin^-CD34^+CD38^-CD45RA^-CD49f^-$), MLP ($lin^-CD34^+CD38^-CD7^-CD10^+$) and MMP ($lin^-CD34^+CD38^+CD7^-CD135^+$) populations as shown in Fig. 7A, we observed no significant differences among total numbers of MPPs in any of the samples (Fig. 7B). Total numbers of MLPs were slightly increased on average in $ARID3a^H$ samples compared to $ARID3a^L$ samples, and total numbers of MMPs were significantly increased in $ARID3a^H$ samples versus those in healthy controls (Fig. 7B). As we predicted, numbers of $ARID3a^+$ cells in $ARID3a^H$ MPP and MLP subsets were significantly higher than those observed in either healthy control of $ARID3a^L$ samples (Fig. 7C). However, numbers of $ARID3a^+$ cells were also increased in the MMP subset in $ARID3a^H$ samples compared to the other samples (Fig. 7C). We therefore examined ARID3a expression in the slightly more mature MMP subset, which includes common lymphoid progenitors (CMP), granulocyte-monocyte progenitors (GMP) and megakaryocyte-erythrocyte progenitors (MEP) subsets, and found that ARID3a expression was also increased in MMPs in $ARID3a^H$ samples compared to both $ARID3a^L$ and healthy

control samples (Fig. 7C). Therefore, SLE patients with increased numbers of ARID3a$^+$ HSPCs show increased numbers of ARID3a$^+$ cells in multiple progenitor subsets.

To assess functional attributes *in vivo*, we asked if CD34$^+$ HSPCs from ARID3a^H versus ARID3a^L SLE patient samples differed in their engraftment potential. We took advantage of the humanized NOD-*scid IL2rγ*^{null} mouse model system described by Notta et al. (24), because these mice were reported to generate multilineage human hematopoietic cells, including antibody producing B cells (30–33). CD34$^+$ HSPCs from 6 ARID3a^H and 6 ARID3a^L samples were injected into NSG mice and analyzed for engraftment potential according to the diagram shown in Fig. 8A. One mouse in the ARID3a^L group failed to recover properly from irradiation and died within a week following HSPC transfer. All other mice showed human engraftment as assessed by the presence of the human marker CD45 on blood cells (Fig. 8B). Mice analyzed at 22 weeks post-transplant (solid symbols) and those sacrificed at 16 weeks (open symbols) showed equivalent engraftment in bone marrow and spleen (Fig. 8C). In addition, we observed no differences in development of B lineage B cells of any subset between ARID3a^H and ARID3a^L recipients in either the bone marrow or spleen (Fig. 7D, E). ARID3a expression within these small subsets was not analyzed because it is not a lineage stable attribute *in vivo* (21). Therefore, numbers of ARID3a$^+$ progenitors did not affect the engraftment potential of HSPCs in this humanized mouse model.

ARID3a expression in mouse B cells was associated with autoantibody production (19). Therefore, we examined sera from each of the recipient mice for the presence of antinuclear antibodies (ANAs). We found that each of the ARID3a^H progenitor recipients (n=6) showed positive HEp-2 ANA staining, while only 1 of the ARID3a^L progenitor recipients (n=5) showed positive staining (Fig 9 and Table I). Further, Fisher's Exact analysis showed these data are statistically significant (p=0.015). Although the sample number is small, these data suggest that SLE HSPCs with increased numbers of ARID3a$^+$ progenitors may preferentially give rise to autoantibody producing B cells in humanized mice, compared to SLE HSPCs with lower numbers of ARID3a$^+$ progenitors.

DISCUSSION

Although ARID3a is critical for normal hematopoiesis in mice (16), nothing was known regarding its expression, or function, in human hematopoietic progenitors. The data presented here demonstrate that, as was observed in the mouse (16), ARID3a expression is only apparent in a subset of hematopoietic stem cells in peripheral blood obtained from healthy controls. ARID3a expression was not limited to hematopoietic progenitors, but was also apparent in varying fractions of cells, generally <10% per population, within each of the other hematopoietic progenitor subsets we examined. Similar to our observations in SLE B lineage cells (21), numbers of ARID3a$^+$ cells within hematopoietic progenitors in SLE patients ranged from normal numbers to nearly 100 times normal in some patient samples. Functional studies comparing SLE samples that varied in numbers of ARID3a$^+$ HSPCs indicated that SLE samples with higher numbers of ARID3a expressing progenitors exhibit retention of CD34 in B lineage cells *in vitro* and may be prone to generation of autoantibody producing cells *in vivo*. Together, these data suggest that levels of ARID3a expression in

human hematopoietic progenitors contribute to their developmental potential. Our data may also have particular impact on autologous transplants in patients with SLE.

Although others reported that SLE patients had decreased numbers of progenitor cells in their peripheral blood (5, 6), in our cohort, we observed increased numbers of peripheral blood hematopoietic progenitor cells in many patients as compared to healthy controls (Figs. 1B, 2B). This disparity could be a reflection of differences in selection criteria for participants in those studies. In both of those studies, patients had low disease activity, were of European descent and were on minimal immunomodulatory medications. Our study included patients from multiple ethnic backgrounds and did not limit participants based on disease severity (SLEDAI scores ranged from 0 to 15) or moderate immunomodulatory medications. Further, in mouse models of SLE, HSC expansion as well as increased HSPC mobilization into the periphery was reported (7, 34–36). Strikingly, many of the medications that are used at low doses to decrease inflammation are also implicated in bone marrow mobilization at higher doses (37,38). We hypothesize that the increased numbers of progenitors in our patients is due to new waves of hematopoiesis resulting from inflammatory responses and/or immunomodulatory drug usage. Regardless of their origin, the group of patient samples with high ARID3a expression had similar numbers of HSCs as those with normal levels (Fig. 2E). These data suggest that SLE patient peripheral blood samples provide a rich source of hematopoietic progenitors for analysis.

We observed abnormal colony formation *in vitro* in all SLE samples, compared to healthy controls, consistent with previous studies that reported abnormal colony development and defects in the capacity of SLE HSPCs to expand *in vitro* (5, 6). HSPCs in a mouse SLE model showed skewed hematopoiesis with a bias for the myeloid lineage and increased self-renewal capacity (7). Although our data showed total numbers of cells were similar in control and ARID3a^{H} cultures, ARID3a^{H} cultures contained smaller colonies (Fig. 3B). Many ARID3a^{H} cells retained the progenitor marker CD34 after four weeks in culture (Fig. 5A), and some of those cells also expressed the B lineage marker, CD19 (Fig. 6). ARID3a^{L} SLE HSPCs showed impaired proliferation *in vitro* relative to both ARID3a^{H} and control HSPCs (Fig. 4A). We hypothesize this may be due to lower expression levels of the surface receptor for IL-7 at the initiation of culture (Fig. 4B). ARID3a CHiP-seq analyses of K562 cells available from the ENCODE group indicate a potential binding site in the promoter of the IL7R, implicating ARID3a as a potential regulator of that signaling pathway. Together, our data suggest that CD34^{+} HSCs from SLE patients can be heterogeneous in their functional attributes *in vitro*.

An alternative pre-pro B cell population defined by Sanz et al. in cord blood progenitors was hypothesized to represent the first wave of B lineage development (29), similar to progenitors of the mouse B1 B cell lineage that arise from CD19^{+} progenitors rather than B220^{+} cells (39). These alternative pre-pro B cells expressed immunoglobulin repertoire characteristics suggesting that they could be precursors of cells destined to secrete polyreactive antibodies (29). This is intriguing as mouse B-1a cells preferentially generate autoreactive antibodies (reviewed in 40). Autoantibody generating cells are characteristically present in SLE patients and it is interesting to suppose that expansions of progenitors of autoreactive B cells might also occur in these patients. We have no direct

evidence that the cells that co-express CD34 with CD19 in our ARID3a^{H} cultures (Fig. 6) are alternative pre-pro B cells associated with autoreactive immunoglobulin repertoires. However, over-expression of ARID3a in B lineage cells in transgenic mice resulted in autoreactive antibody production (19, 28), and the humanized mouse model data presented herein also suggest that autoantibodies may be preferentially generated in mice reconstituted with ARID3a^{H} versus ARID3a^{L} cells (Table 1). Using a gating strategy characterized by Griffin et al. (41, 42), we identified small numbers of putative human B1-like B cells in the spleens of our chimeric mice. However, numbers of these cells did not differ between ARID3a^{H} and ARID3a^{L} recipients. Thus, the reason for differences in autoreactivity between the ARID3a^{H} and ARID3a^{L} samples remains unknown.

We previously showed that ARID3a enhances transcription of a subset of IgH promoters after B cell activation (11–14, 43, 44), and that it can act as a repressor of pluripotency genes, including Oct4, in somatic cells (45, 46). However, the gene targets and mechanisms by which ARID3a functions in HSPCs are unknown. Others reported that forced expression of ARID3a in somatic cells reduced negative regulators of the cell cycle by affecting the activity of p53 and p16 (INK4a) (47, 48). Therefore, some of the effects we observed in the ARID3a^{H} versus ARID3a^{L} lupus samples could be directly related to increased expansion of CD34^{+} cells in those *in vitro* cultures (Fig. 5A). The potential for ARID3a to function similarly in more differentiated progenitors could also account for increased total numbers and ARID3a^{+} cell numbers in MPP, MLP and MMP populations (Figs. 7B–C). Furthermore, several studies revealed that hematopoietic progenitors from both mice and humans have the capacity to respond to innate signals through toll-like receptors (reviewed in 49). One such study in mice showed that chronic stimulation of TLR4 with LPS led to altered hematopoiesis (50). Our unpublished data suggest that increases in ARID3a expression in B lymphocytes can be induced by innate signaling. We hypothesize that chronic inflammatory responses, and stimulation of innate pathways, may contribute to both ARID3a expression and functional abnormalities observed in SLE HSPCs. Experiments to address these hypotheses are ongoing.

Autologous hematopoietic stem cell transplants have been used as a treatment for severe refractory SLE for approximately 15 years, with varying success. Reported survival rates following transplant, range from 62%–84%, with relapse rates ranging from 32–66% (51–53). In most cases, transplantation uses total lineage negative CD34^{+} cells as a source of progenitors (reviewed in 54). Our data suggest that those progenitors may differ functionally based on numbers of cells that express ARID3a. Our data did not indicate that numbers of ARID3a^{+} cells affected engraftment in humanized mice, but ARID3a expression was not limited to hematopoietic stem cells in our studies (Fig. 7). Previously, we showed the importance of ARID3a expression for the development of erythrocytes in ARID3a deficient mice (16), and other recent reports suggest roles for ARID3a in some activated T cell subsets (55). In addition, ARID3a transcripts have been reported in mouse neutrophils (56), a hematopoietic subset associated with disease activity and IFNα expression in SLE patients (57–59). However, whether ARID3a is produced in human neutrophils or what its function might be in those cells is unclear. Intriguingly, we observed expression of ARID3a in the myeloid progenitors that give rise to neutrophils (Fig. 7C). Together, our data underscore the

potential importance of ARID3a in normal and SLE immune function. Additional experiments to better define the roles of ARID3a in SLE and normal hematopoiesis in humans will be important.

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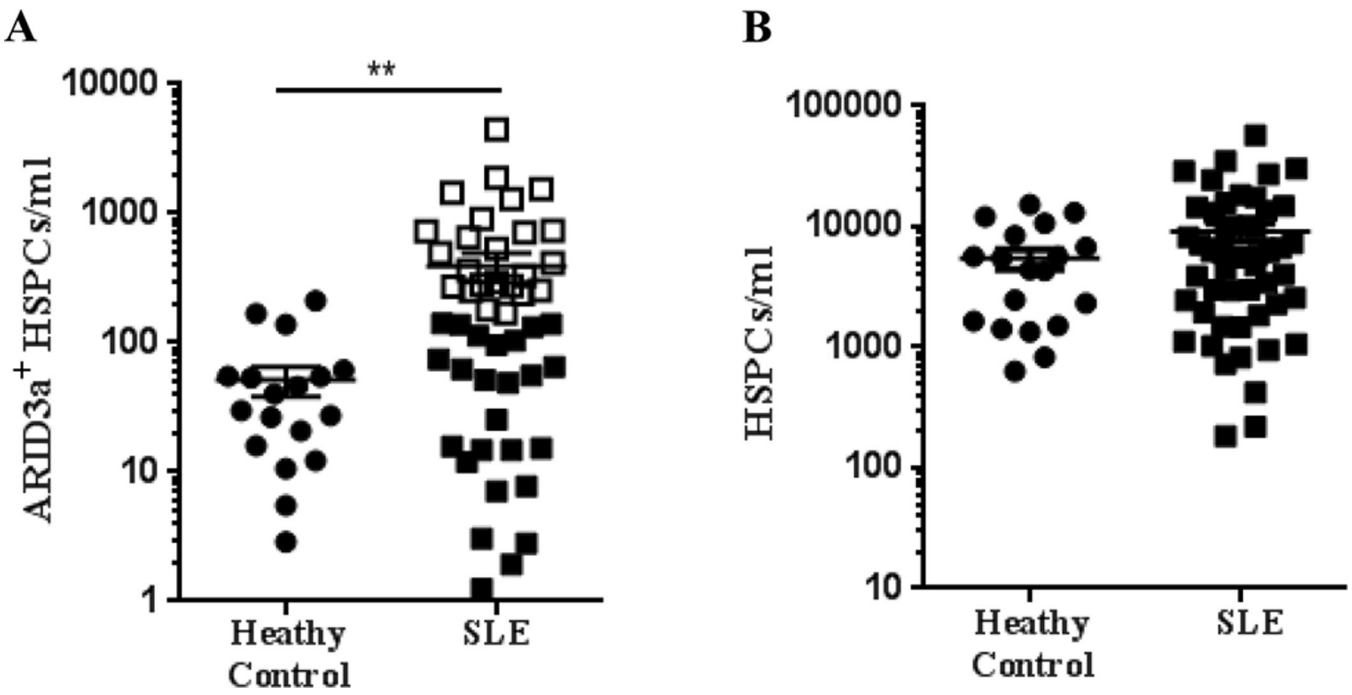

Figure 1. ARID3a expression is increased in HSPCs from SLE patients versus healthy controls

PBMCs from 19 healthy control and 51 SLE patient samples were assessed for numbers of $CD34^+$ HSPCs and ARID3a expression by flow cytometry. A. Numbers of HSPCs in individual healthy controls and SLE patient samples are shown. Averages and standard error bars are indicated. Open squares indicate those samples with numbers of $ARID3a^+$ HSPCs >2 SD above the mean of the healthy control (designated $ARID3a^H$). SLE samples with fewer $ARID3a^+$ cells (closed squares) are designated $ARID3a^L$ B. Numbers of $ARID3a^+$ cells in healthy control and SLE HSPCs are indicated. Statistical significance was determined by Mann Whitney U test: **, p=0.0043.

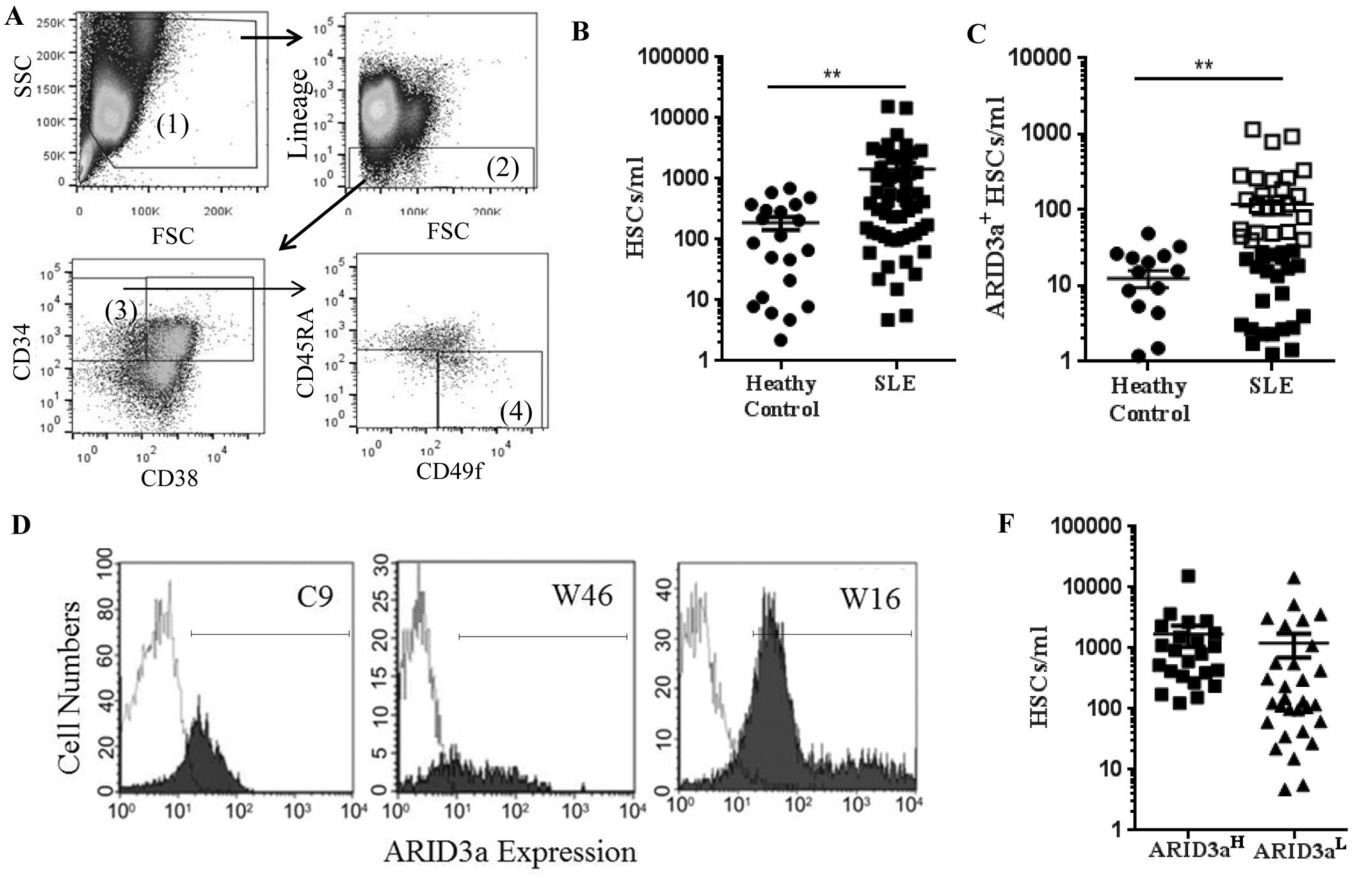

Figure 2. ARID3a expression is increased in HSCs from many SLE patients

PBMCs from 19 healthy control and 51 SLE patient samples were assessed for numbers of HSCs and ARID3a expression by flow cytometry. A. Representative data indicate the gating strategy used for analysis of HSCs. Numbered populations indicated are: (1) live cells, (2) lineage negative: $CD2^-$ $CD3^-$ $CD14^-$ $CD16^-$ $CD19^-$ $CD56^-$ $CD235a^-$, (3) $CD34^+CD38^-$ parent cells, (4) HSCs: $CD34^+CD38^-CD45RA^-CD49f^+$. HSCs typically represented 9% of the parent population. B. Numbers of HSCs in individual healthy controls and SLE patient samples are shown. Averages and standard error bars are indicated. C. Numbers of $ARID3a^+$ cells in healthy control and SLE HSCs are indicated. Open squares indicate those samples with numbers of $ARID3a^+$ HSPCs >2 SD above the mean of the healthy control (designated $ARID3a^H$). SLE samples with fewer $ARID3a^+$ cells (closed squares) are designated $ARID3a^L$. D. Representative histograms show increased ARID3a expression (shaded area) in HSCs from two SLE patient samples (W46 and W16) versus a representative healthy control (C9). Gating for ARID3a+ cells, indicated by bars, was set according to isotype controls (open peaks). E. Total HSCs in $ARID3a^H$ SLE versus $ARID3a^L$ SLE samples are shown. Statistical significance was determined by Mann Whitney U test: **, $p<0.004$.

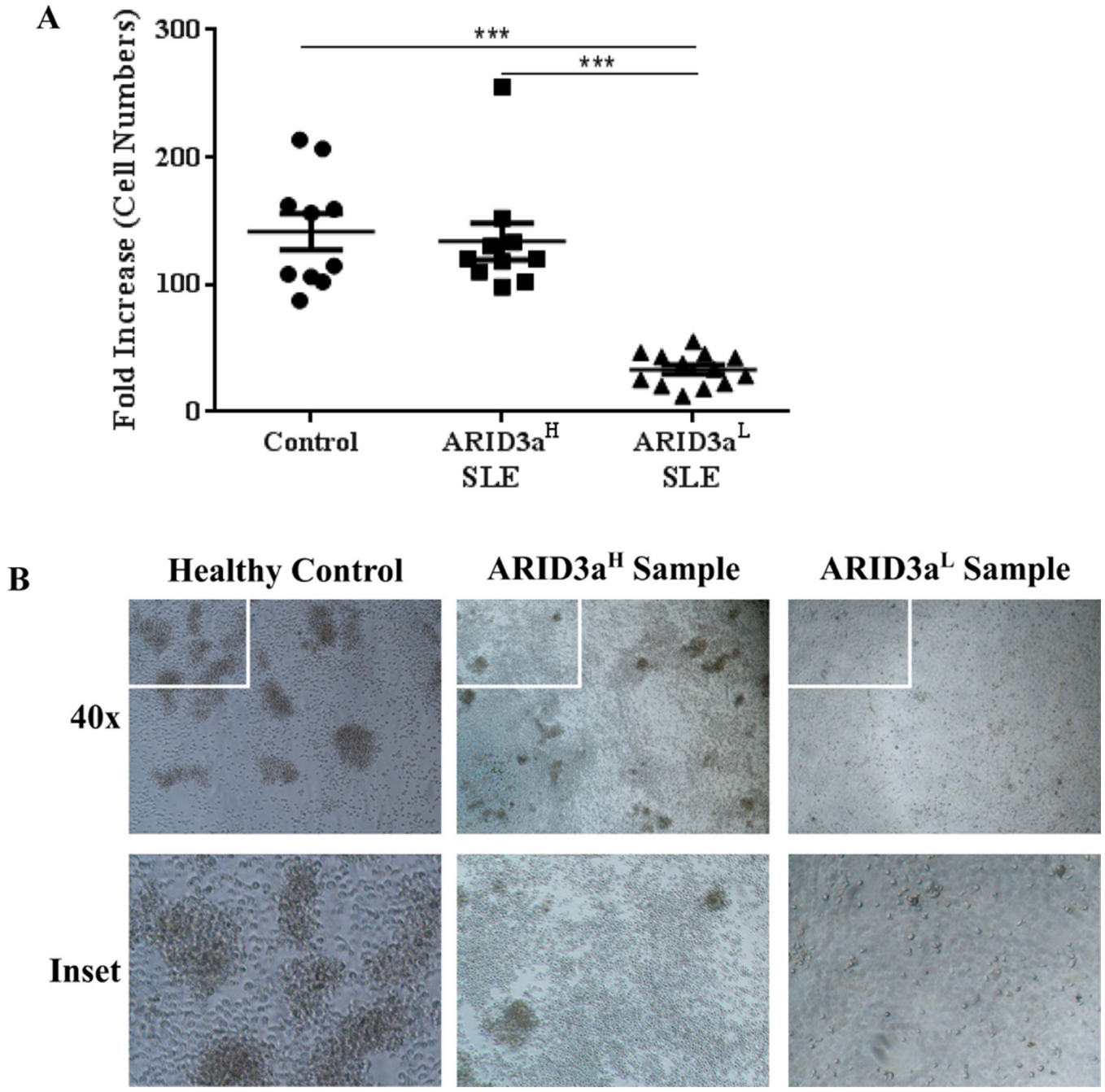

Figure 3. Hematopoietic progenitors from SLE samples differ functionally from healthy control progenitors *in vitro*

B lymphocyte cultures were initiated in triplicate with 10,000 $CD34^+$ lineage negative HSCPs each from 10 healthy control, 10 $ARID3a^H$ and 13 $ARID3a^L$ SLE patient samples and media supplemented with conditioned feeder media, G-CSF, IL-7, SCF, and FLT3. A. Total cell numbers were counted after 4 weeks of *in vitro* culture and are expressed as a fold-increase over the initial number cultured. Means and standard error bars are shown. $p<0.0001$ for three group comparison (Kruskal-Wallis) with significance on $ARID3a^L$ compared with control and $ARID3a^H$ ($p=0.001$ and $p=0.002$, respectively on Dunn multiple

comparison test) B. Representative light microscopic images of 4-week old cultures of each type are shown at two magnifications.

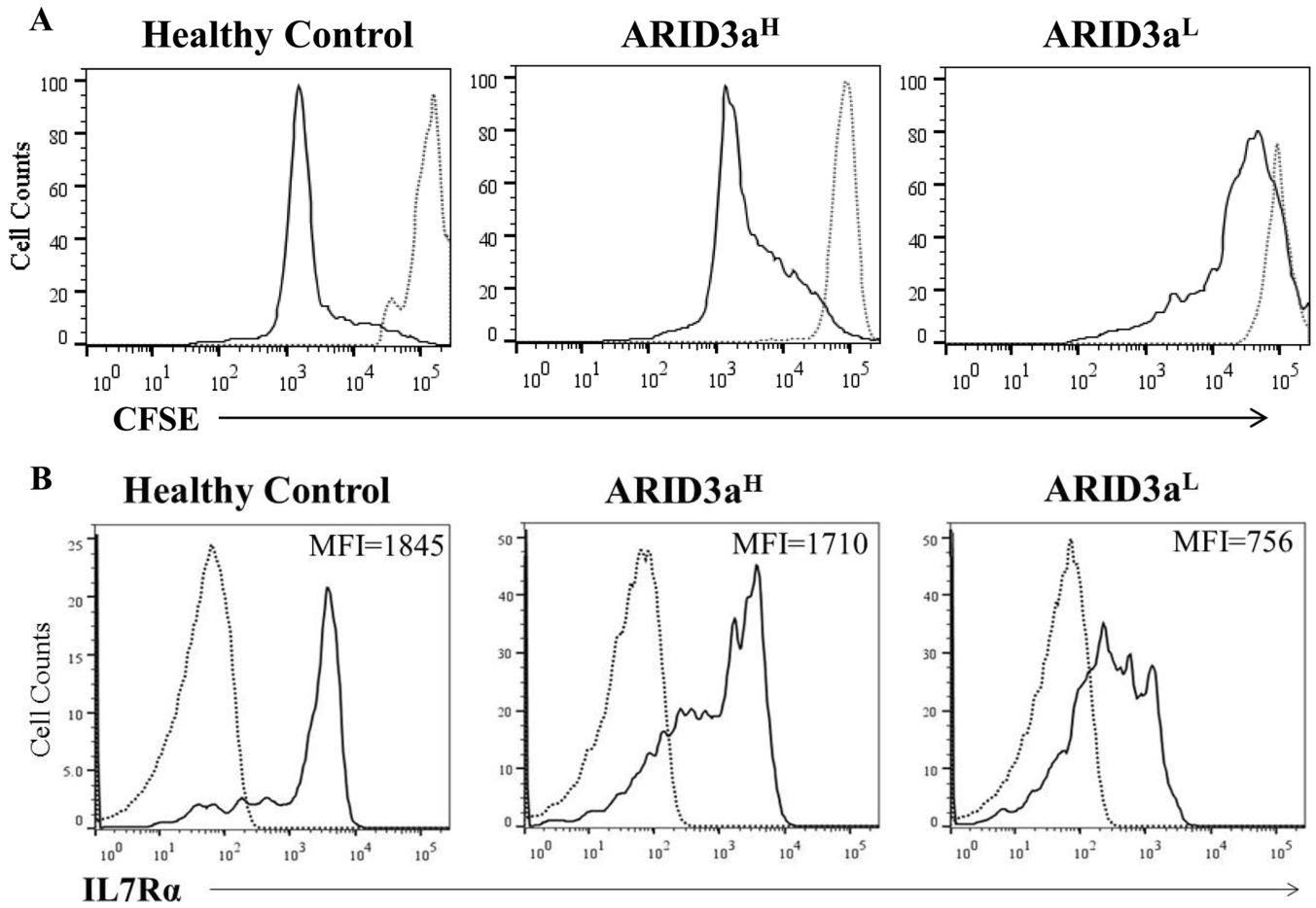

Figure 4. ARID3a^{L} SLE HSPC cultures show reduced proliferation and IL7R expression compared to healthy control and ARID3a^{H} SLE HSPC cultures

HSPCs from healthy control, ARID3a^{H} SLE and ARID3a^{L} SLE samples were analyzed for proliferative capacity and IL7Rα expression levels via flow cytometry. A. Representative histograms of proliferative responses of 10,000 CD34^{+} HSPCs loaded with CFSE and grown for six days are shown. B. Representative histograms of IL7Rα expression at the initiation of culture are shown. Data are representative of three each of healthy control, ARID3a^{H} SLE, and ARID3a^{L} SLE samples.

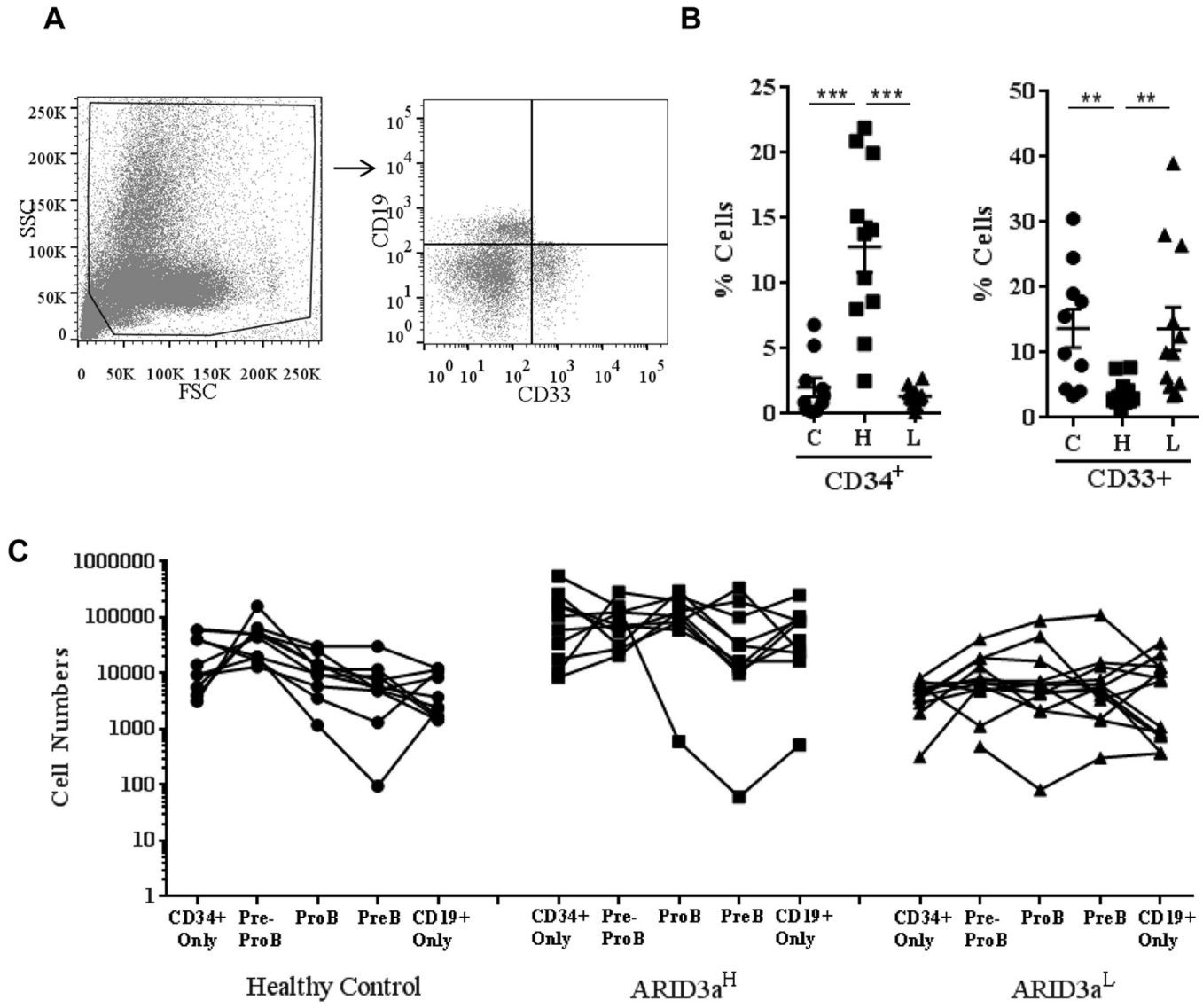

Figure 5. ARID3a^{H} cultures differ phenotypically from healthy control or ARID3a^{L} cultures

In vitro cultures of healthy control, ARID3a^{H} and ARID3a^{L} HSPCs were analyzed for B lineage and myeloid surface markers by flow cytometry after 4 weeks of culture. A. Representative gating for B lineage and monocyte lineage cells of a healthy control culture. B. Percentages of cells retaining surface CD34 (CD34^{+} CD33^{-} CD19^{-} CD10^{-}) and CD33^{+} (CD33^{+} CD19^{-} CD10^{-}) myeloid lineage cells in healthy control (C), ARID3a^{H} (H), and ARID3a^{L} (L) cultures are shown. Means and error bars are shown. C. Individual cultures of each type were assessed for CD34 alone and B lineage subset markers defined as pre-pro B cells (CD34^{+} CD10^{+} CD19^{-} CD33^{-}), pro B cells (CD34^{+} CD33^{-} CD10^{+} CD19^{+}), pre-B cells (CD34^{-} CD33^{-} CD10^{+} CD19^{+}), and CD19^{+} B cells (CD34^{-} CD33^{-} CD10^{-} CD19^{+}). Each point represents one sample. Lines connect B lineage cells at individual developmental stages within a single sample. $p<0.0001$ and $p=0.0014$ for three-group analysis (Kruskal-Wallis) of CD34^{+} and CD33^{+} populations respectively with significance on H compared

with C and L (***p=0.0007 and 0.0002 respectively for $CD34^+$ and **p=0.0056 and0.0045 respectively using Dunn multiple comparison test).

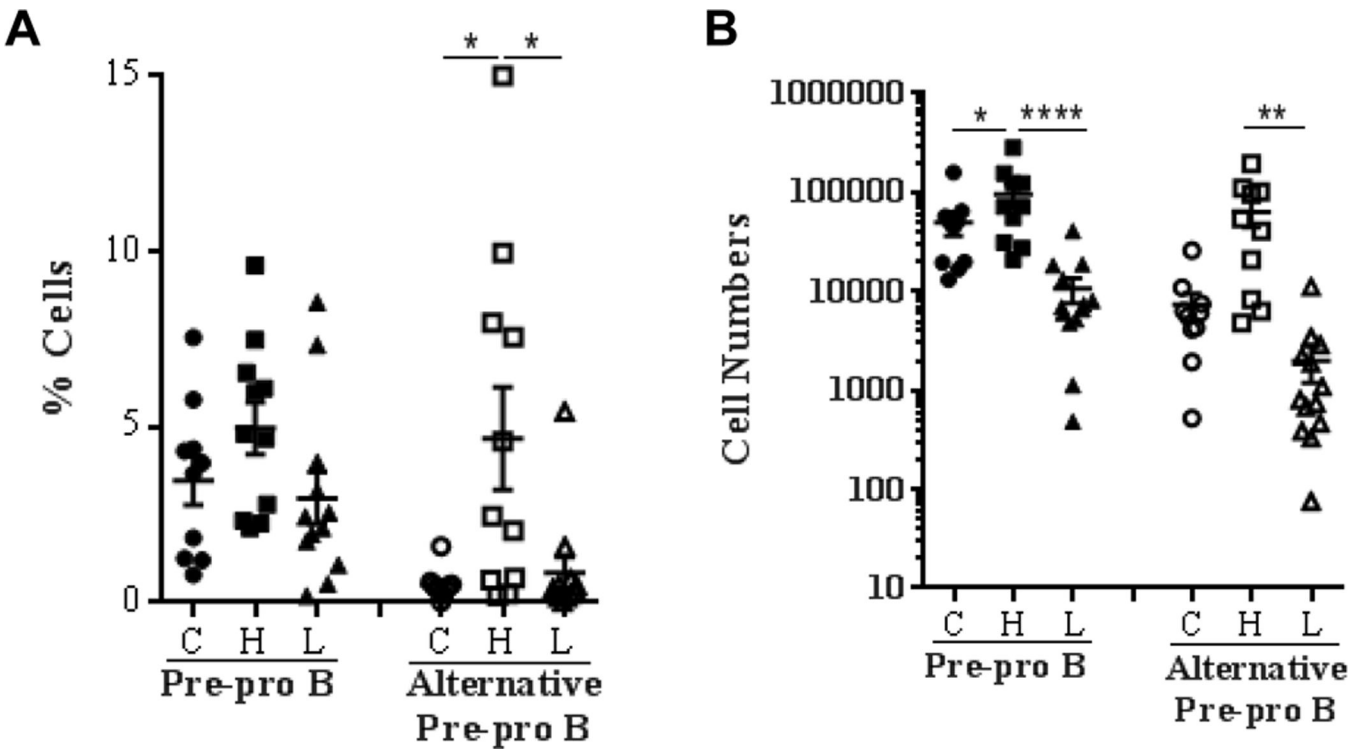

Figure 6. ARID3a^{H} B lineage cells can retain CD34 expression

Early B lineage cells from *in vitro* cultures of healthy control (C), ARID3a^{H} (H) and ARID3a^{L} (L) HSPCs were analyzed for alternative populations expressing CD34. A. Percentages of pre-pro B (CD34^{+} CD33^{-} CD10^{+} CD19^{-}) and alternative pre-pro B (CD34^{+} CD33^{-} CD10^{-} CD19^{+}) cells in each culture were assessed. B. Total numbers of alternative and conventional pre-pro B cells in each culture are shown. Means and standard error bars are shown. $p<0.0001$ for three-group analysis (Kruskal-Wallis) of all populations with significance by Dunn multiple comparison test on H compared with C and L in percentage of alternative pre-pro B cells (*p=0.0214 and 0.016 respectively) and pre-pro B cell numbers (*p=0.04 and ****p<0.0001 respectively), as well as on H compared with L for alternative pre-pro B cell numbers (**p-0.0036).

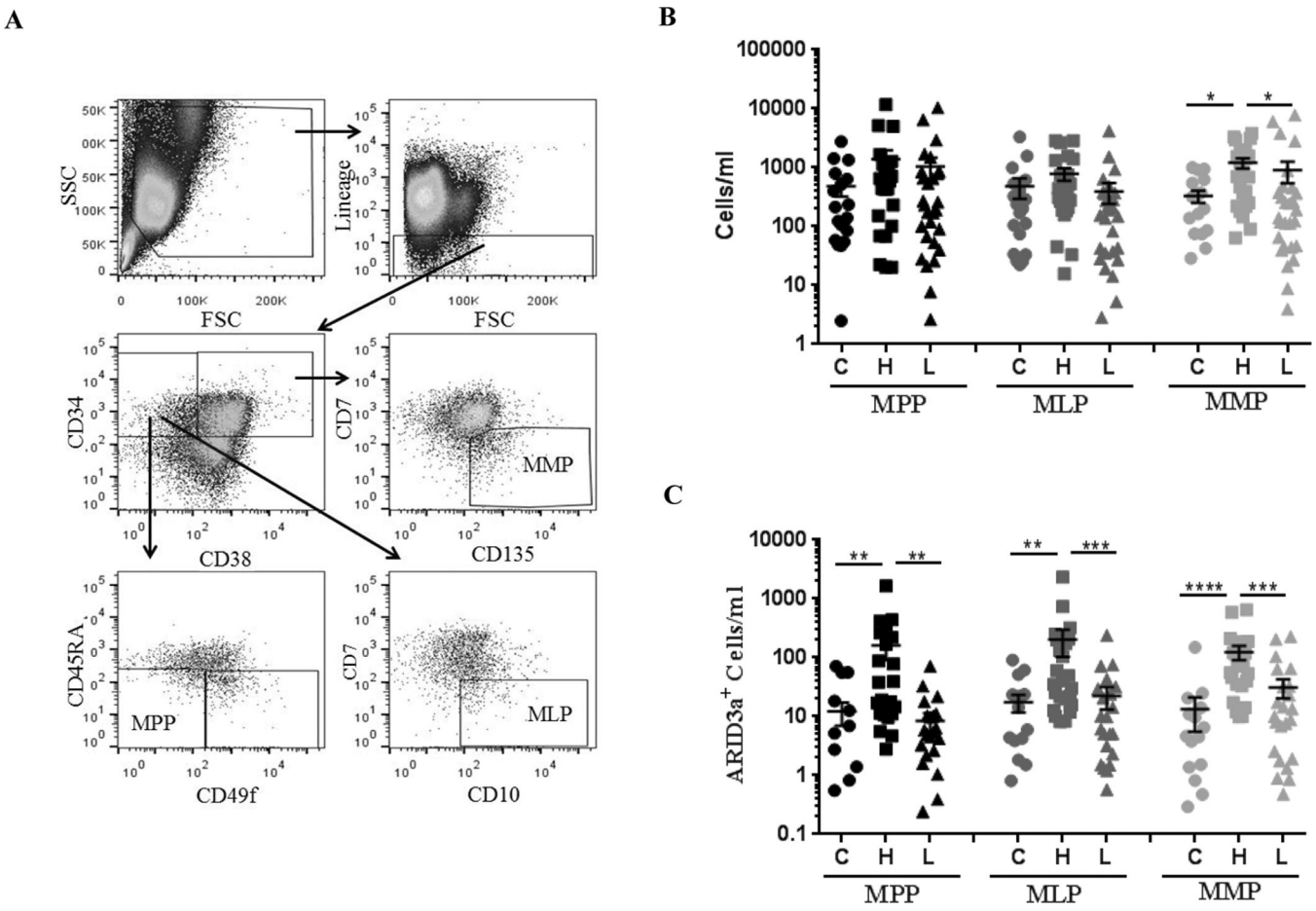

Figure 7. ARID3a is variably expressed within multiple subsets of hematopoietic precursors

Healthy control and SLE patient PBMCs were stained for hematopoietic progenitor markers and analyzed by flow cytometry. A. Representative data shows the gating strategy used to identify multi-potent progenitors (MPP), multi-lymphoid progenitors (MLP), and multi-myeloid progenitors (MMP). Typical percentages of each of these subsets were 15, 4 and 7, respectively. B. Total numbers of progenitor subsets are indicated for 19 healthy controls (C) and for SLE patient samples divided into ARID3a^{H} (H) (n= 24) and ARID3a^{L} (L) (n=27) groups based on ARID3a expression in HSCs in Figure 1 are shown. C. Numbers of ARID3a-expressing cells within each progenitor subset are indicated. Means and standard error bars are given. p=0.0066, p=0.0001, p=0.0001, and p<0.0001 for three-group analysis (Kruskal-Wallis) of cell numbers MPP and of ARID3a^{+} cell numbers of MPP, MLP, and MMP respectively with significance using Dunn multiple comparison on H compared with C and L for numbers of MPP (*p=0.0495 and *p=0.0241 respectively) and ARID3a^{+} cell numbers of MPP (***p=0.0006 and **p=0.0012 respectively), MLP (**p=0.0011 and ***p=0.0002 respectively) and MMP (****p<0.0001 and ***p=0.0002 respectively).

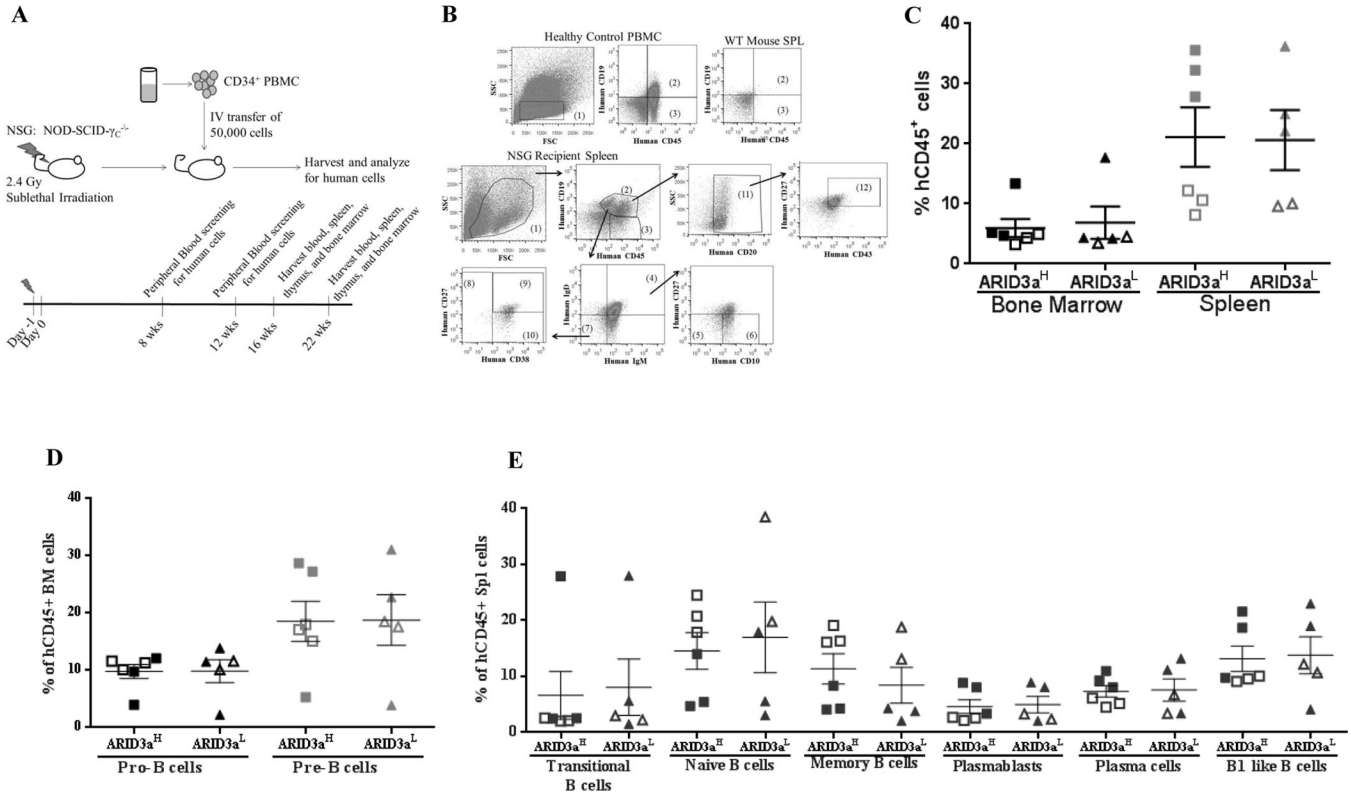

Figure 8. Numbers of ARID3a$^+$ progenitors did not influence engraftment or B cell development

Sublethally irradiated NSG mice were engrafted with equal numbers of CD34$^+$ PBMCs from ARID3a^H and ARID3a^L SLE patients. A. A schematic diagram indicates engraftment protocol and analysis time points. B. Representative data show the flow cytometric gating strategy used to identify human B lineage populations in NSG recipient mice. Numbered populations indicate: (1) lymphocytes, (2) human B cells (hCD19$^+$), (3) human non-B cells (hCD19$^-$), (4) IgM$^+$IgD$^+$, (5) naïve, (6) transitional, (7) IgM$^-$IgD$^-$, (8) memory, (9) plasmablasts, (10) plasma cells, (11) CD20$^+$, and (12) B1-like B cell subsets. C. Spleen and bone marrow from NSG recipient mice were analyzed by flow cytometry for total numbers of engrafted human cells for recipient mice that received ARID3a^H cells (■) and ARID3a^L cells (▲). Open symbols indicate recipients analyzed 16 weeks following human CD34$^+$ cell transfer versus 22 weeks (closed symbols). C. Percentages of human pro-B and pre-B cells in the bone marrow of recipient mice are shown. D. Analyses of percentages of engrafted B cell subsets in the spleen are shown. No differences in percentage of B lineage cells were seem between 16 and 22 weeks. Means and standard error bars are shown. Statistical analyses were performed by Mann Whitney t test.

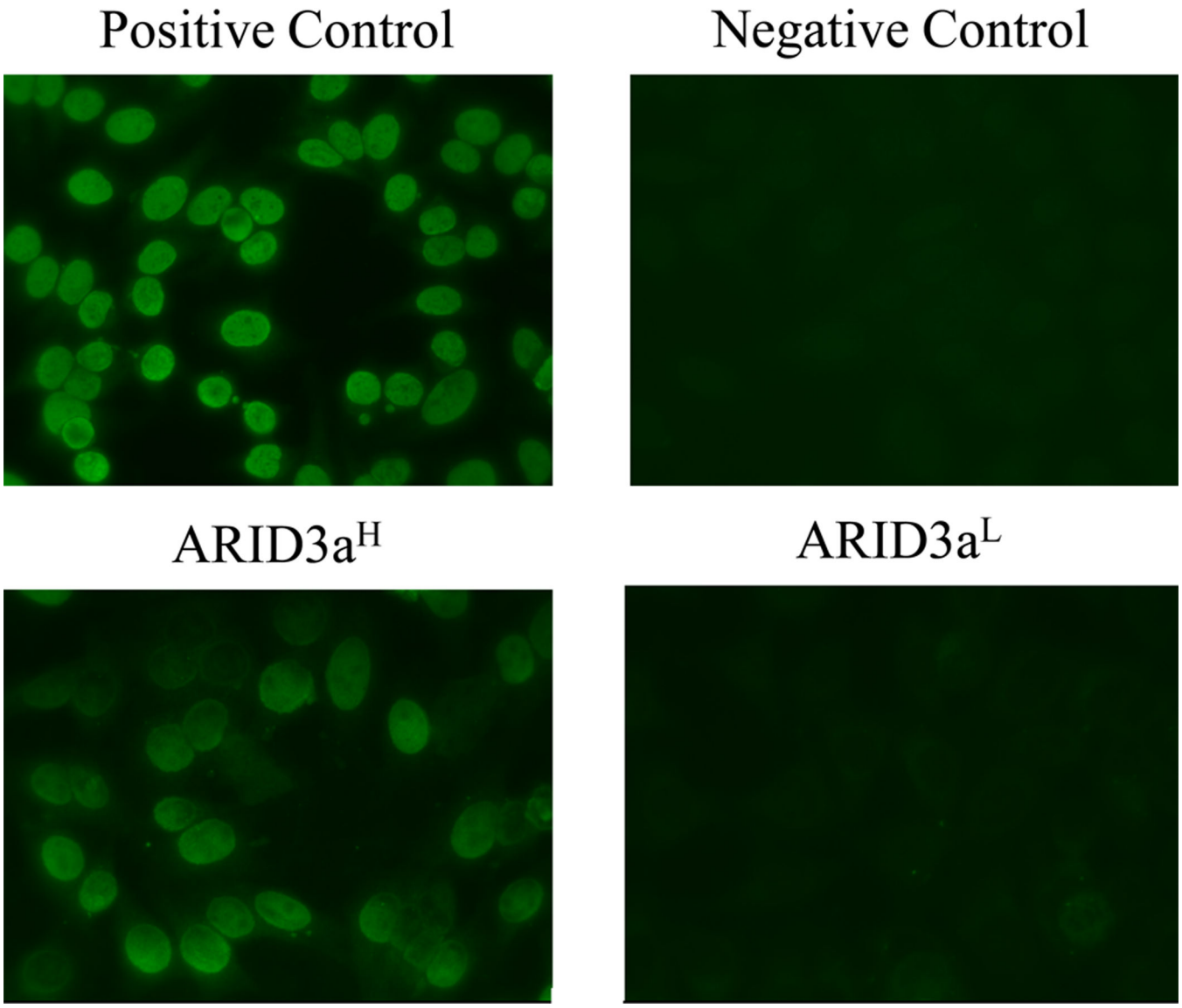

Figure 9. ARID3a^{H} progenitor engrafted mice were more likely to produce autoantibodies than those engrafted with cells from ARID3a^{L} progenitors

Representative fluorescent microscopic images of ANA staining produced by plasma samples from ARID3a^{H} versus ARID3a^{L} engrafted mice are shown with positive and negative controls.

Table I

Increased ANA Production in Mice Engrafted with ARID3a^{H} versus ARID3a^{L} cells[a].

ARID3a^{H} Patient	ANA	ARID3a^{L} Patient	ANA
W26[b]	+	W6	-
W36	+	W12	-
W45	+	W41	-
91[c]	+	25	+
W9	+	67	-
W37	+		

[a] ANA staining patterns produced by plasma samples from ARID3a^{H} versus ARID3a^{L} engrafted mice

[b–c] Individual patient samples obtained under IRB compliance #09-07 and #06-19