Published in final edited form as:
Stem Cells. 2014 September ; 32(9): 2550–2556. doi:10.1002/stem.1778.

SERPIN SPI2A AS A NOVEL MODULATOR OF HEMATOPOIETIC PROGENITOR CELL FORMATION

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Abstract

Prime regulation over hematopoietic progenitor cell (HPC) production is exerted by hematopoietins (HP's) and their Janus kinase-coupled receptors (HP-R's). For HP/HP-R studies, one central challenge in determining specific effects involves the delineation of non-redundant signal transduction factors and their lineage restricted actions. Via loss-of-function (LOF) studies, we define roles for an HP-regulated *Serpina3g*/Spi2A intracellular serpin during granulomyelocytic, B-cell and HSC formation. In granulomyelocytic progenitors, GMCSF strongly induced *Serpina3g* expression with Stat5-dependency. Spi2A-KO led to 20-fold decreased CFU-GM formation, limited GMCSF-dependent granulocyte formation, and

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Author contributions: In generating data, LL together with SB were prime contributors. NR, SS EJ, AA, DD, PA-R and DMW each also significantly contributed to investigations. Data analysis was via major efforts by LL, SB, PA-R and DMW. All authors contributed to manuscript construction (with lead efforts by LL and DMW). Study designs were via DMW and PA-R, together with LL and SB. Manuscript approval is in place for all authors.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST
No conflicting interests exist for any authors.

compromised neutrophil survival upon TNF-α exposure. In B-cell progenitors, Serpina3g was an IL7 target. Spi2A-KO elevated CFU-preB >6-fold, and altered B-cell formation in competitive BMT, and CpG challenge experiments. In HSCs, *Serpina3g*/Spi2A expression was also elevated. Spi2A-KO compromised LT-HSC proliferation (as well as LSK cell lysosomal integrity), and skewed LSK recovery post 5-FU. Spi2A therefore functions to modulate HP-regulated immune cell, and HSC formation post 5-FU challenge.

Keywords

Hematopoiesis; serpins; granulomyelocytes; neutrophils; GMCSF; B-cells; HPC; HSC

INTRODUCTION

The formation of hematopoietic progenitor cell (HPCs) is regulated, in part, by hematopoietins (HP's), and their cell surface receptors (HP-R's). This includes extended sets of interleukins and colony-stimulating factors that target HPCs and regulate their growth, development and/or cellular functions [1–3]. HP-R's typically are unique for their cognate ligands [2–4], but are differentially expressed among HPC lineages and/or developmental stages [2–6]. With regards to HP-R signaling events, substantial commonalities nonetheless exist (eg, PI3K/AKT and MEK/ERK modules) which further can be blended via HP-R crosstalk [7, 8]. To understand (and modify) specific HP actions, it therefore becomes important to define signals that HP-R's differentially transduce to coordinately regulate hematopoiesis.

Via transcriptome analyses of erythroid progenitors, we recently discovered the intracellular serpin (serine protease inhibitor) *Serpina3g*/Spi2A as a novel EPOR/JAK2 target [9]. Within maturing erythroblasts, Spi2A proved to cytoprotect against oxidative stress in part by inhibiting leached lysosome cathepsins [9]. Guided by observed *Serpina3g* expression profiles, we presently employ our unique Spi2A-KO model [9] plus primary HPC analyses to provide new insight into HP-regulation of *Serpina3g*, and roles of Spi2A in modulating the formation of defined granulomonocytic, B-cell and HSC populations.

MATERIALS AND METHODS are detailed for all approaches in supplemental text.

RESULTS

Serpina3g expression profiles, and Spi2A as an agent of GMCSF-dependent granulomyelocytic cell formation

Serpina3g/Spi2A action studies in HPCs were prompted by predominant expression observed among hematopoietic cells, including granulomyelocytic progenitors (Fig-1A) (together with B-cells and HSC, see below). In a LOF approach to gain functional insight, effects of Spi2A deletion on granulomyelocytic cells were assessed. At steady-state, Spi2A-KO mice maintained normal peripheral blood cell levels (Fig-S1). However, clonal colony-forming unit analyses of bone marrow HPCs (Fig1B–D) revealed a marked 22.7 ± 3.4-fold deficit in CFU-GM due to Spi2A-KO. CFU-M and CFU-G were less affected (~1.4-fold effect on CFU-G, p=0.05), but total myeloid CFU were diminished. GMCSF effects on CFU

formation also were studied. When GMCSF was included together with permissive HP's (IL3, IL6, SCF), numbers of wild-type CFU-G increased (as expected) but Spi2A-KO HPCs were non-responsive to this GMCSF effect (Fig-1D). For CFU-GM, GMCSF inclusion somewhat increased overall numbers, as well as the difference in CFU-GM observed due to Spi2A-KO (each by ~125%)(data not shown).

Based on faltered Spi2A-KO CFU-GM formation, *Serpina3g* regulation by GMCSF was assessed. JAK2-plus-Stat5 are key GMCSF-R mediators [10], and Stat5 has been implicated in *Serpina3g* expression [9]. GMPs from wild-type as well as Stat5a/b-KO bone marrow therefore were analyzed. HP's were withdrawn, cells were challenged with GMCSF. RNA was isolated, and effects on *Serpina3g* transcript levels were determined. In wild-type GMP, GMCSF induced *Serpina3g* expression ≥7.8 fold, while Stat5a/b-KO blocked this response (Fig-1E). Within the *Serpina3g* locus, several candidate Stat5 elements were observed within 5′, 3′ and intronic regions (Fig-1F). For a proximal promoter consensus element, ChIP analyses confirmed Stat5 occupancy (using T-cell extracts to provide for known positive Stat5-target genes) (Fig-S2).

Possible GMCSF effects on granulomonocytic cell formation from Spi2A-KO vs wild-type Lin^{neg} HPCs next were analyzed ex vivo (Fig-2). For neutrophils and with GMCSF as a singular HP, no significant Spi2A-KO effects were observed. For IL3, IL6, SCF plus GMCSF dosing, however, neutrophil formation from Spi2A-KO progenitors was compromised 4.2-fold (Fig-2B,C) emphasizing functional roles for Spi2A as an important GMCSF transducer in GMP (and/or granulocytic cells). (For observed increases in monocyte levels, this was a relative frequency effect, and overall cell numbers were largely unaffected due to Spi2A-KO). Possible Spi2A effects among developing neutrophils also were assessed using TNF-α as a relevant pro-apoptotic cytokine [11, 12]: TNF-α-exposed Spi2A-KO neutrophils exhibited significantly heightened apoptosis (Fig-2D–F).

Roles for Spi2A during B-cell formation

B-cell progenitor formation depends upon IL7/IL7R actions (and JAK1/3-plus Stat5 signaling) [3, 13]. In bone marrow-derived $B220^{pos}$ progenitors expanded ex vivo, IL7 proved to induce *Serpina3g* expression ≥9-fold, with elevated transcript levels also observed among B-cells, lymph nodes and spleen (Fig-3A,B). CFU analyses revealed heightened pre-B cell levels due to Spi2A-KO (Fig-3C and S4). Spi2A effects on B-cell formation also were observed in competitive BMT studies (Fig-3D). Here, CD45.2 LSK cells from either Spi2A-KO, compound Spi2A-KO plus Cathepsin B-KO, or wild-type BM were co-transplanted with competing CD45.1-marked wild-type donor cells to CD45.1-plus-CD45.2 co-marked recipients. Consistent with CFU-preB findings, Spi2A-KO proved (in this competitive format) to enhance contributions to $B220^{pos}$ B-cell formation, and this phenotype was partially reversed by Cathepsin B-KO. These effects also were observed albeit less markedly among $CD3^{pos}$ T-cell populations. Findings indicate apparent compensatory increases in progenitor B-cell populations upon Spi2A deletion. When later stage $B220^{pos}$ B-cells were propagated ex vivo, significant deficiencies in ex vivo expansion (Fig-3E) and increases in apoptosis upon CpG challenge (to activate TLR-9) [14] (Fig-3F), nonetheless, were observed.

Spi2A expression, and functional effects, in HSC populations

Serpina3g/Spi2A expression in HSCs was high-level (Fig-4A). In the absence of Spi2A, HSC populations were maintained at steady-state (data not shown), and also in long-term competitive BMT studies (Fig-S5). In vivo BrdU incorporation rates, however, indicated compromised LT-HSC cell cycle progression (Fig-4B). Based on recently observed lysosomal compromise in Spi2A-KO erythroblasts [9], lysosome numbers and integrity also were analyzed. In Spi2A-KO LSK cells, numbers of intact lysosomes were decreased, and extra-lysosomal distributions of Cathepsin-B were increased (Fig-4C) suggesting that observed impairments in cell cycle progression may be due to a loss of protection from LMP. In 5-fluorouracil challenge studies, further effects of Spi2A-KO on rebound LSK formation were observed. Specifically (and as analyzed at d7 post-5FU) Spi2A-KO LSK levels proved to be significantly elevated (Fig-4D). This was observed among all replicates (Fig-S6), and may reflect engagement of compensatory HP effects, spatial-temporal increases in HSC niche residence, and/or possible induction of a surrogate clade-B serpin.

DISCUSSION

Serpins are a family of ~45 kDa protease inhibitors plus orthologues (eg, Maspin tumor suppressor, MENT chromatin factor) that exert diverse biochemical and cellular effects [15, 16]. Many target catalytic serines (eg, chymotrypsin, elastase), while others cross classes (eg, cysteine protease inhibitors). Most are secreted with one example as PAI serpin inhibition of tPA during fibrinolysis [17]. A second involves GCSF repression of secreted Serpina-1 and -3 [18]. This reverses inhibition of bone marrow proteases which then promote $CD34^{pos}$ progenitor release. For the present studies, *Serpina3g*/Spi2A's observed lineage-selective expression, intracellular localization [9, 19] and HP-regulation prompted analyses of Spi2A's hematopoietic roles.

Among granulomyelocytic cells, deficiencies in CFU-GM formation due to Spi2A-KO, together with the defining of *Serpina3g* as a GMCSF plus Stat5 target, first underscore important effects on GM(P) expansion (Figs 1,2). Ex vivo, Spi2A-KO further compromised neutrophil formation, with GMCSF-dependency. Here, roles for co-acting IL3, IL6 and SCF HP's in engaging Spi2A's effects point to early GM progenitors as a Spi2A-regulated cohort. This also underscores GMCSF as a prime *Serpina3g* inducer, but in suggested synergism, induction by IL3 has been reported (in BaF3 cells, NCBI GEO GDS3349). Spi2A also proved to cytoprotect developing neutrophils against TNF-α-induced apoptosis (Fig-2). TNFa can heighten ROS, but unlike the case in erythroid progenitors, ROS levels in neutrophils were not significantly altered due to Spi2A-KO (negative data not shown). This, nonetheless, does not discount hypothesized effects linked to lysosomal compromise. Via CFU, competitive BMT and ex vivo analyses, roles for Spi2A also were defined during B-cell formation (Fig-3). For CFU-pre-B, compensatory effects are implicated by observed substantial increases due to Spi2A-KO. BMT studies likewise reveal apparent enhancement of $B220^{pos}$ cell formation. In isolated ex vivo settings, Spi2A-KO proved to compromise later stage $B220^{pos}$ B-cell growth, and survival.

In HSCs, Spi2A levels were maximal. Spi2A disruption compromised LT-HSC BrdU incorporation rates, and LSK lysosomes also leached Cathepsin B (Fig4A–C). Spi2A-KO

HSC levels nonetheless were sustained (including post long-term BMT). Post 5-FU exposure, however, rebound LSK formation was skewed due to Spi2A-deficiency with ~200% increases observed (Fig-4D, S6). As for CFU-preB, this implicates compensatory mechanisms potentially involving HP effects (eg, Tpo/Mpl), HSC niche remodeling, and/or surrogate clade B serpin induction. The extent to which such 5-FU and stress-induced increases in Spi2A-KO LSK cells give rise to durable peripheral blood cell progeny will be of interest to assess in future investigations.

CONCLUSIONS

As a GMCSF, IL7 and Stat5 target, *Serpina3g*/Spi2A is shown via LOF studies to regulate GMP pools, neutrophil formation plus survival, and pre-B pools plus B-cell formation. Novel roles for Spi2A therefore are implicated in cellular immunity, and potentially the leukemogenic effects recently shown to be exerted by GMCSF [20], and the IL7R [21]. Spi2A also appears to modulate LT-HSC cell cycle progression, lysosome integrity and LSK expansion post-5FU myeloablation. Figure 4E provides a summary outline of these diverse and meaningful hematopoietic roles of Spi2A.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Studies were supported by NIH R01 DK089439 (DMW), NIH R01 HL044491 (DMW) and NIH R01 AI40608 (PA-R).

For flow cytometry and transcript(ome) analyses, the authors gratefully acknowledge core facility resources (Progenitor Cell Analysis Core, Molecular Phenotyping Core) as those supported in part by NIH/NIGMS P30 GM106391 (DMW, PI). For transcriptome analyses of purified GMP, we acknowledge the generous contributions of Drs. D. Yamaji and L. Hennighausen (NIH/NIDDK). In ChIP analyses, contributions of Drs. L Wang and D Heathcote (Imperial College London) are gratefully recognized. We also acknowledge the Mouse Transgenic Core Facility for assistance with mouse colonies (supported in part by NIH/NIGMS P30 GM103392).

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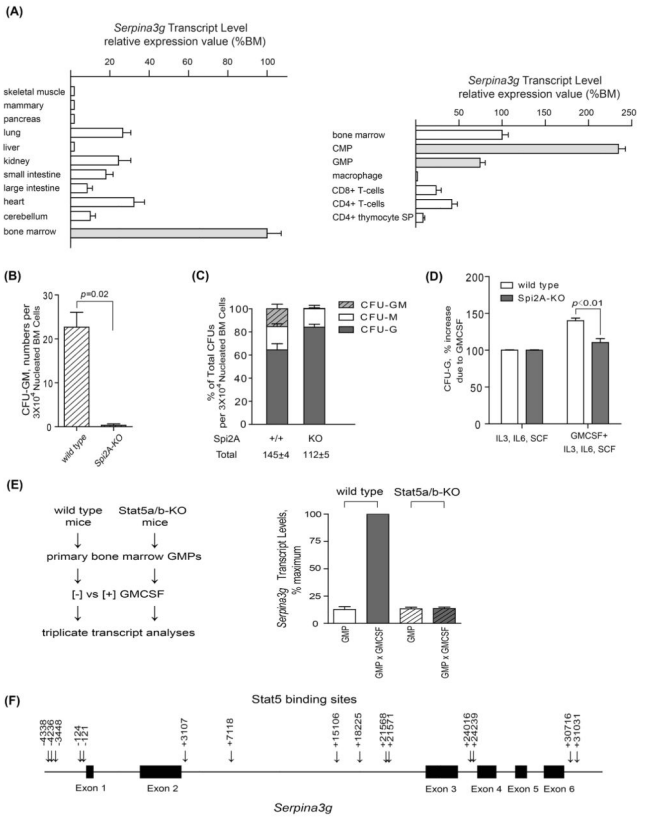

Figure 1. Regulated Spi2A expression, and function in supporting granulomyelocytic CFU formation

A] Heightened *Serpina3g* expression in bone marrow, and among granulomonocytic progenitor cells: Values are mean *Serpina3g* transcript expression values (+/− SE) as normalized to levels in murine bone marrow. **B]** Spi2A deficiency markedly compromises CFU-GM pools: Via methylcellulose colony-forming assays, numbers of CFU-GM in bone marrow preparations from Spi2A-KO and wild-type mice were determined (means +/− SE, n=3). **C]** Effects of Spi2A deletion on CFU-G, CFU-M and CFU-GM composite pools: In assessing frequencies of granulomyelocytic HPC populations in Spi2A-KO mice vs wild-type controls, bone marrow CFU-GM, CFU-G and CFU-M colony-forming units were assayed. Values are means +/− SE (n=3). **D]** Spi2A-deficiency limits GMCSF-supported CFU-G colony formation: CFU-G assays were performed in the absence vs presence of GMCSF (10 ng/mL). Values are normalized means +/− SE (n=3). **E]** GMCSF regulates *Serpina3g* expression via Stat5: GMP were isolated from either wild-type or Stat5a,b-KO mouse bone marrow. For each, biological triplicate GMP preparations were then incubated for 5 hours in the absence of HP's, and challenged with GMCSF. At 180 minutes, RNA was isolated and was used in transcriptome analyses to determine GMCSF and Stat5a, b effects on *Serpina3g* expression. Values are mean relative expression levels +/− SE. **F]** Consensus Stat5a/b binding site distribution at the *Serpina3g* locus: In silico analyses predict the occurrences of five upstream, two downstream and several intragenic consensus Stat5 binding sites within the *Serpina3g* gene.

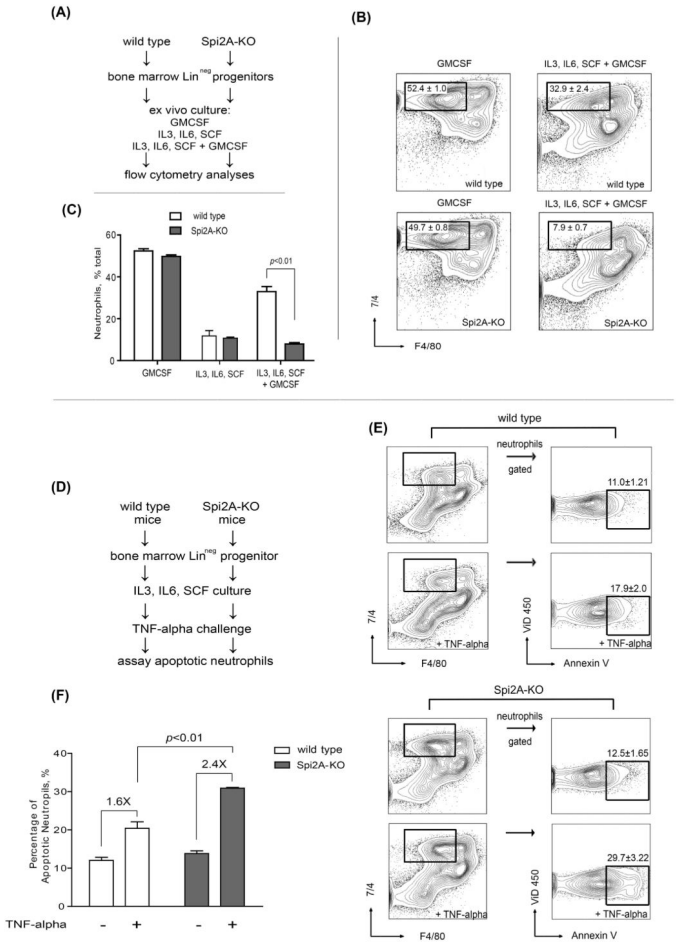

Figure 2. GMCSF-dependent development of HPCs to neutrophils falters due to Spi2A deficiency, and Spi2A cytoprotects developing neutrophils against TNF-α induced apoptosis

A–C] Spi2A-KO HPCs exhibit deficits in GMCSF-dependent neutrophil formation: The approach used for analyses of HP-dependent granulomyelocyte formation from Lin^{neg} HPCs is outlined (A), and effects on neutrophil formation of Spi2A disruption are illustrated (B), and summarized (mean values +/− SE, n=4) (C). **D–F]** Developing Spi2A-KO neutrophils exhibit heightened sensitivity to TNF-α-induced apoptosis: Lin^{neg} progenitors were isolated from wild-type or Spi2A-KO bone marrow, and expanded ex vivo (IL3, IL6, SCF). At day 3 of expansion, cells were exposed to TNF-α (25ng/mL, 12 hours). Frequencies of apoptotic cells then were determined (Annexin V-positivity) Representative primary flow cytometry analyses are shown (E) together with summary data (F) [means +/− SE (n=3)].

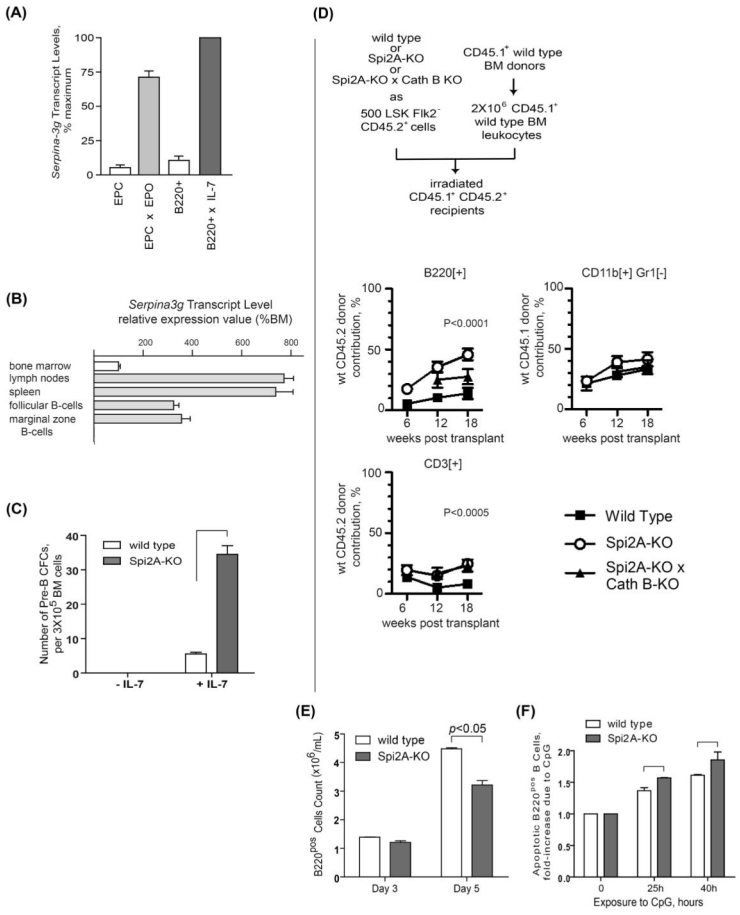

Figure 3. Regulated Spi2A/*Serpina3g* expression, and function, in B-cells

A] IL7 induction of *Serpina3g* in primary bone marrow-derived B220^{pos} progenitors: Bone marrow HPCs were isolated and expanded in the presence of IL7, SCF and OP9 stromal cells. B220^{pos} B-cell progenitors were then isolated, cultured for 6 hours in the absence of HP's (without stromal cells) and challenged with IL7 (±10ng/mL). At 90 minutes, cells were lysed (Trizol reagent), and RNA plus cDNA were prepared. *Serpina3g* levels then were determined by quantitative PCR (normalized mean relative expression levels +/− SE, n=3). For comparison, EPO-induction of *Serpina3g* in primary erythroid progenitor cell preparations was analyzed in parallel. **B]** *Serpina3g* expression levels among B-cells, and B-cell resident tissue: values are relative expression levels (means +/− SE) normalized to *Serpina3g* transcript levels in bone marrow. **C]** CFU-preB levels are multi-fold elevated due to Spi2A-KO: Steady-state pre-B colony forming unit levels in wild-type and Spi2A-KO mice are graphed (means +/− SE, n=4). **D]** CD45.2-marked LSK cells from either wild-type, Spi2A-KO, or compound Spi2A-KO Cathepsin B-KO bone marrow preparations were competed in transplantation experiments with wild-type CD45.1-marked bone marrow leukocytes. At weeks 6, 12 and 18, frequencies of engrafted donor-derived CD45.2 cells as co-marked by B220^{pos}, CD3^{pos}, or CD11b^{pos} then were determined. **E]** B220^{pos} B-cell formation from Spi2A HPCs falters ex vivo: Primary bone marrow HPCs from wild-type or Spi2A-KO mice were plated (1.5 × 10^6 cells/mL) onto OP9 feeder cells in the presence of

IL7 (10 ng/mL). At day-2, medium was refreshed. At days 3 and 5, numbers of viable $B220^{pos}$ cells were determined (mean values +/− SE). **F]** Spi2A-KO primary $B220^{pos}$ B-cells exhibit heightened sensitivity to CpG-induced apoptosis: At day-2.5 of culture, B-cell cultures were exposed to a CpG TLR-9 ligand (2 μM). At day-5, effects on apoptosis were determined (APC-Annexin V) (means +/− SE, n=3).

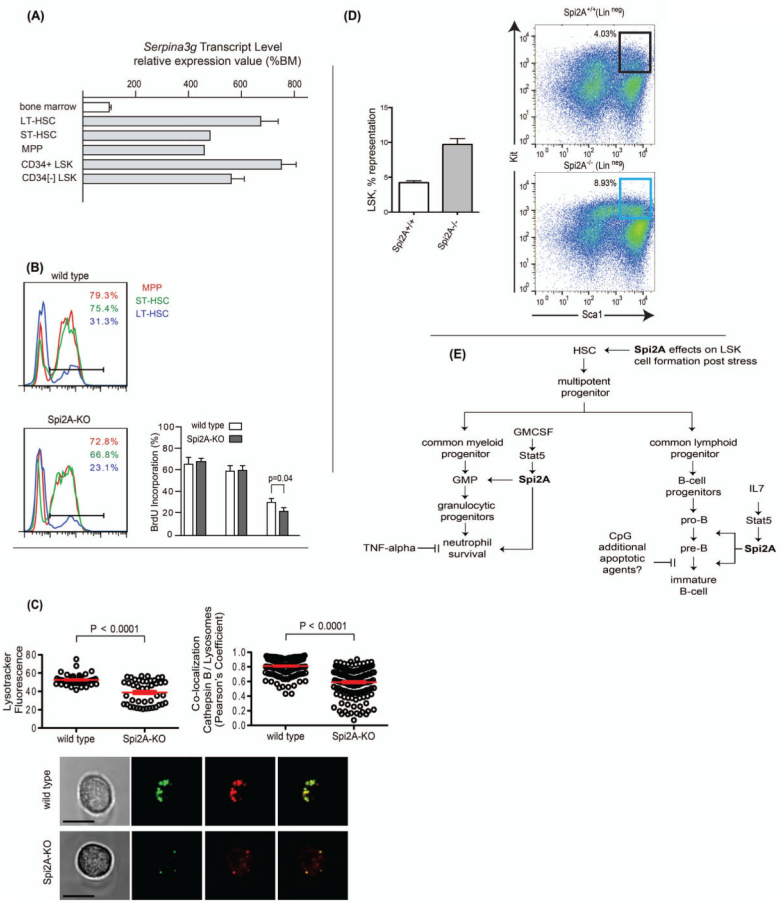

Figure 4. *Serpina3g*/Spi2A expression, and actions in hematopoietic stem cells

A] *Serpina3g* transcript expression levels among HSC/HPC subpopulations. Values and means (+/− SE) for relative expression levels (NCBI GEO and Ensembl databases) as normalized to *Serpina3g* levels in murine bone marrow. **B]** LT-HSC proliferation (based on BrdU incorporation rates) is compromised due to Spi2A-KO: At 20hr post-BrdU dosing, bone marrow was isolated from wild-type and Spi2A-KO mice, and assayed for HSC markers plus BrdU incorporation via flow cytometry. Left panel: Primary flow cytometry histograms for BrdU incorporation in bone marrow progenitor subsets as MPP (red) Lin^{neg} $Sca1^{+}$ c-Kit^{+} $Flk2^{+}$ $CD34^{+}$; ST-HSC (green) Lin^{neg} $Sca1^{+}$ c-Kit^{+} $Flk2^{-}$ $CD34^{+}$; LT-HSC (blue) Lin^{neg} $Sca1^{+}$ c-Kit^{+} $Flk2^{-}$ $CD34^{-}$. Gating and percentage of BrdU+ cells for each HSC subset are indicated. Right panel: Mean percentage BrdU+ cells ± SEM (n = 5 mice). Results are representative of three independent experiments. **C]** Compromised lysosome numbers, and heightened cathepsin-B leaching, in LSK cells due to Spi2A deletion: Among LSK cells, frequencies of lysosomes (green fluorescence) were decreased due to Spi2A disruption (left, and lower panels). Confocal IF analyses of lysosomes plus Cathepsin-B (red fluorescence) further indicated leaching of Cathepsin-B from lysosomes within Spi2A-KO LSK cells (right, and lower panels). **D]** Spi2A-deficiency skews the rebound formation of LSK HSC's post 5-fluorouracil challenge: Spi2A-KO and wild-type $Spi2A^{+/+}$ control mice were dosed with 5FU (150mg/kg). At d7 post-5FU, bone marrow was isolated and levels of $Lin^{neg}Sca1^{pos}Kit^{pos}$ LSK HSC's were analyzed. Values are means +/− SE (n=4 plus 4).

Representative primary flow cytometry data also are shown (please also see Supplemental Figure S6). **E]** Summary model outlining apparent roles for Spi2A serpin during HSC, granulomonocytic and B-cell formation.