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Research:

Amphiregulin confers protection to murine hematopoietic progenitor cells from apoptotic death

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Abstract

Amphiregulin (Areg) is a unique ligand of epidermal growth factor family. The functions of Areg in hematopoiesis remain to be elucidated. Using mouse bone marrow (BM) regeneration model and *in vitro* culture systems, we were able to show that Areg is a potent anti-apoptotic factor that rescues hematopoietic stem and progenitor cells (HSPCs) from apoptotic stimuli. Like other growth factor genes, *Areg* was found to be up-regulated in the stromal compartment (CD45$^-$) of BM. The role of *Areg* was determined by loss-of-function study using shRNA-mediated *Areg* knock-down M210B4 (M210B4$^{Aregfk/dl}$) stromal cell line. Co-culture experiments of BM Lin$^-$Sca-1$^+$c-Kit$^+$ (LSK) cells with wild-type or M210B4$^{Aregfk/dl}$ stroma revealed that a significant fraction of LSK cells in knock-down stroma progressed towards apoptosis due to auto-activation of death receptor CD95R and subsequent activation of pro-apoptotic (*Bad*, *Bax*) and suppression of anti-apoptotic (*Bcl-xL*) genes, and activation of caspases-3 & 7. The role of Areg was confirmed by reconstitution assay where M210B4-conditioned medium significantly recovered LSK cells from apoptotic death. All these cellular changes that occurred in M210B4$^{Aregfk/dl}$ cells-supported culture of LSK cells were due to suppression of Stat5 activation. Overall, this preliminary study suggests that initially ErbB/Areg activation protects LSK cells in culture from apoptosis; later the hematopoietic cytokines are probably involved in proliferation and differentiation.

Keywords: Amphiregulin; Apoptosis; HSCs; shRNA; Stat5.

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Introduction

Self-renewal, differentiation and apoptosis of hematopoietic stem cells (HSCs) are fate determining factors for maintaining homeostasis in hematopoietic system. In normal physiological conditions HSCs pool of healthy individual is maintained; whereas, its imbalance may cause hematopoietic insufficiencies leading to acute cytopenia or hematological malignancies [1]. The mechanisms that govern apoptosis and self-renewal of HSCs are not clearly understood, emerging literature suggested the roles of both intrinsic and extrinsic factors [2-4]. In adult, transcription factors like *Zfx* and *Foxo3a* have been found critical for self-renewal of HSCs. In mouse, deletion of *Zfx* resulted in up-regulation of stress inducible genes and impairment of self-renewal leading to apoptosis of HSCs [5]. *Foxo3a*-deficient mice also showed decline in HSCs number and their long-term engraftment potential [6].

Earlier different hematopoietic growth factors are shown to be involved in prevention of apoptosis in HSCs. In mouse, prevention of apoptosis seems require signals from both Bcl-2 and stem cell factor (SCF) [7]. The HSCs of H2K-Bcl2 transgenic mice were better protected against many apoptotic stimuli than wild-type HSCs, when cultured in the presence of SCF. This study probably suggest that even in the presence of strong proliferation signal of SCF, the self-renewal of HSCs does require anti-apoptotic signal or else cells die. Cord blood $CD34^+$ cells were found to be protected from apoptosis by activating cAMP using thrombopoietin (TPO), granulocyte-colony stimulating factor (G-CSF) or SCF. In this case, anti-apoptotic properties were conferred by precluding cells from mitochondrial transmembrane potential, Bcl-xL down-regulation and caspase-3 activation [8]. Additionally, Flt3-L signaling pathway prevented spontaneous apoptotic death of cord blood stem and progenitor cells, which was believed to occur due to up-regulation of a cell survival factor, Mcl-1 [9]. The activation of canonical Wnt/b-catenin signaling pathway has been found indispensable during development of many tissues, including HSCs [10]. However, in adult systems, conditional activation of β-catenin increased apoptosis in HSCs and progenitors through the induction of intrinsic mitochondrial pathway [11]. Interestingly, double mutant of Wnt/β-catenin and PI3K/Akt mice showed self-renewal and expansion of HSCs with long-term functional capacity [12]. Other mouse studies showed that the activation of Notch pathway by Jagged 1 or by ectopic expression of notch intracellular domain (NICD) promotes HSCs self-renewal [13]. However, human HSCs showed opposite results; where ectopic expression of constitutively active Notch 1 led to cell cycle arrest and apoptosis through up-regulation of p21 and BCL2L1 [14].

Amphiregulin (Areg) is a heparin-binding molecule and a unique epidermal growth factor (EGF) family member. It acts as an autocrine growth factor in human lung bronchial epithelial cells and keratinocytes [15,16]. Like estrogen receptor a, Areg is involved in proliferation of mammary epithelium, terminal buds formation and ductal elongation in pubertal mice [17]. Areg signaling confers survival of many cancers, including colon, breast, ovarian, lung, liver, prostate, and pancreas [18-22]. Areg acts mainly through ErbB1, leading to the activation of various pathways like Ras/Raf/MEK/ERK, PI3K/Akt, NF-kB and STAT1, 3 and 5 depending on the target cells [23]. It also harbors a mitogenic activity and is involved in several biological processes like cell cycle progression, inflammatory response, invasion, nerve generation, bone formation, as well as the outgrowth

and branching of several tissues such as lung, kidney and prostate [24].

The role of Areg in hematopoiesis is not studied well; human basophils were found to express Areg in response to T cells for eliminating nematode infection [25]. Areg has been proposed as a molecular marker discriminating between AML and B-ALL leukemia [26]. Here, we have conducted genome wide search to identify a novel function of Areg in hematopoiesis. We show that Areg protects mouse LSK cells in culture from apoptosis in a Stat5-dependent manner.

Materials and Methods

Animals: Six to eight weeks old C57BL/6J [Ptprcb (Ly5.2)] and C57BL6.SJL [Ptprcc (Ly5.1)] mice were used in this study. Mice were obtained from The Jackson Laboratories and maintained in the experimental animal facility of the institute. Mice were kept in individual ventilated cages and fed with autoclaved acidified water and irradiated food *ad libitum*. All experiments using mice were conducted following the approved protocols of the Institute Animal Ethics Committee, National Institute of Immunology, New Delhi.

Isolation of marrow cells and transplantation: Thirty thousand $Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$ (LSK) BM cells of CD45.2 mouse were transplanted in each mouse (CD45.1) through lateral tail vein injection [27]. After 10 days of transplantation, mice were sacrificed and BM stromal cells ($CD45.1^{-}$) and LSK cells of donor origin were sorted using a customized FACS AriaIII (BD Biosciences, San Jose, CA). It is needless to mention that $CD45.1^{-}$ cells were a heterogeneous population of fibroblasts, osteoblasts, MSCs and endothelial cells, but devoid of erythrocytes. As control, stromal cells of healthy mice were used.

Isolation of total RNA: Above cells were spin-down in RNase-free siliconized tubes. Cell pellet was disrupted in 300 ml Trizol Reagent (Sigma Chemical, St. Louise), to that 60 ml of chloroform was added and the tube was centrifuged at 14,000 rpm for10 min. RNA present in the aqueous phase was further purified using a RNeasy Mini-Kit (Qiagen, Valencia, CA) following the manufacturer's RNA Clean-up protocol. The quantity of total RNA and the integrity was checked using a bioanalyzer. The RNA samples with a RIN score 7.0 and above were considered for hybridization reactions.

Global gene expression analysis: In brief, three hundred nano-gram of total RNA was applied for hybridization in Agilent Platform (Agilent Technologies, CA) using Agilent whole mouse genome 8 × 60k array chips (AMADID: 26986) and data were analyzed using GeneSpring GX software by Genotypics Pvt Ltd, Bangalore, India. Microarray fluorescence (Cy3) images were scanned on an Agilent Gene Array Scanner with a 560-nm filter. Fold change of gene (≥ Log2) expressions were calculated by comparing with that in healthy mouse cells. Raw data are deposited in GEO data base (accession ID: GSE75294).

Quantitative real-time PCR: Quantitative RT-PCR analysis was performed in Thermal cycler (Eppendorff Realplex Instrument, Hamburg, Germany) using power SYBR Green Supermix (Thermo Fisher Scientific, Waltham, MA). The details of the procedure and primers used are provided in the Supporting Methods. Fold change of gene expression was calculated using formula $2^{-\Delta\Delta Ct}$.

Cloning of shRNA (against *Areg* gene) in a pLKO.1-puromycin vector and establishment of *Areg*-k/d cell line: The pLKO.1-TRC cloning vector (Addgene, Cambridge, MA) containing a 1.9 kb stuffer was released upon digestion with *EcoR*1 and *Age*1. The

Areg-shRNA oligo was flanked by sequences that were compatible with the sticky ends of *EcoR*1 and *Age*1. Forward and reverse oligos were annealed and ligated in the pLKO.1 vector, producing a final plasmid that expresses the shRNA of interest. The cloning was performed by transforming *E.coli*, the right clone was selected on the basis of restriction digestion. This construct was used along with packaging and envelop vectors to generate lentiviral particles in HEK293 cell line. Murine stromal cell line M210B4 was transduced with these virus particles, and the knock-down clone was selected in the presence of puromycin (3 mg/ml).

Cell culture: Mouse hematopoietic stroma cell line, M210B4 (a kind gift of Prof. Connie Eves, Terry Fox Institute, Vancouver, Canada), M210B4 (*Areg*-k/d) cell line [M210B4$^{Areg(k/d)}$] and mouse LSK cells were used for this study. Stromal cell lines were routinely cultured in RPMI-1640 supplemented with 10% FBS. One day before initiating co-culture, stromal cell lines were irradiated at 100 cGy and cultured on 6-well plates in the same medium. After 24 h 15,000 LSK cells (CD45.2)/well were over-layered and cultured in the presence of CellGro-SCGM (Cell Technologie Transfer GmbH, Germany) supplemented with 0.5 × ITS (Gibco Laboratories, Grand Island, NY) and 5% ES cells certified serum (Biological Industries, Israel) for 2 to 10 days. In a separate experiment, medium was further supplemented with 10 ng/ml SCF (PeproTech Asia, Rehovot, Israel).

Caspase-3 & 7: Caspase activity was determined in sorted CD45.2^{+}LSK cells from co-culture experiment using Apo-one Homogeneous Caspase-3/7 assay kit (Promega Corporation, Madison, MI).

Immunocytochemistry: Sorted CD45.2^{+}LSK cells were cyto-spinned on poly-L-lysine coated glass slides. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% saponin in room temperature for 20 min. The washed cells were stained with pSTAT5 primary antibodies (Cells Signaling, Denver, MA) for overnight at 4°C, washed and further stained with Alexafluor 488 conjugated secondary antibodies (Molecular Probes Inc., Eugene, OR) for 1 h. Nuclear staining was done with DAPI; cells were mounted with ProLong^{R} anti-fade (Molecular Probes Inc.) and examined by Zeiss LSM 510 META confocal laser-scanning microscope using a Plan-Apochrom at 63 ×/1.4 oil objective. LSM 510 software was used for acquisition of images. The images were processed by Zeiss LSM Image browser, version 4.2.0.121.

Areg neutralization assay: Ten thousand THP1 cells/100 ml were taken in triplicate wells of 96 well plates. Cells were cultured in the presence of increasing concentrations (0, 3, 9, 27 mg/ml) of Areg-neutralization antibody (PeproTech Asia) in a CO_2 incubator for 48 h. The effect of Areg-neutralization on proliferation of cells was determined by MTT assay.

Flow cytometry: Antibodies used for this investigation were CD45.1/PECy5, c-Kit/APC (eBisciences, San Diego, CA), biotinylated lineage cocktail antibodies (Miltenyi Biotech, Gladbach, Germany), Sca-1/FITC, CD45.2/ V450, CD45.2/RPE, streptavidin-APCCy7, anti-CD95 (BD Pharmingen). Cell were incubated with antibodies for 30 min on ice and then washed with phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA). For biotinylated lineage cocktail antibodies, after washing cells were further incubated with streptavidin-APCCy7 for an additional period of 30 min. Washed cells were analyzed with FACS AriaIII. Dead cells were identified by counter staining of the cells with Sytox green/red dye. Apoptotic cells were detected by staining with Annexin V/PE kit (BD Pharmingen).

Statistical Analysis: Results of multiple experiments

were reported as mean ± SEM. Student's t test was carried out to calculate the significance between the means of both groups and $p < 0.05$ was considered as significant. All analyses were carried out using Graph pad Prism software, Version 5.02.

Results

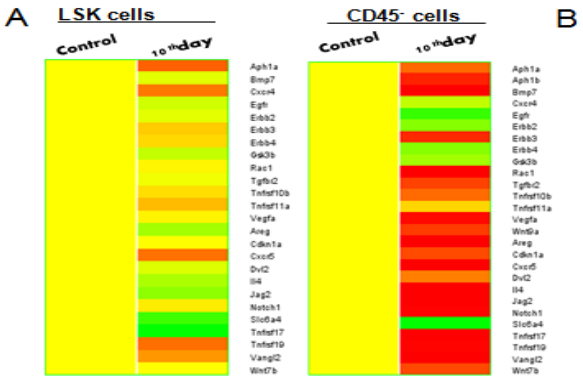
Transcriptome analyses of LSK and stromal cells

To study differential gene expressions, cells were sorted from control (LSK and $CD45^-$ cells at day 0) and test mice (donor LSK and recipient $CD45^-$ cells after 10 days of transplantation) and corresponding mRNAs were subjected to global gene expression analyses. The heat-map of up-regulated and down-regulated genes in LSK and $CD45^-$ compartments of Notch, Wnt, VEGF and ErbB pathways are shown (Figure 1A).

were differentially expressed in the test mice $CD45^-$ compartment as compared to control was found to be 27, of which few up-regulated genes were filtered and corresponding genes in LSK cells have been shown in (Figure 1B). As *Jag2*, *Vegf*, *Areg* genes were exclusively expressed in $CD45^-$ compartment, we anticipated their paracrine effects on LSK cells. The transcripts of few selected genes in stromal compartment were validated by quantitative RT-PCR analysis. It may be seen that in many genes both microarray and RT-PCR data were comparable (Figure 1C). Since the function of Areg in haematopoiesis is not detailed yet, we investigated that.

Generation of M210B4$^{Areg(k/d)}$ cell line

Given that Areg is responsible for ErbB receptor activation in LSK compartment, we wanted to know its' effect on LSK cells.



Path-way	Genes	$CD45^-$ cells	LSK cells
Notch	*Aph1a*	2.76	1.24
	Aph1b	2.01	0.55
	Bmp7	4.0	-0.22
	Jag2	2.37	-0.88
Wnt	*Rac1*	1.21	0.07
	Vangl2	2.67	0.81
	Fosl1	1.04	0.08
VEGF	*Vegf*	1.93	0.24
	IL4	2.48	-0.64
	Koh	1.3	0.18
ErbB	*Areg*	2.37	-0.71
	Cdkn1a	1.42	0.01
	ErbB3	1.68	0.4

Figure 1: Microarray analyses of selected genes in the regenerating BM. (A) Sub-lethally irradiated mice received 3×10^4 CD45.2LSK cells. After 10 days of transplantation CD45.2LSK and $CD45^-$ BM cells were sorted for gene expression analysis. Heat-maps show number of genes (Notch, Wnt, VEGF, ErbB pathways) that were up-regulated/down-regulated in both the cellular compartments as compared with respective controls. (B) Comparison of few selected up-regulated genes between $CD45^-$ and LSK cells. (C) Validation of few microarray gene expressions by real-time RT-PCR. Number of experiments: 2-4.

To begin with, we designed and cloned the *Areg*-

shRNA possessing sticky ends of EcoRI and AgeI in pLKO.1-puromycin vector. Following transformation in *E. coli* we obtained 12 clones, out of which clone #8 was found to be the positive after restriction digestion with Sal1, the unique restriction site added in the *Areg*-shRNA (Figure 2A & 2B). The *Areg*-shRNA transduced and puromycin selected M210B4 cells were examined by PCR to determine the extent of knocked-down of *Areg* gene. The results showed about 80% suppression of *Areg*-mRNA expression as compared to parental M210B4 cells (Figure 2C). The knock-down cell line was named as M210B4$^{Areg(k/d)}$, which was further validated by determining protein expression. Immunocytochemical analysis confirmed significant suppression of Areg protein expression in knock-down cells as compared to the wild-type cells (Figure 2D). Knock-down of *Areg* gene may lead to change in expression patterns of key hematopoietic growth factors.

To examine that few important transcripts (*SCF, IL3, VEGF, IL-6*) were analyzed by quantitative RT-PCR and found to be unaffected in M210B4$^{Areg(k/d)}$ cells (Figure 2E). Overall, the cellular characterization revealed that gene manipulation leads to major shut-down of Areg expression in M210B4$^{Areg(k/d)}$ cells, but the expression of other growth factor genes were unaffected.

M210B4$^{Areg(k/d)}$ stromal cells suppress hematopoietic activity and promote spontaneous apoptosis in HSPCs

In order to study the role of Areg in hematopoiesis, GFP$^+$LSK cells were co-cultured in the presence of sub-lethally irradiated M210B4 (parental) and M210B4$^{Areg(k/d)}$ stroma cells for 5 and 10 days. No decrease of over-layered cells was observed in case of parental stroma-supported culture, whereas the cell number was significantly declined in case of knock-down stromal cell line (Figure 3A).

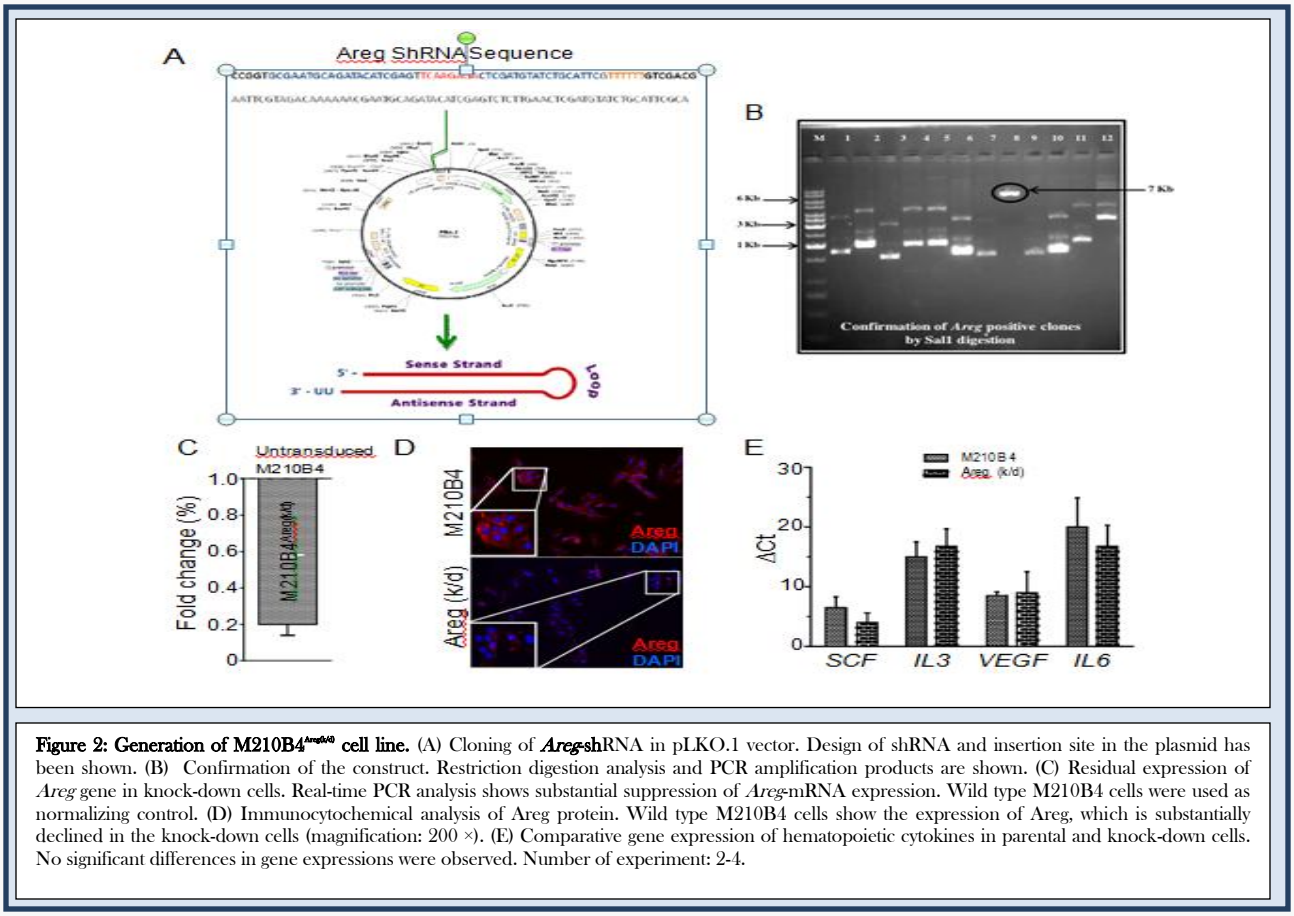


Figure 2: Generation of M210B4$^{Areg(k/d)}$ cell line. (A) Cloning of ***Areg*-sh**RNA in pLKO.1 vector. Design of shRNA and insertion site in the plasmid has been shown. (B) Confirmation of the construct. Restriction digestion analysis and PCR amplification products are shown. (C) Residual expression of *Areg* gene in knock-down cells. Real-time PCR analysis shows substantial suppression of *Areg*-mRNA expression. Wild type M210B4 cells were used as normalizing control. (D) Immunocytochemical analysis of Areg protein. Wild type M210B4 cells show the expression of Areg, which is substantially declined in the knock-down cells (magnification: 200 ×). (E) Comparative gene expression of hematopoietic cytokines in parental and knock-down cells. No significant differences in gene expressions were observed. Number of experiment: 2-4.

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To examine colony forming ability, methycellulose colony assay was performed in 5 days grown cells. Surprisingly, colony forming potential of over-layered cells recovered from M210B4$^{Areg(k/d)}$ culture was severely compromised as compared to parental cells (Figure 3B). Since the support for hematopoiesis by M210B4$^{Areg(k/d)}$ stromal cells was extremely poor, we anticipated an early effect on LSK cells. Therefore, subsequent experiments were conducted after 2 days of co-culture. As SCF is a potent growth and anti-apoptotic factor of HSPCs, we performed short term culture experiments in the presence and absence of SCF. In Areg-k/d stroma-supported culture, the apoptotic cell population was increased by a factor of 4 to 5 as compared to parental stroma-supported culture (29.7 ± 4.0 vs 6.9 ± 1.7%, $p < 0.01$), which could not be reversed by the addition of SCF (Figure 3C).

Further analysis revealed that the over-layered cells retained 90% LSK phenotypes, which was declined to 30-40% in knocked-down stroma culture, in spite of the presence of SCF in the culture medium. The decline of LSK cells was associated with lineage commitment in M210B4$^{Areg(k/d)}$ stroma-supported culture (data not shown).

As SCF could not protect cells from apoptosis, we hypothesized that lack of Areg in M210B4$^{Areg(k/d)}$ stroma supported culture was responsible for induction of apoptosis in LSK cells. To confirm that, the same experiment was performed, but instead of SCF the medium was supplemented with 50% M210B4 parental cells' conditioned medium. A significant (47.2 ± 8.0 vs 31.3 ± 0.9, $p < 0.05$) reduction in apoptotic cells was evidenced in knocked-down stroma-supported culture but in the presence of conditioned medium (Figure 3D).

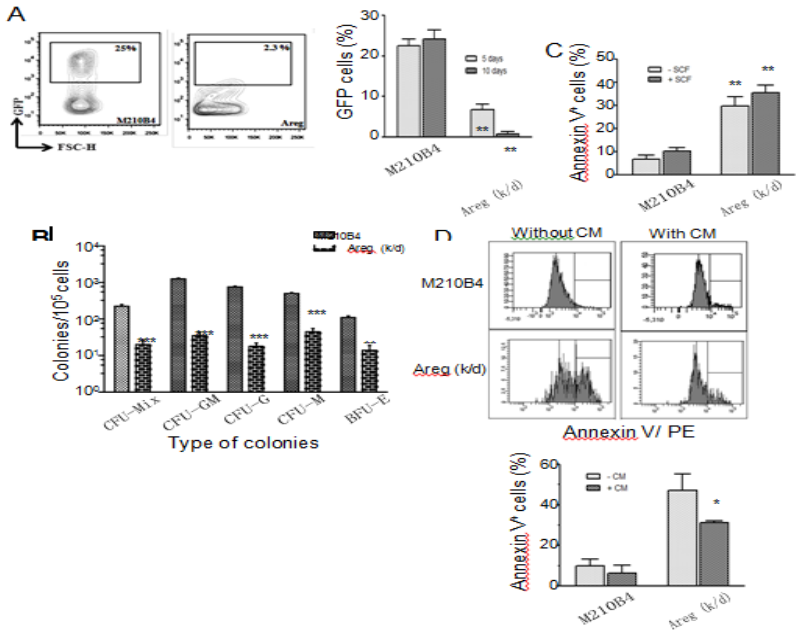

Figure 3: Suppression of hematopoietic activity in co-culture with M210B4$^{Areg(k/d)}$ cell line. (A) Representative contour plot show percentage GFP$^+$LSK cells after 5 and 10 days days of culture on both stromal cells. Bar diagram shows a significant drop in GFP$^+$ cells in culture with knock-down cells. (B) Bar diagram shows a significant reduction of colony forming potential of LSK cells after culture for 5 days with *Areg* knock-down stromal cells. (C) Bar diagrams shows a significant increase of Annexin-V$^+$ (apoptotic) LSK cells within 2 days of co-culture with knock-down stroma in absence or presence of SCF. (D) Culture reconstitution assay. Histogram show representative results of the recovery of LSK cells from apoptosis in the presence of 50% M210B4-CM supplemented culture with knock-down cells. Bar diagram shows significant decrease in apoptosis of LSK cells. Number of experiment: 3. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

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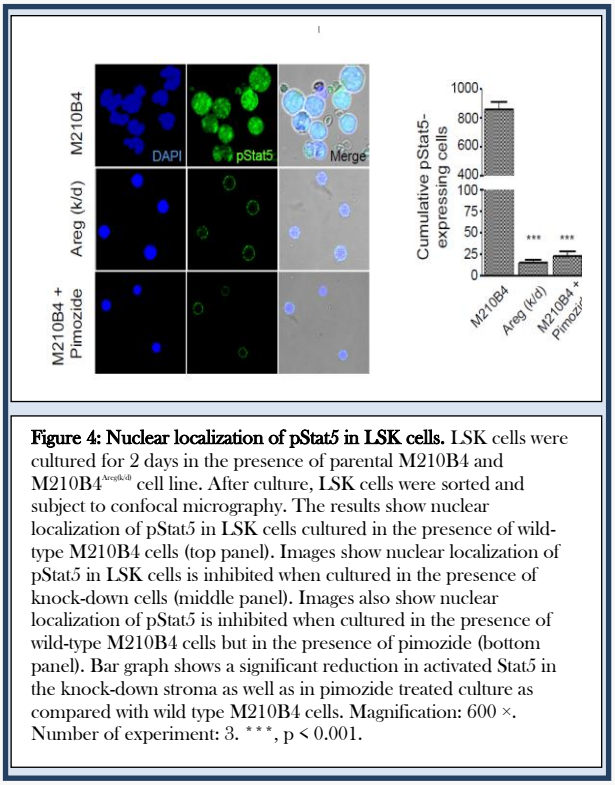

Figure 4: Nuclear localization of pStat5 in LSK cells. LSK cells were cultured for 2 days in the presence of parental M210B4 and M210B4$^{Areg(k/d)}$ cell line. After culture, LSK cells were sorted and subject to confocal micrography. The results show nuclear localization of pStat5 in LSK cells cultured in the presence of wild-type M210B4 cells (top panel). Images show nuclear localization of pStat5 in LSK cells is inhibited when cultured in the presence of knock-down cells (middle panel). Images also show nuclear localization of pStat5 is inhibited when cultured in the presence of wild-type M210B4 cells but in the presence of pimozide (bottom panel). Bar graph shows a significant reduction in activated Stat5 in the knock-down stroma as well as in pimozide treated culture as compared with wild type M210B4 cells. Magnification: 600 ×. Number of experiment: 3. ***, $p < 0.001$.

Thus, above results implicated the role of Areg in preventing apoptosis and lineage commitment of LSK cells in short-term cultures.

Inhibition of Stat5 pathway lead to apoptosis in M210B4$^{Areg(k/d)}$ stroma-supported culture of LSK cells

Stat5 is one of the important transcription factors activated upon engagement of ErbB receptor with the ligands. To know whether Stat5 activation in LSK cells was inhibited in *Areg*-k/d stromal culture, we determined nuclear localization of pStat5 in LSK cells in both type of support cultures. LSK cells were also cultured in wild-type stromal cells but in the presence of pimozide, a potent inhibitor of Stat5 phosphorylation. This served as second control of ErbB pathway inhibition. Immunocytochemical analysis confirmed nuclear localization of pStat5 in almost 80% of LSK cells in M210B4 parental stroma-supported culture, whereas its' activation was significantly suppressed both in M210B4$^{Areg(k/d)}$ stroma or pimozide-supplemented cultures (Figure 4). These results suggest a potential role of Areg in activation of ErbB pathway and subsequent nuclear localization of pStat5.

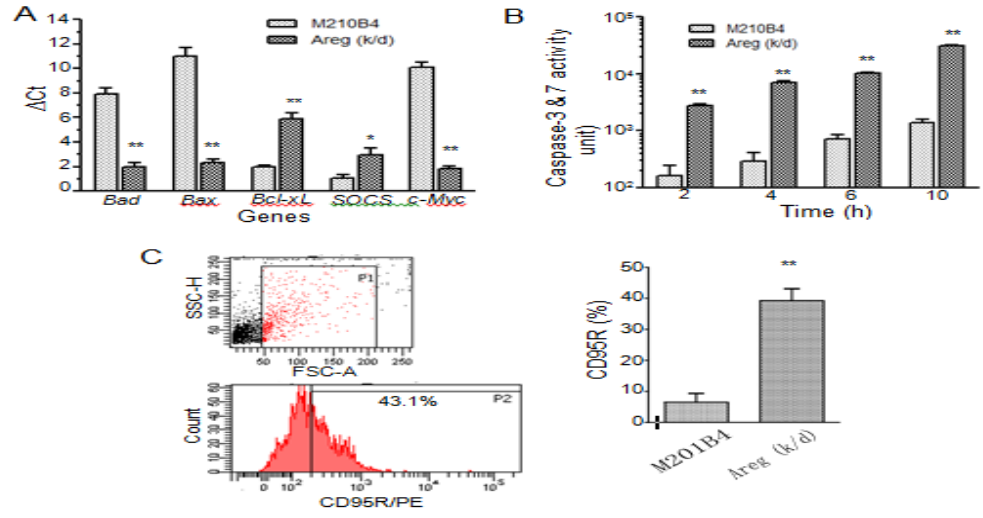

Figure 5: Activation of apoptotic genes. LSK cells were cultured in the presence of parental M210B4 and M210B4$^{Areg(k/d)}$ cell line. After 2 days LSK cells were sorted and subject to the analysis. (A) Pro-apoptotic genes (*Bax, Bad, c-Myc*) were up-regulated and anti-apoptotic genes (*Bcl-xL, SOCS*) were down-regulated in culture with knock-down cells. (B) Caspases- 3 & 7 was significantly activated in LSK cells cultured with knock-down cells. (C) Representative histogram shows the expression of CD95R (Fas) in LSK cells in culture with knock-down cells. Bar diagram shown CD95R is significantly expressed in these LSK cells. Number of experiment: 3. *, $p < 0.05$; **, $p < 0.01$.

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Since many survival genes are down-stream targets of Stat5, we conducted their analysis by real-time RT-PCR. The pro-apoptotic genes (*Bad*, *Bax* and *c-Myc*) were significantly upregulated in M210B4$^{Areg(k/d)}$-supported LSK cells as compared to parental cells (Figure 5A). Furthermore, anti-apoptotic gene (*Bcl-xL* and *SOCS*) expressions were down-regulated in same cells in the absence of *Areg* (Figure 5A). Together, gene expression analyses suggested that LSK cells became sensitive to apoptotic signals in the absence of activated Stat5. To further confirm that LSK cells underwent apoptosis, post-sorted cells, previously co-cultured for 48 h on parental and *Areg*-k/d M210B4 cells, were tested for caspases-3 and -7 activity. It was evidenced from the assay that caspase activity was increased by 20 folds in M210B4$^{Areg(k/d)}$-supported culture than parental cells (Figure 5B).

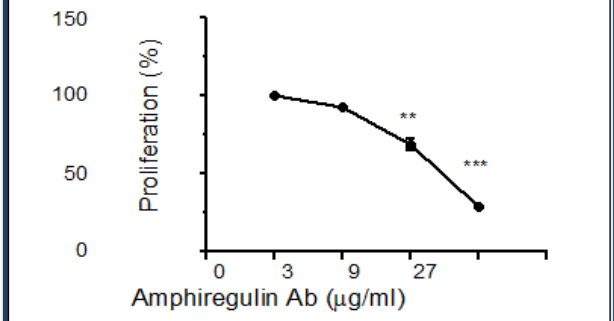

Figure 6: proliferation of THP-1 cells in the presence of Areg-neutralizing antibody. Dose-dependent growth inhibition is observed in THP-1 cell line. Number of experiment: 3. **, $p < 0.01$; ***, $p < 0.001$.

It is possible that the death receptor Fas (CD95R) was activated in LSK cells within 2 days of culture in *Areg*-k/d stromal environment, which leads to apoptosis. To confirm that we co-cultured LSK cells in the presence of both stromal cell lines for 2 days and determined the auto-activation of the death receptor. It was observed that CD95R was highly expressed in LSK cells (39.3 ± 3.85% vs 6.95 ± 2.85%, $p < 0.01$) when co-cultured with M210B4$^{Areg(k/d)}$ than that with parental M210B4 cells (Figure 5C). Overall, this study proposes that in the absence of Areg in culture the apoptotic pathway was activated in LSK cells by clustering of CD95R. Apoptosis can be avoided by supplementing *Areg*, which induces the expression of anti-apoptotic genes in a Stat5-dependent manner.

Areg induces proliferation of THP-1 through autocrine loop

Epithelial tumor cells are known to secrete *Areg* for self-proliferation. It is not known whether the proliferation of human acute monocytic leukemic cell, THP-1 is *Areg*-dependent. So, we performed the proliferation assay of THP-1 cells in the presence of different concentrations of Areg neutralization antibody. A dose-dependent inhibition of growth was observed in 48 h culture, probably indicating that THP-1 secretes Areg for self- proliferation (Figure 6). At higher concentrations of neutralizing antibody, the bioavailability of secreted Areg in culture medium was significantly depleted leading to the death of cells.

Discussion

Bone marrow transplantation (BMT) is commonly practiced in many non-malignant and some malignant hematological disorders [26]. The purpose of transplantation is to reconstitute/rejuvenate hematopoietic system for continuous supply of blood lineages and to induce graft versus leukemic (GVL) reaction. Though HSCs are the major players to induce physiological changes in the recipients, the cellular microenvironment is found to play a critical role for engraftment, proliferation and differentiation of these cells. Earlier, we have shown that in irradiated host, the donor LSK cells can proliferate better as compared to the recipient LSK cells [25]. In the

present study we have identified Areg, a ligand of epidermal growth factor (EGF) family, in regenerating bone marrow stromal cells which protects LSK cells from apoptotic death thus allowing their proliferation and differentiation.

Global gene expression analysis was performed in sorted CD45$^-$ (stromal) cells, and on the basis of microarray analyses we identified eight genes among those were up-regulated during marrow regeneration. *Areg* gene was selected for further investigation as this was meagerly studied in the context of hematopoiesis. *Areg* is typically know as a factor involved in proliferation of mammary epithelial cells [15], besides many epithelial tumor cells are also known to secrete Areg for their proliferation [16-20]. In this study, we have reported a novel function of Areg to protect mouse LSK cells from apoptosis. Earlier report described about pre-requisition of two separate signals (Bcl2 and c-Kit) for prevention of apoptosis in HSCs, but it may be noted that the investigators used Bcl2 over-expressing system [5]. As most of the HSCs are present in quiescent state *in vivo* and only a few are actually in an active state [27], it is expected that in normal physiological conditions the major fraction of HSCs should remain alive. Apoptosis of cells is believed to occur intrinsically for regulating the size of the HSPC pool. In the present study, however LSK cells were isolated from bone marrow microenvironment and cultured *in vitro*. Though, anoikis, a form of programmed cell death, has only been reported in case of epithelial and endothelial cells [28], it is possible that same mechanism may follow in case of LSK cells once excluded from the marrow microenvironment. Anoikis is mediated through intrinsic (mitochondria) or extrinsic (triggering of cell surface death receptors) pathways [29]. In the present study, it is difficult to affirmatively conclude which pathway had a major role in apoptosis of BM-derived LSK cells, the circumstantial evidences suggested that in M210B4$^{Areg(k/d)}$-supported culture both pathways had contributions. This was based on the fact that along with CD95R up-regulation and caspases-3 & 7 activation, we also observed up-regulation of pro-apoptotic genes *Bad* and *Bax* and at the same time down-regulation of anti-apoptotic gene *Bcl-xL* in HSCs [30,31]. Here, it is important to note that the cellular apoptosis was not controlled by native anti-apoptotic genes present in LSK cells and supplementation of medium with SCF.

In vitro culture studies have shown that combination of many cytokines can prevent apoptosis in hematopoietic cells, most important of them are Flt3-L, SCF, IL-3, CSFs [32-35]. These cytokines change the balance of expression of apoptosis inducing (*Bax*) and suppressing (*Bcl2*/*Bcl-xL*) genes thereby protect cells [36]. Despite parental M210B4 cells over-express IL-3, SCF, G-CSF [37], interestingly co-culture of LSK cells with Areg-k/d stroma did not protect cells from apoptosis. Thus this study proposes the role of Areg in protection of LSK cells against activation of apoptotic pathways. It also suggested that though *Areg* gene was expressed in LSK cells their protein expression was low (data not shown). Furthermore, due to low affinity of Areg towards its' receptor, there must be a threshold concentration of ligand for biological activity that probably was much lower in case of M210B4$^{Areg(k/d)}$-supported culture [38]. MSCs of BM stromal cells are known to produce Areg to enhance tumor growth and metastasis of lung cancer cells [39]. The present data suggests that HSPCs may not synthesize enough Areg, upon differentiation and/or neoplastic transformation or upon infection of basophils [22] its' expression is enhanced for cells' survival, as in the case of THP-1 seen in this study.

In hematopoietic cells, Stat5 can be activated by multiple hematopoietic cytokines; however the downstream effects may not be identical. Stat5 activation in CD34LSK murine HSCs by TPO led to expansion of multi-potent progenitor, HSCs self-renewal and onset of myeloproliferative disease [40]. GM-CSF and IL-3 were found to induce Bcl-xL expression in myeloid progenitors in Stat5-dependent manner to rescue them from apoptosis [41]. While other STATs can mediate pro-apoptotic or anti-apoptotic signals depending on the conditions of cells' stimulation, STAT5 mostly exhibits pro-survival signal [42]. Here we propose that Areg confers protection to LSK cells by over-expressing anti-apoptotic genes and reducing pro-apoptotic genes in Stat5-dependent manner. The role of *Areg* in hematopoiesis *in vivo* may not be such critical due to the presence of alternate pathways for activation of Stat5, which needs to be investigated in *Areg*$^{-/-}$ mouse. We propose that once LSK cells escape apoptosis, they can respond to hematopoietic cytokines for proliferation and differentiation. This preliminary study suggests that incorporation of Areg in stroma-free *ex vivo* culture of LSK cells or HSPCs would be promising to establish a protocol for the expansion of cells. Improvement of HSPC expansion protocol may pave the way for advancement of cell therapy. The expanded human HSPCs may be used for clinical applications, such as in case of anemia or thalassemia, and in gene therapy where the success has been limited due to low number of cells.

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