HHS Public Access

Author manuscript

Blood Cells Mol Dis. Author manuscript; available in PMC 2022 June 24.

Published in final edited form as:

Blood Cells Mol Dis. 2021 November ; 91: 102594. doi:10.1016/j.bcmd.2021.102594.

CXCL15/Lungkine has Suppressive Activity on Proliferation and Expansion of Multi-potential, Erythroid, Granulocyte and Macrophage Progenitors in S-Phase Specific Manner

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Abstract

Cytokines/chemokines regulate hematopoiesis, most having multiple cell actions. Numerous but not all chemokine family members act as negative regulators of hematopoietic progenitor cell (HPC) proliferation, but very little is known about such effects of the chemokine, CXCL15/Lungkine. We found that *CXCL15/Lungkine* −/− mice have greatly increased cycling of multi cytokine-stimulated bone marrow and spleen hematopoietic progenitor cells (HPCs: CFU-GM, BFU-E, and CFU-GEMM) and CXCL15 is expressed in many bone marrow progenitor and other cell types. This suggests that CXCL15/Lungkine acts as a negative regulator of the cell cycling of these HPCs *in vivo*. Recombinant murine CXCL15/Lungkine, decreased numbers of functional HPCs during cytokine-enhanced *ex-vivo* culture of lineage negative mouse bone marrow cells. Moreover, CXCL15/Lungkine, through S-Phase specific actions, was able to suppress *in vitro* colony formation of normal wildtype mouse bone marrow CFU-GM, CFU-G, CFU-M, BFU-E, and CFU-GEMM. This clearly identifies the negative regulatory activity of CXCL15/Lungkine on proliferation of multiple types of mouse HPCs.

Keywords

CXCL15/Lungkine; *CXCL15* −/− Mice; Hematopoietic Progenitor Cell; Colony Formation and Ex Vivo Assays

Introduction

Numerous cytokines and chemokines regulate the production and proliferation of hematopoietic stem (HSCs) and progenitor (HPCs) cells and hematopoiesis *in vitro* and *in vivo* (1–8). Many members of the CXC and CC family of chemokines have demonstrated suppressive regulatory actions *in vitro* and *in vivo* on the proliferation of HPCs (4–20).

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ELR$^+$ chemokine CXCL15 was first designated as Lungkine, as it was expressed in the lung (21), but it has also been called WECHE (22) and was later found to be expressed in other organs/tissues (22,23), although its expression in bone marrow (BM) was not noted. CXCL15/Lungkine has activities associated with immunology and host defense systems (23,24), but very little is known of its effects on hematopoiesis other than what appeared to be selective effects on erythroid progenitor cells (BFU-E) *in vitro* (22). We thus undertook studies evaluating proliferation of HPCs using *CXCL15/Lungkine* knock-out (−/−) mouse BM and spleen, and exogenous effects of recombinant murine CXCL15/Lungkine on *ex-vivo* culture of cytokine stimulated normal wildtype (WT) BM cells and on colony formation by WT BM cells in order to better understand a role for CXCL15/Lungkine on subsets of functional multi cytokine-stimulated granulocyte macrophage (CFU-GM), granulocyte (CFU-G), macrophage (CFU-M), BFU-E, and multipotential (CFU-GEM) progenitor cells. CXCL15 expression was found in many HPC subsets and other cells in BM. BM and spleen HPCs from *CXCL15/Lungkine* −/− mice were greatly increased in HPC cell cycling, and recombinant murine CXCL15 had myelosuppressive effects on *ex-vivo* culture of functional WT BM HPCs and on colony formation by normal WT BM HPCs. These suppressive effects were S-Phase specific *in vitro* for functional HPCs, as assessed by colony assessment and high specific activity tritiated thymidine kill assay.

Materials and Methods

Mice.

CXCL15/Lungkine −/− mice on a C57Bl/6 strain background (22) were provided by Sergio Lira, Columbia University. Age and sex matched control WT mice were from Jackson Laboratories (Bar Harbor, ME) and our U54 CCEH and IU Simon Comprehensive Cancer Center Animal Core Facilities at the Indiana University School of Medicine (IUSM). Experiments were done per Institutional (IACUC) Guidelines at the IUSM.

Myeloid Progenitor Cell Assays and Assessment of Cell Cycle Status for *LUNGKINE* −/− Mice.

Absolute numbers and cycling status of CFU-GEMM, BFU-E, and CFU-GM from femoral bone marrow (BM) and spleen of *LUNGKINE* −/− and wildtype control (+/+) mice were assessed as described previously (9, 25–27). In short, unseparated marrow and spleen cells were pulse treated in complete medium (IMDM, 10% FBS) with either control medium or high specific activity tritiated thymidine for 30 minutes prior to washing cells 2x with complete medium. Cells were plated at 5×10^4 BM and 5×10^5 spleen cells/ml in 1.0% methylcellulose culture medium with 1 U/ml recombinant human erythropoietin (EPO), 50 ng/ml recombinant murine steel factor (stem cell factor, SCF), 5% vol/vol pokeweed mitogen mouse spleen cell conditioned medium (a source of IL-3, GM-CSF and other hematopoietic growth factors) and 0.1 mM Hemin. CFU-GEMM-, BFU-E- and CFU-GM-colonies were scored after 7 days incubation at 5% CO_2 and lowered (5%) O_2. This combination of growth factors allows detection of the more immature subsets of these progenitors. Each mouse was individually assessed for absolute numbers of progenitors calculated from the cellularity per organ and the number of colonies formed per number

of cells plated. The cycling status (percent progenitors in S-Phase of the cell cycle) was estimated by the high specific activity tritiated thymidine assay (9, 25–27).

CXCL15 Expression in BM.

For *Cxcl15* mRNA expression analysis, microarray normalized log2 expression value data was downloaded from the BloodSpot (28–30) database and plotted in graphpad prism. Normalization for these data has been previously described (28–30). For CXCL15 protein expression analysis, BM cells were harvested in separate experiments from wildtype mice (n=4) and stained for cell surface markers to define different HSC/HPC subpopulations (Suppl. Table 1, Suppl. Fig. 1). Cells were washed 3 times. Cells were then fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences), then stained with anti-CXCL15-FITC antibody (Bioss Antibodies bs-2554R-FITC) or isotype-FITC control (Bioss Antibodies bs-0295P-FITC). Cells were washed 3 times, then analyzed by flow cytometry for CXCL15 expression. See Legend to Fig. 2 and Suppl. Fig. 1 for additional information on expression of CXCL15 in subsets of HPCs identified by phenotypic analysis.

Effects of recombinant mouse CXCL15/LUNGKINE on colony formation *in vitro* of normal WT BM cells.

Recombinant murine CXCL15 (Cat # 442-LK-002) was purchased from R&D Systems (Minneapolis, MN) See: https://en.wikipedia.org/wiki/CXCL15 for general information on CXCL15. It was assayed on WT C57Bl/6 BM cells as noted in the Figure Legends. Colony formation by CFU-GM (stimulated with GM-CSF −/+ SCF), CFU-G (stimulated by G-CSF −/+ SCF), CFU-M (stimulated by M-CSF −/+ SCF), in agar cultures, and BFU-E (stimulated by EPO −/+ SCF), and CFU-GEMM (stimulated by EPO, SCF, PWMSCM and 0.1mM Hemin) cultured in methylcellulose were scored after 6–7 days incubation at 5% O_2. Use of a CSF plus SCF detects the more immature, and use of only a CSF detects the more mature, subsets of functional HPCs (31). All colony stimulating factors were purchased from R&D Systems (Minneapolis, MN) and EPO (Epogen, Amgen, Thousand Oaks, CA) was purchased through McKesson Medical. Cell cycle analysis to determine S-phase cycling of functional HPCs as assessed by BM colony formation was performed after pulse treatment with high specific activity ^{3}HTdr and prior to addition of cytokines and CXCL15 to the culture plates.

Effects of recombinant CXCL15/LUNGKINE on cytokine stimulated *ex vivo* expansion of normal WT BM cells

WT mouse BM was harvested and lineage depleted (Milltenyi 130–110-470). Lineage depleted cells were grown for 4 days in liquid culture (RPMI1640 supplemented with 10%FBS and 100ng/mL recombinant murine (rmu)SCF, rmuTPO, rhuFLT3L (FL)) with or without 20ng/mL recombinant CXCL15. Cells were collected on Day 0 and Day 4 and plated for colony formation analysis as described in the previous section. Cells were collected on Day 0 and Day 4 and stained with fluorophore conjugated anti-mouse lineage cocktail, anti-CD117, anti-Ly6A/E, anti-CD34, anti-CD135, anti-CD150, and anti-CD16/32 (BioLegend). Numbers of HSCs/HPCs, etc. were determined by flow cytometry.

Statistics

Original Figures, except Figure 1, are the averages of 3–4 different experiments each shown as a fold or percentage change, while the individual experiments shown with actual numbers are found in the Supplemental Figures. Significant differences between 2 groups was assessed by Student's t test with a P value <0.05; considered statistically significant. Three plates were scored per point for all colony forming experiments.

Results

Myeloid Progenitor Cell Assays (*CXCL15/LUNGKINE* −/− and +/+).

There are numerous occasions in which changes in absolute numbers of mature leukocytes were not noted in marrow or circulating blood of gene-knockout mice or after normal mice were administered cytokines. However, analysis of functional progenitor cells picked up clear differences in these very important immature cell types in either absolute numbers and/or in the cycling status of these progenitors (9, 27, 32–36) which suggested a regulatory role for the deleted gene product or administered cytokine. For this reason, and since others have suggested a role for WECHE/Lungkine as an inhibitor of BFU-E (22), we evaluated the absolute numbers and cycling status of CFU-GEMM, BFU-E, and CFU-GM in the marrow and spleen of *CXCL15/LUNGKINE* −/− and +/+ mice. While there were no significant differences in nucleated cellularity (Fig. 1A) or in absolute numbers of these progenitors in either marrow or spleen except for the modest decrease in BM CFU-GEMM (Fig. 1Bc), there were highly significant differences in the cycling status of these progenitors in both organs (Fig. 1C). Myeloid progenitors in BM are usually cycling and in S-phase of the cell cycle while those in spleen are usually in a very slow or non-cycling state. In contrast, progenitors in BM and spleen of *CXCL15/LUNGKINE* −/− mice were cycling at a much higher rate than those in WT control mice HPC from BM and spleens of *CXCL15/LUNGKINE* −/−. These results strongly suggest a negative regulatory role *in vivo* for CXCL15 on proliferation of CFU-GEMM, BFU-E and CFU-GM in BM and spleen.

Expression of CXCL15 in subsets of HPCs in normal mouse BM.

Because the expression of CXCL15 in murine BM is not well defined, we examined whether CXCL15 is expressed in stringently defined primitive and mature hematopoietic cells. As shown in Fig. 2 and Suppl. Fig. 1, multiple subsets of BM HPCs, identified by phenotypic analysis as assessed by Flow Cytometry, expressed CXCL15. First, we mined data from the BloodSpot database (28–30) to show that CXCL15 mRNA is expressed in all examined primitive and mature hematopoietic cells, including LT-HSC, ST-HSC, and various mature immune cells (Fig. 2A). Next, we examined whether and to what degree CXCL15 protein is expressed in different mouse BM cell subpopulations. Cell populations were defined using cell surface immunophenotype (Suppl. Table 1, Suppl. Fig. 1). Cells were permeabilized and stained with anti-CXCL15 to examine intracellular expression of CXCL15 (Fig. 2B). CXCL15 is expressed in 71% (+/− 6.1%) of total murine bone marrow cells (Fig. 2C). Interestingly, CXCL15 is expressed in a higher percentage of more primitive HSC/HPC, such as LT-HSC which are 93.6% (+/−1%) positive for CXCL15 expression compared to more mature BM populations like Lin+ cells which are 69.7% (+/−6.5%) positive for CXCL15 expression (Fig. 2C). CXCL15 also exhibits highest relative expression levels

as measured by fluorescence intensity of the CXCL15 stain in more primitive HSC/HPC compared to more mature cell populations such as Lin+ and Lin-Kit- cells (Fig. 2D). Taken together, these data demonstrate that CXCL15 is produced in the BM, and particularly in HSC/HPC populations.

Influence of CXCL15 on Colony Formation on WT HPCs.

Based on our description that CXCL15 *in vivo* plays a role on proliferation of CFU-GM, BFU-E, and CFU-GEMM, and as described by others that CXCL15 (WECHE) had myelosuppressive effects *in vitro* on mouse BM BFU-E, with approximately 50% inhibition at 100ng/ml (22), we evaluated effects of rmu CXCL15 on colony formation in methylcellulose media stimulated by EPO, SCF, PWM and hemin (Fig. 3, average of 3 experiments shown as percent change from control media and Suppl. Figs. 2–4 with the actual individual experiments) by CFU-GM (Fig. 3Ai), BFU-E (Fig. 3Aii) and CFU-GEMM (Fig. 3Aiii) and on BFU-E stimulated with only EPO (Fig. 3Aiv (top)), or with EPO plus SCF (Fig. 3iv (bottom)). CXCL15 demonstrated dose-dependent inhibitory effects with maximum inhibition (~50%) by 20ng/ml and 10ng/ml CXCL15, and some loss of inhibition at 5ng/ml for CFU-GM, BFU-E, and CFU-GEMM, and with similar results for BFU-E stimulated by EPO plus SCF. There was no effect on BFU-E stimulated with only EPO (Fig. 3Aiv; top). EPO/CSF plus SCF detect the more immature subsets of CFU-GM, BFU-E, and CFU-GEMM, while the cells stimulated without SCF detect the more mature subsets of these HPCs (2, 31). Other inhibitory chemokines are only suppressive on the earlier less mature subsets of HPCs in multi cytokine-stimulated colony formation that includes the potent co-stimulati ng cytokine SCF (2, 3, 6, 35, 36).

In order to delve further into the immature subsets of CFU-GM, CFU-G, and CFU-GM, we respectively analyzed these cells in WT normal mouse BM stimulated respectively with GM-CSF, G-CSF, and M-CSF −/+ SCF. CXCL15 which elicited a dose suppressive influence of CXCL15 on the immature subsets of CFU-GM, CFU-G, and CFU-M stimulated with a CSF (respectively GM-CSF, G-CSF, or M-CSF) plus SCF, but had no effect on the more mature subsets of these cells stimulated with a CSF without SCF (Fig. 3B and Suppl. Figs. 2–4).

Effects of CXCL15 on *Ex-Vivo* Expansion of Cytokine Stimulated Normal WT Mouse BM Cells.

Culturing Lin-negative normal WT BM cells *ex-vivo* with SCF, TPO, and FL enhances HSC and HPC numbers (1–4). As shown in Fig. 4, this combination of growth factors enhanced numbers of nucleated cells, and phenotypically defined LT-HSCs, and short term HSCs, and HPCs (Fig. 4A, Suppl. Fig. 5) with no effect on phenotypically defined HSCs and HPC on output numbers at day 4 for CXCL15 vs. PBS. However, increased numbers of CFU-GM, BFU-E, and CFU-GEMM were decreased by CXCL15 (Fig. 4D–E; Suppl. Fig. 6). That one can see an effect of CXCL15 at Day 4 of *ex-vivo* on functional, but not on phenotypically-defined, HPCs is not necessarily surprising as phenotype of these cells does not always recapitulate functional activity of HSCs/HPCs (37).

Suppressive Effects of CXCL15 on Colony Formation *In Vitro* by Mouse BM HPCs are S-Phase Specific.

We demonstrated that effects of CXCL15 *in vitro* on colony forming cells is S-phase specific by noting that cells pretreated first with high specific activity tritiated thymidine pulse (rendering HPCs in S-phase replication incompetent) are not inhibited by CXCL15 (Fig. 5, Supp. Fig. 6). CXCL15 does not inhibit colony formation by pre-^{3}HTdr pulse treatment, but does inhibit colony formation by control treated cells. Hence, suppression of immature subsets of CFU-GM, BFU-E, and CFU-GEMM by CXCL15 is S-phase specific, consistent with studies on CXCL15/Lungkine −/− mice (Fig. 1).

Discussion

Our results now demonstrate that CXCL15 is expressed by multiple subsets of phenotypically-expressed BM HPCs and the *in vivo* suppressive effects of *CXCL15/LUNGKINE* on multi-cytokine stimulated responsive immature subsets of CFU-GM, BFU-E, and CFU-GEMM, as assessed by increased proliferation of these HPCs in BM and spleen of *CXCL15/LUNGKINE* −/− mice, and of inhibition of multi cytokine-stimulated *ex-vivo* expansion of CFU-GM, BFU-E, and CFU-GEMM and *in vitro* suppression of colony formation by CXCL15 of multi cytokine-stimulated CFU-GM, CFU-G, CFU-M, BFU-E, and CFU-GEMM. This adds extensive new information on the suppressive effects of CXCL15, noted previously for only BFU-E (22). Moreover, we demonstrate that these CXCL15 effects are S-Phase specific for these immature sub-populations of responsive HPCs. It is not clear if the results reported for suppression of BFU-E *in vitro* by WECHE/Lungkine, were specific for BFU-E, as the authors did not report effects on CFU-GM, CFU-G, CFU-M, and CFU-GEMM, although they presented some data on suppression by WECHE of colony formation by fetal liver high proliferative progenitor (HPP) cells (22). While of some interest, it is not entirely clear at present where HPP cells fit within the HSC/HPC hierarchy. They were once considered to be in the HSC category, but this may not be entirely true, because HPP are not known to give rise to CFU-GM, BFU-E, CFU-GEMM or other HPCs. That exogenous administration of CXCL15 to BM cells, that already express CXCL15 has a suppressive effect on these cells may suggest at least in part that *in situ* levels of CXCL15 in BM cells is not expressed at suppressive levels. Alternatively, or in addition the *in situ* expressed CXCL15 may not be in a suppressive form (26).

Why the *CXCL15/LUNGKINE* −/− mouse BM demonstrated greatly enhanced cycling of CFU-GM, BFU-E, and CFU-GEMM, without a concomitant increase in absolute numbers of these HPCs is not clear, but may possibly reflect *in vivo* compensatory effects of *CXCL15/LUNGKINE* in the knockout mice. We previously noted for *CCR2* (9) and *Fanconi anemia complementation C group* (11) null mice, that enhanced cycling status of myeloid progenitors without increases in the absolute numbers of these cells in BM, was at least in part associated with enhanced apoptosis of these cells. Whether or not apoptosis of HPCs may be a reason for lack of increases in absolute numbers of CFU-GM, BFU-E, and CFU-GEMM in *CXCL15/LUNGKINE* −/− mice when these progenitors are highly cycling is not known, but does not take away from the more important observation of increased cell cycling, consistent with the *in vitro* effects of recombinant CXCL15 that we noted on HPCs.

CXCL15 is found in lung, and lung contains HPCs (38), so it is possible that CXCL15 may have suppressive effects on cycling HPCs in other organs. Exactly how CXCL15 mediates its negative effects on suppression of HPC cell cycling, other than that the effects are S-phase specific remains to be determined. Intracellular signaling induced by cytokines/chemokines is an ongoing effort with regards to regulation of hematopoiesis (2–4). Similarities and differences of CXCL15 induced signaling to that of other cytokines/chemokines (31) remains to be determined. Whether or not CXCL15 has future clinical translational possibilities is yet to be evaluated, especially in patients with hyperproliferative hematopoiesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by US Public Health Service Grants from the National Institutes of Health R35 (Outstanding Investigator Award) HL139599 and U54 DK106846 Cooperative Center of Excellence in Hematology (CCEH) to HEB. JR was supported by NIH T32 DK007519 to H.E.B. We thank Dr. Sergio Lira, Columbia University for the *LUNGKINE* −/− mice.

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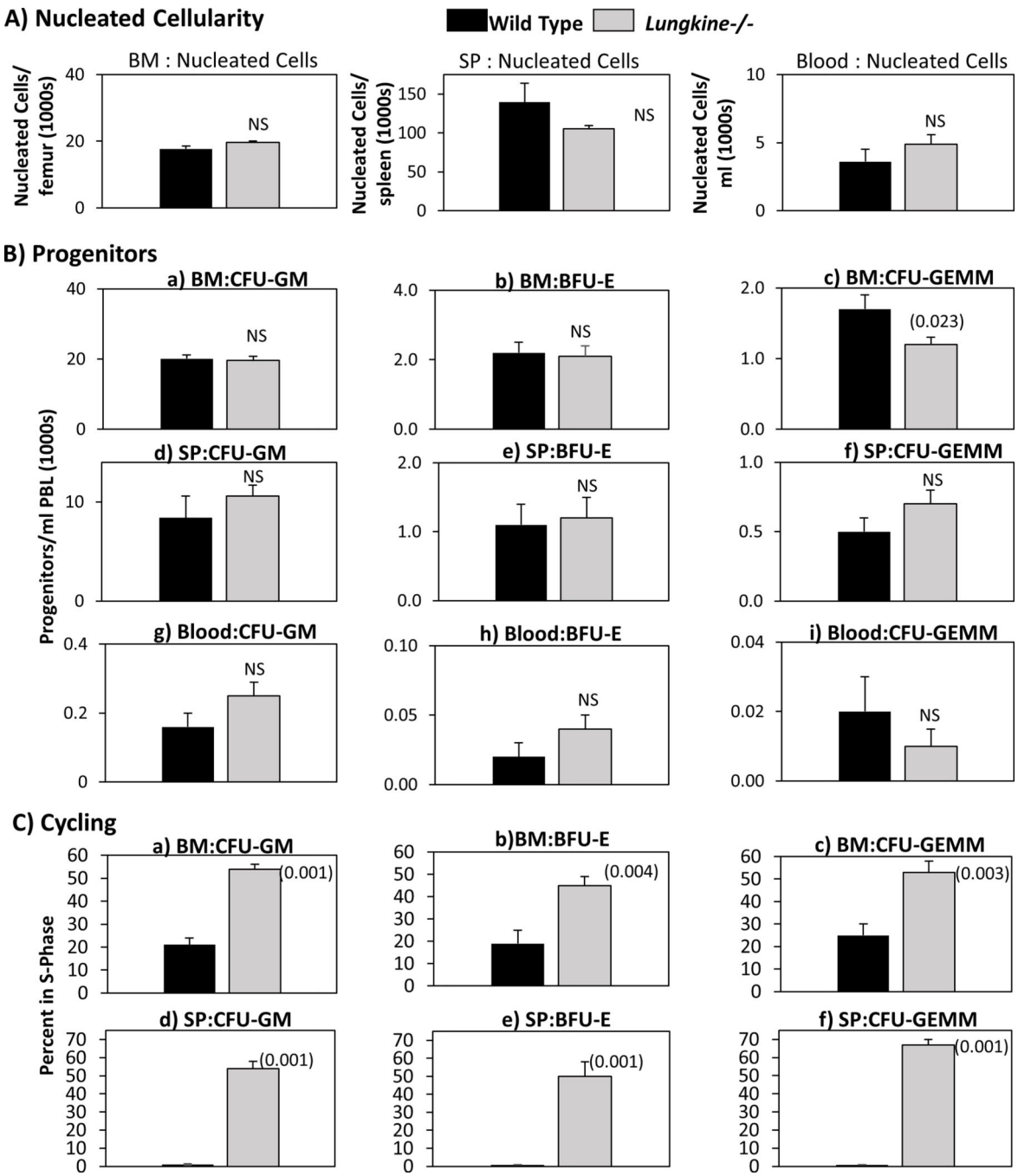

Figure 1.

Bone marrow (BM), Spleen (SP), and Peripheral Blood Nucleated Cellularity (A) and Absolute numbers (B) and cycling (C) of CFU-GM, BFU-E and CFU-GEMM in WT and *lungkine* −/− mice (B, C). Cells were plated in methylcellulose culture medium with EPO, SCF, PWMSCM, and hemin. Cycling status of CFU-GM, BFU-E, and CFU-GEMM in BM (Ca-c) and Spleen (Cd-f) in WT and *lungkine* −/− mice are shown. Statistical differences between WT and *lungkine* −/− are shown on Figure. NS = non-significant (P>0.05) for WT vs. lungkine −/− mice.

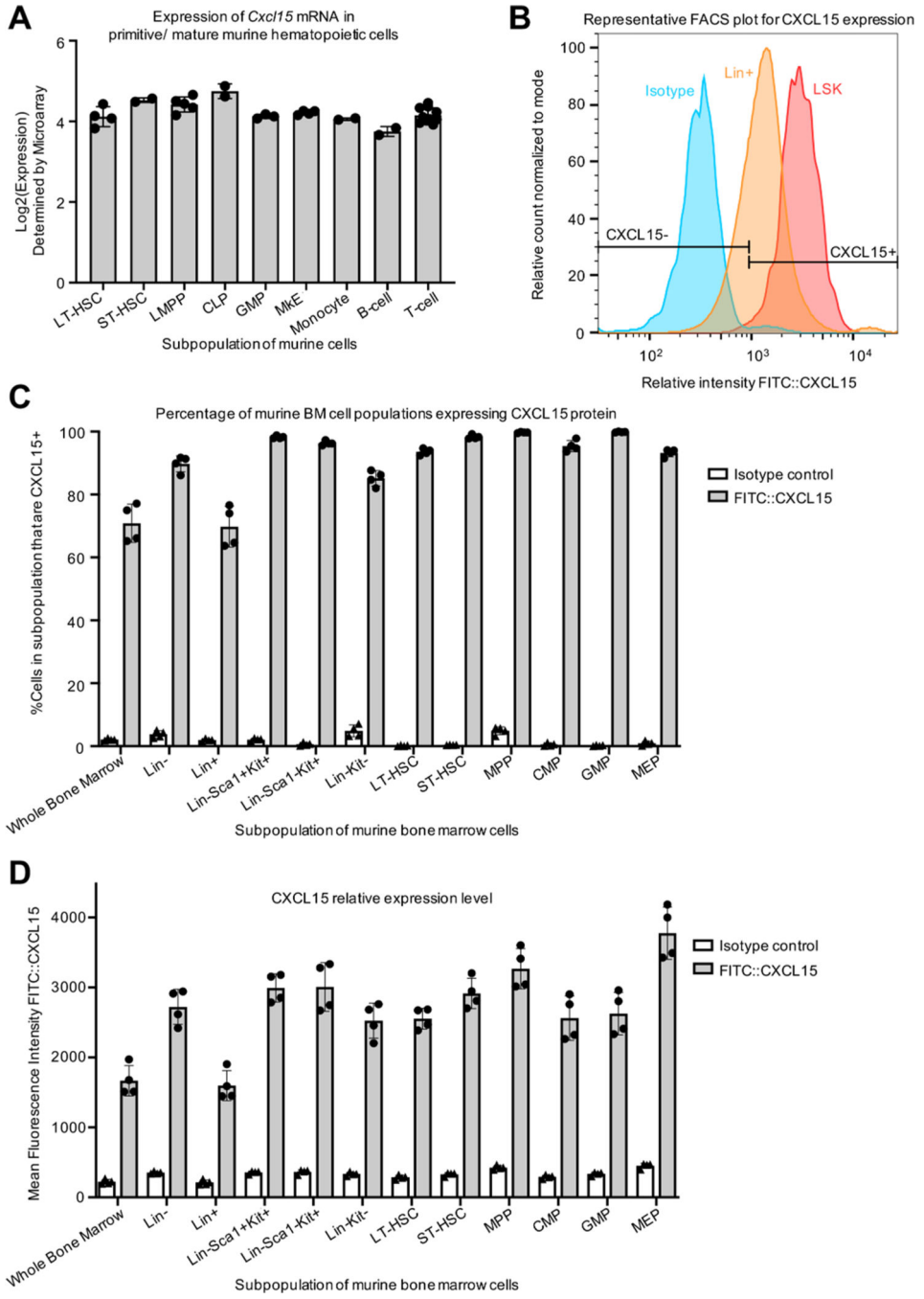

Figure 2:

Expression of CXCL15 by phenotyped HSC and HPC subsets in normal mouse BM. A) Expression of *Cxcl15* mRNA as analyzed by microarray for selected primitive and mature mouse hematopoietic cells was obtained from the BloodSpot database (28–30). Data shown is for microarray probe 1456428_at. LMPP=lymphoid primed multipotent progenitors; CLP = common lymphoid progenitor; MkE = megakaryocyte erythroid progenitor. B-D) Wildtype mouse BM was harvested, cells were stained for cell surface markers to define specific subpopulations of HSC/HPC, and cells were permeabilized and stained

with FITC-anti-CXCL15 antibody. B) Representative FACS plot demonstrating CXCL15 protein expression in Lin+ or LSK cells from murine BM. Isotype control is shown. C) Percentage of cells in the indicated subpopulations that are positive for CXCL15 protein expression using an isotype control to gate CXCL15+ versus CXCL15- cells. D) Calculated mean fluorescence intensities for FITC (conjugated to anti-CXCL15) for the indicated subpopulations of cells. Example of Flow analysis shown in Suppl. Fig. 1.

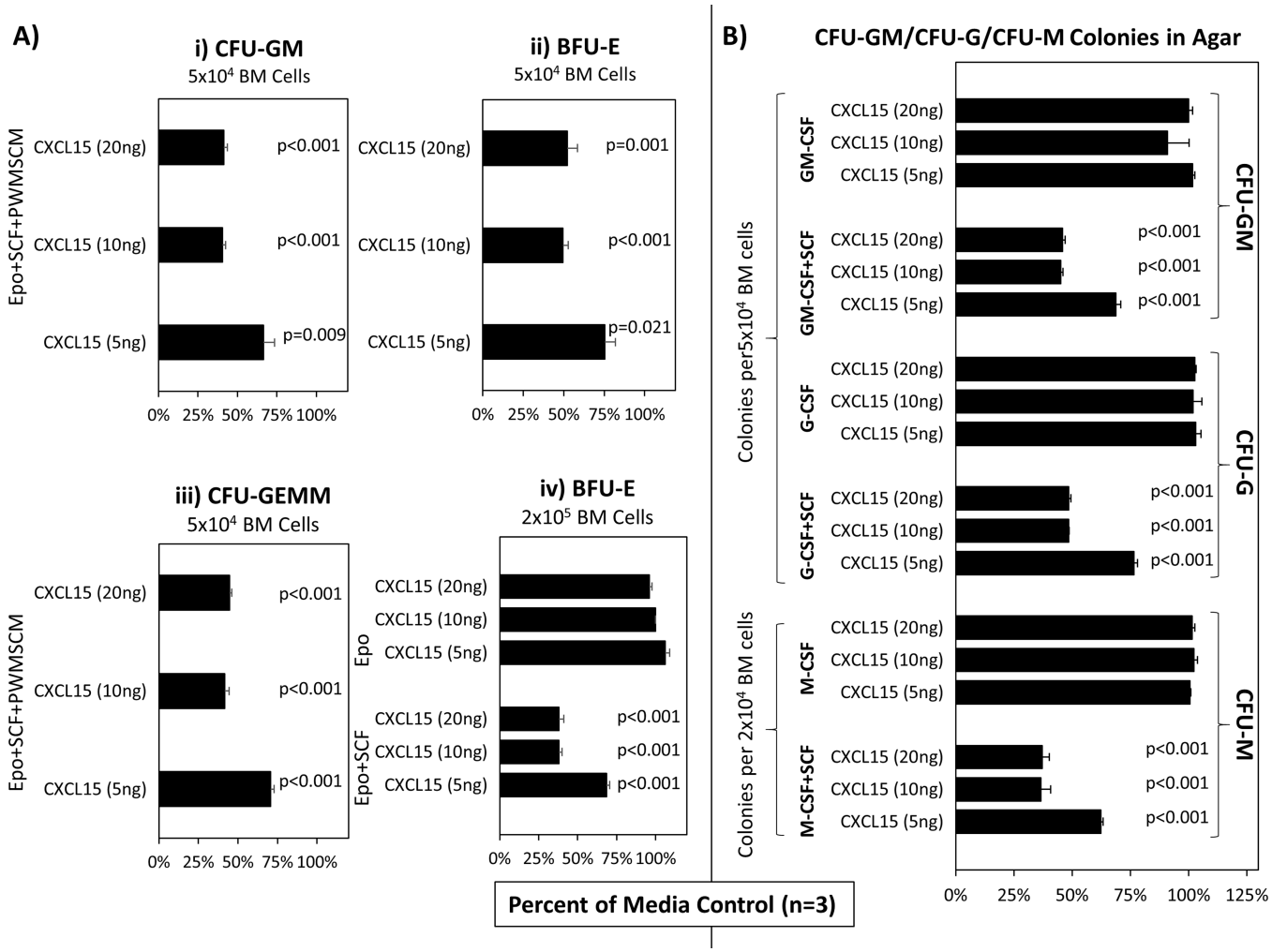

Figure 3.

Effects of Varying Dosages of Recombinant Murine CXCL15/Lungkine on Colony Formation by CFU-GM (Ai), BFU-E (Aii, Aiv), and CFU-GEMM (Aiii) on WT BM Cells stimulated with noted cytokines in Methylcellulose Culture Medium at the Indicated cell plating numbers, and on CFU-GM, CFU-G, and CFU-M (B) in Agar Culture Medium as Indicated. Data is shown as mean percent change +/− SEM for 3 separate experiments. See Suppl. Figs. 2–4 for the actual numbers of each experiment.

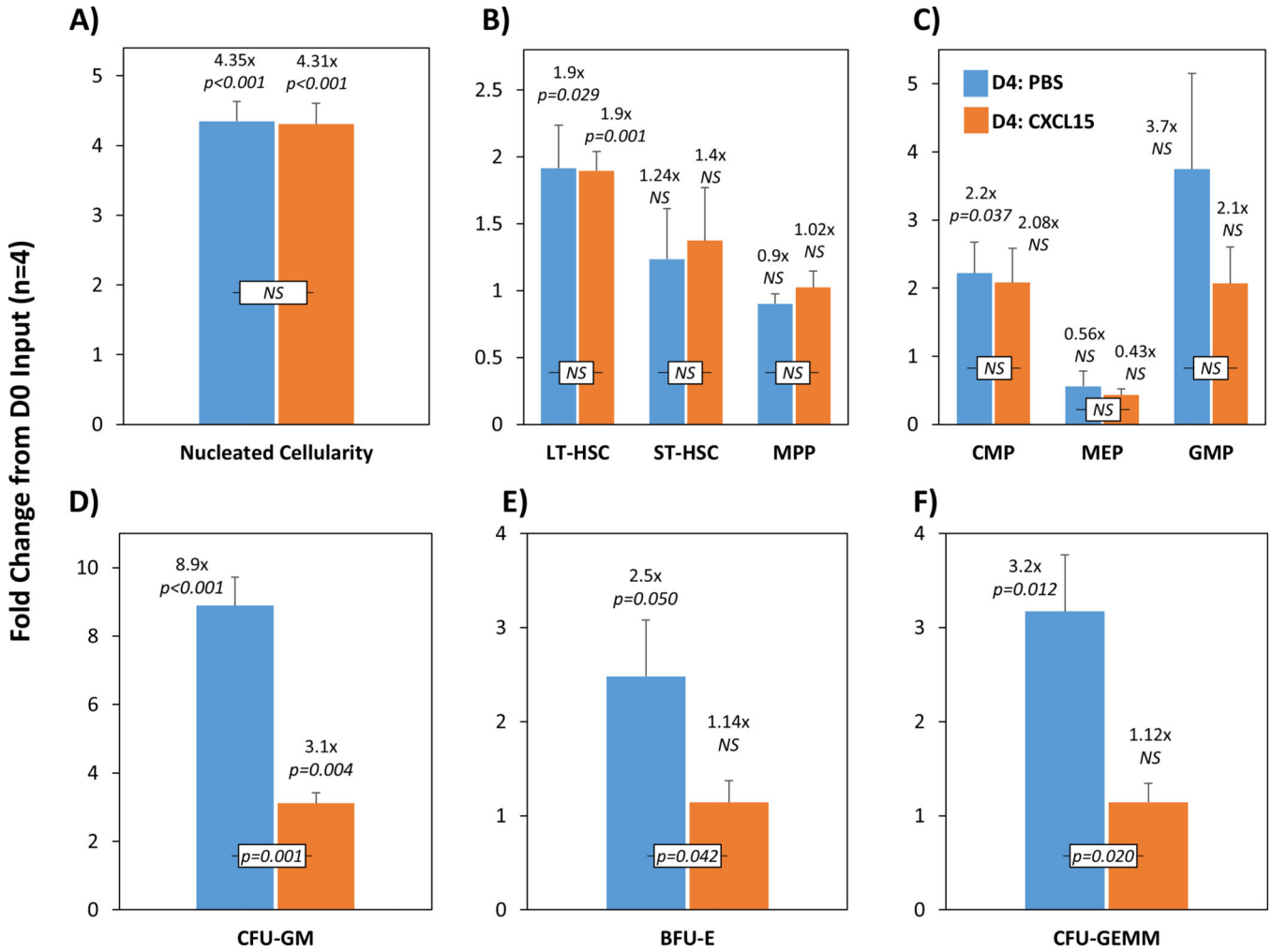

Figure 4:

Effects on CXCL15 on ex-vivo expansion of phenotyped HSC/HPC (B, C) and functional HPCs (D-F). Cells were harvested from wildtype BM and enriched for the lineage negative (Lin-) population. Lineage depleted cells were plated at a density of 100,000 cells/mL/well in IMDM supplemented with 10%FBS and 100ng/mL rmTPO, rmSCF, and rhFLT3L in the presence or absence of 20ng/mL CXCL15. At day 4 of expansion, cells were harvested for Day 4 expansion analysis. A-C) On Day 0 prior to culturing cells for expansion, lineage depleted cells were analyzed by flow cytometry to determine the input number of the indicated HSC/HPC subpopulations per expansion well. On Day 4, expanded cells were analyzed by flow cytometry to determine the expanded number of the indicated HSC/HPC subpopulations per expansion well. Shown are the fold-change values of expanded cells relative to input cells per well. D-F) On Day 0 prior to culturing cells for expansion, lineage depleted cells were plated in methylcellulose for determination of input HPC (type of CFU is indicated in the figure). On Day 4, expanded cells were plated in methylcellulose for determination of expanded HPC (type of CFU is indicated in the figure) numbers per expansion well. Shown are the fold-change values of expanded cells relative to input cells per well. Results are provided as mean fold change +/− SEM for 4 experiments from Day 0

input values. See Suppl. Figs. 5 and 6 respectively for the individual numbers of phenotyped HSC and HPC, and functional HPCs. The latter determined by colony assays.

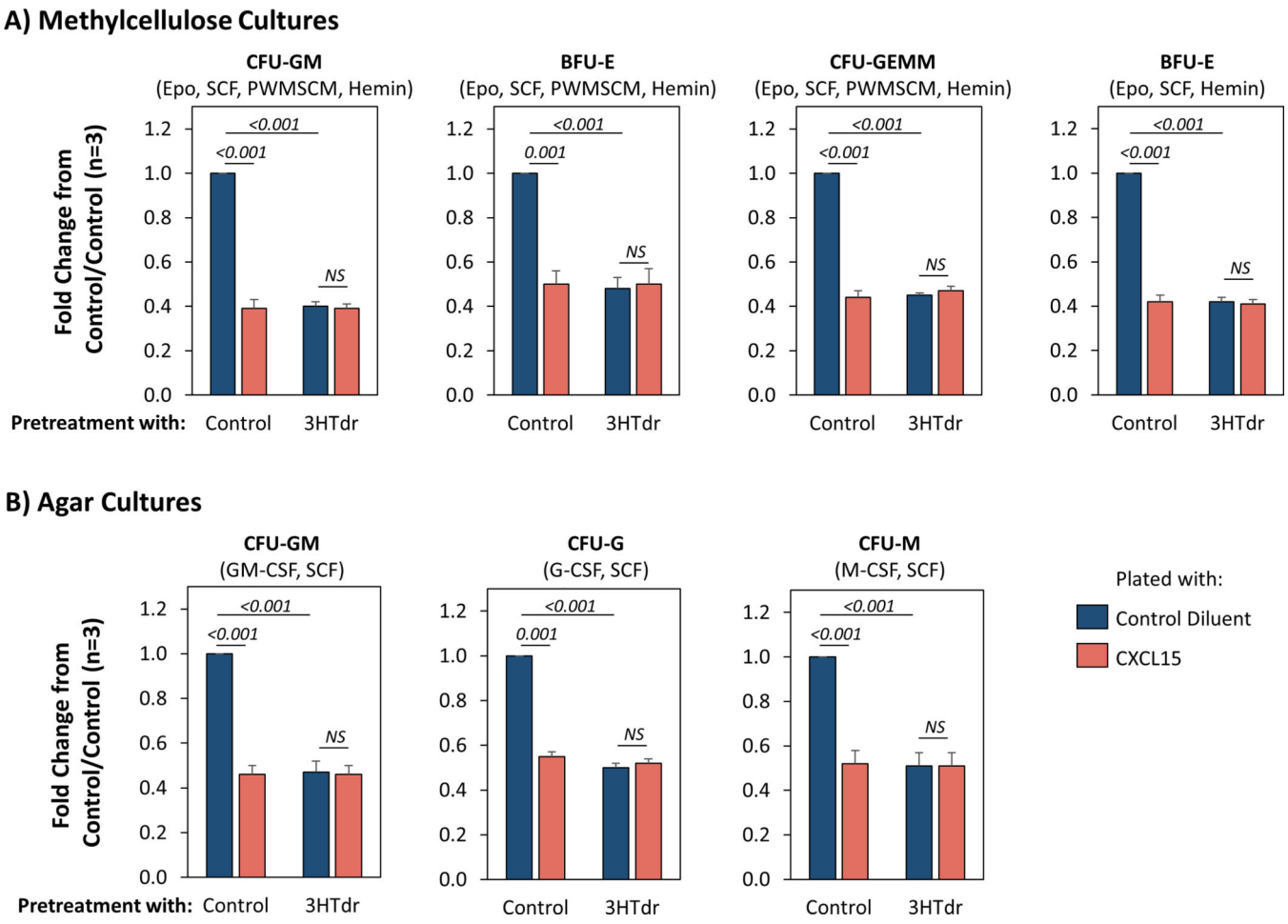

Figure 5.

S-Phase Specific Inhibitor Effects of Recombinant Murine CXCL15 on Colony Formation of Different HPCs *In Vitro*. This is the average fold change of 3 separate experiments expressed as a mean +/− SEM compared to PBS control minus CXCL15, and without high specific activity 3HTdr pretreatment. See Suppl. Fig. 7 for data of each experiment in terms of actual colony numbers.